Biological Reference Data on CD(SD) IGS Rats - 2002/2003

CD(SD)IGS Study Group

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PREFACE

This is the fifth publication of Biological Reference Data on CD(SD)IGS Rats-2002/2003 and is the last volume to be published by CD(SD)IGS Study Group in Japan. Since the first publication in 1998, our study group has published four volumes (1998, 1999, 2000, and 2001), which provided useful information on biological characteristics of CD(SD)IGS rats. These data books, known as "Blue Books," consist of three major chapters - General Toxicology, Reproduction Toxicology, and Carcinogenicity. These three categories are essential to evaluate toxicity of new drugs and chemicals.

It is well known that CD(SD)IGS rats were produced from CD(SD) rats by using the international genetic standard (IGS) system in Charles River Laboratories from 1992. This system includes the standardization of four basic elements; health, genetics, quality assurance, and operations. The Foundation Colony was established using 100 pairs of breeder rats of the CD(SD) strain from Charles River colonies located throughout the world. We believe that standardized methods for breeding animals provide laboratory rats with uniformity and high quality, contributing to the evaluation in various toxicity studies. There was little available biological reference data on CD(SD)IGS rats prior to 1995, when supply of CD(SD)IGS rats has begun domestically. Therefore, it was necessary to immediately compile the reference data as soon as possible. Consequently, the members of the CD(SD)IGS Study Group decided to collaborate biological reference data on CD(SD)IGS rats.

Hence, the CD(SD)IGS Study Group has been organized in collaboration with many safety research facilities for drugs, agrochemicals, and other chemicals and the breeding facility, Charles River Japan, Inc., in 1997. The study group has a policy that information of basic biological characteristics on CD(SD)IGS rats should be standardized among the researchers using the animals. We believe that the international and commercial use of CD(SD)IGS rats in toxicology area would contribute the human health and welfare. The CD(SD)IGS Study Group consists of three expert working groups, the General Toxicology Group, the Reproduction Toxicology Group, and the Carcinogenicity Group. These three working groups correlate to three major chapters of the "Blue Books." Each expert working group has worked independently and/or collaborated together. They have collected and organized the reference data from the members of their specialty area, which has been represented at the annual meeting of the study group. The Carcinogenicity group, in which the group leader is Dr. Hijiri Iwata, has held a satellite meeting on the basic data on CD(SD)IGS rats for carcinogenicity study, such as survival rate, tumor incidence, food restriction, and tumor histopathology at the Annual Meeting of the Japanese Society of Toxicologic Pathology. Moreover, each expert working group has had Editorial Board for publishing any reference data in the "Blue Books."

The last board of the CD(SD)IGS Study Group has been organized in 2002, and we felt "the time is coming." Accumulation of the biological reference data on CD(SD)IGS rats has reached a plateau and it is difficult to collect new data for publishing in the "Blue Books." We decided that the CD(SD)IGS Study Group should finish its role, after the last publication of the "Blue Book" and the last Annual Meeting in the end of 2003. In the future, however, it may be necessary to confirm newly-collected biological reference data on CD(SD)IGS rats after the Forward Migration.

Grateful acknowledgement is made to Dr. Hiroyuki Inoue, the former Chairman, Dr. Toshiaki Matsuzawa, the former Editor-in-chief, and all steering and editing members of the CD(SD)IGS Study Group for their supervision. We thank Messrs. Goro Shimaya, Yoshifumi Chazono (former secretariat), and Yasuyuki Ii, and Mrs. Eiko Hattori, the Secretariats and/or Editorial Secretariats, for their excellent administration work. And I wish to express my thanks to Dr. Kohichi Kojima, the Vice-Chairman, and Dr. Yasuyuki Maeda, the Editor-in-chief, for their support in the CD(SD)IGS Study Group.

In the summer of 2003
Kazumoto Shibuya, D.V.M., Ph.D., Chairman

PREFACE

CD(SD)IGS Study Group was founded in 1997 with a mission to elucidate the characteristics of the IGS rat. To accomplish this end, the Study Group set a goral to publish reference data book on the IGS rat.

Through the tireless efforts of the editors beginning with Dr. Hiroyuki Inoue, the first chairman and Dr. Toshiaki Matsuzawa, the first editor in chief, the first reference data book was published in 1998 and went through several editions, four in total by 2001. I would like to express my gratitude to all members for their invaluable contributions.

Though continuous time effort was taken in 2002, the 5th edition of the data book had not been issued due to various unforeseen circumstances. As a concerned editor, I sincerely apologize to the members.

Finally, we can introduce the 5th edition of 2003 data book to you. I would like to express my profound gratitude to the members for their contribution of numerous data. This invaluable data book on the IGS rat will be issued for the first time in 2 years. As an editor, it is my fondest pleasure that it will be widely utilized by researchers in the years to come.

The Study Group has been quite active for six years now since its foundation. Now our task is complete. I would like to express my profound gratitude to all concerned in completing this final edition, especially Dr. Matsuzawa, the bureau and editors for their guidance down the long, hard road that got us to our final destination.

This data book is a unique compilation of collected papers on the IGS rat. I hope you utilize these 5 data books well, wisely and often.

In the summer of 2003
Yasuyuki Maeda, D.V.M., Ph.D. (Editor-in-Chief)

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Additionally, we received the benefit of the labors of secretaries and support staff of Charles River Japan, Inc., Mr. G. Shimaya, Mr. Y. Ii, and Ms. E. Hattori. We again express our sincere appreciation for this service.

Regarding the publication of this work, we are happy to acknowledge the tireless efforts of Mr. Y. Tsudome and other staff of Best Printing, Co., Ltd., thanks to whose support this volume was able to progress smoothly throughout the edition process.

The present publication is the last edition of Biological Reference Data on CD(SD)IGS Rats. We acknowledge that this collection is able to exist wholly due to the cooperation of the various organizations to which the study group's members and contributors are affiliated.

Finally, we express our hopes for the continued good fortune and health of our readership.

This volume is delivered into your hands with the fervent wish that it may prove of service.

Yasuyuki Maeda, D.V.M., Ph.D. and Kazumoto Shibuya, D.V.M., Ph.D.

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CHAPTER 1

Introduction

Gender-specific factors in the differential responsiveness of rats to chemicals and stress

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ABSTRACT. The rat has inherent gender differences including: morphology, function and physiology of genital, accessory sex organs and many others. A literature survey was conducted to review and investigate gender differences in the responses of rats used in numerous types of research including metabolism, carcinogenicity, behavioral, and pharmacokinetic studies of drugs and chemicals and the effects of various stresses. Gender differences probably under the control of growth and gonadal hormones and cytochrome P-450 were evident under various conditions. The elucidation of such mechanisms will be required to evaluate gender differences in rats to make meaningful interpretations of safety data. Sex differences should be always be expected and evaluated in any work using the CD(SD®)IGS® rats. — Key words: Gender (sex) differences, rat, male, female

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INTRODUCTION

Strain differences in rats were reviewed in the 2000 annual report of the IGS Rat Study Group [85]. Gender-specific factors of rats in the response to the administration of various drugs and chemicals are discussed in this review. It is well known that the rat exhibits gender differences in the response to treatment with the various pharmaceutical agents. In "Casarett & Doull's Toxicology [68]", the bible for diplomats of toxicology in Japan and U.S.A., only scattered descriptions on gender differences in individual fields can be found, with no section devoted specifically to gender differences. Gender differences in CD(SD)IGS rats are not discussed in this review. Please refer the previous and present publications for historical data on body weight, mortality, etc. concerning gender differences in CD(SD)IGS rats. This review provides numerous examples of gender differences in various strains of rats that have been reported in the literature.

Drug research involving both humans and animals has focused predominately on the study of males [19, 80]. However, it is now apparent that women and men differ on several characteristics of drug addiction. In general, more men than women abuse alcohol and drugs (except cigarettes, tranquilizers, and prescription medications) [25, 33, 36, 62, 73, 78].

During the past three decades, the following facts became widely recognized from reports on gender difference in LD_{50} values and sleeping time in rats. 1) A marked difference between male and female rats in the pharmacologic and toxicologic response to a number of xenobiotics has been noted. For example, female rats sleep considerably longer than male rats when treated with equivalent doses of phenobarbital. 2).Similarly, the widely used organophosphate insecticide parathion is approximately twice as toxic to female rats as to males. These potentiated responses in female rats result from a reduced capacity of their livers to biotransform these as well as other chemicals. In these cases the parent compound has a prolonged biologic half-life in females that leads to a prolonged response. If a metabolite or reactive intermediate produces the biologic response, then male rats will usually show the greater response. This is the reason male rats are more susceptible to hepatic injury produced by carbon tetrachloride, as describe later and halothane. Male rats convert these chemicals to reactive intermediates at faster rates than female rats. Kato and Yamazoe 1992 [64] have reported that sex-related differences in toxicity of various drugs and chemicals in rats, in most cases, were closely connected with the sex-related differences in hepatic drug metabolisms. Their studies indicate the existence of sex-specific cytochrome P450, such as P450-male (2C11) and P450-female (2C12) and P450e0 (3A2) in rat livers, and also show that their expression levels were markedly different between male and female rats The expressions of sex-specific P450s are regulated by growth hormone, thyroid hormone, sex hormones and other chemicals.

F344 rats have been shown to exhibit gender differences in the incidence of spontaneous tumors in the liver, pituitary, adrenals, hematopoetic system, mammary glands and lungs, Tables 1 and 2 [45]. Also, nephroma due to the effects of urinary globulin has been reported in male rats [105]. Epithelial thymoma occurs more frequently in W/Hng female rats and BUF/Mna male rats. Is it possible that this can be classified as a gender difference?

In this review, gender-specific factors of rats in responses to exposure to medications, chemicals, environmental contaminants and toxins are discussed in terms of pharmacokinetics and hormonally mediated changes. In addition, the relationship between stress load and gender differences is discussed. This article was partially presented in the educational forum of the Japanese Society of Laboratory Animals (March, 2003, Tokyo).

LITERATURE REVIEW

1. Biotransformation, metabolism and xenobiotics

The notion that gender-related differences exist in the regulation of metabolism is not new, but the precise nature of the interrelations of sex and metabolism is not understood completely. Considerable information suggests there are differences in intermediary metabolism between males and females.

The balance between male and female sex hormones is important in determining the activity of cytochrome P-450 enzymes. Administration of testosterone to female rats increases their ability to biotransform a number of drugs and other xenobiotics. Following treatment, rates of biotransformation in female rats approach the activity observed in their male

counterparts. Similarly, castration of male rats reduces their capacity to biotransform xenobiotics. Measurements of cytochrome P-450 concentration and NADPH-cytochrome P-450 reductase activity in hepatic microsomes have shown preparations from male rats to contain 20 to 30 percent more of these components (Table 3). It is now established that the differences between the sexes reflect differences in the profile of cytochrome P-450 isozymes in liver microsomes from male and female rats. Both male-specific and female-specific forms of cytochrome P-450 have been identified.

Sex-related differences in mammalian metabolism and pharmacokinetics have been known for the last few decades [91, 125]. In particular, there are major differences in the metabolic potential in rats, which often result from differences in either the amount of enzyme(s) and/or the level of expression of individual members of the cytochrome P-450 (CYP) family of enzymes. For example, male rats express CYP2C11, CYP2C13, and CYP3A2 whereas females express CYP2C12 [125]. These enzymes are important to physiological function and homeostasis in the rat because they catalyze the oxidation of many steroids, xenobiotics, and drugs[120]. Because male and female rats have different levels of expression of these key enzymes, the net result is a significant difference in both the qualitative and quantitative processing of endogenous and xenobiotic agents. Individual example will be described as follows.

Benz[a]anthracene (BA) : Boyle and Craft 2000 [15] have reported sex-specific metabolism of benz[a]anthracene in hepatic microsomes from rats. The cytochrome P450 isoforms responsible for the regio-selective metabolism of BA are poorly defined but as with other polycyclic arornatic hydrocarbons (PAHs) may include members of the CYP2C subfamily. Since the expression of some of these is regulated in a gender-specific manner and may be altered by age or rat strain, the effects of these variables on metabolism of BA to diols were investigated. These studies used hepatic, microsomal membranes from immature and adult Long-Evans rats and adult Hooded Lister rats. The results suggest that CYP2C11 along with a male-specific isoenzyme not regulated by age are important in the formation of BA-10, 11-diol and a component(s) of peak (3/8) in males. CYPs 2B2 and/or 2C6 appear to be involved in formation of BA-5, 6-diol in male and female. Identification of the CYPs involved in the regio-selective metabolisrn of BA may lead to an explanation of the lower carcinogenic potency of this PAH compared to dimethylbenz [a]anthracene and this study provides novel clues concerning the identities of the CYPs, which are important.

cDNAs for two CYP3A genes and growth formone: The cDNAs for two CYP3A genes were isolated from the livers of rats using a reverse transcriptase-polymerase chain reaction (RT-PCR) approach with CYP3A subfamily-specific primers [106]. Sequence analysis revealed these cDNAs to be identical to CYP3A9, which had previously been isolated by them from rat brain and nasal epitheliuln and the recently described CYP3A18. The hepatic expression of both genes was sexually dimorphic. Thus CYP3A18 mRNA levels were 25-fold higher in male livers compared to females, while CYP3A9 showed a reverse pattern with 6-fold higher expression in the liver of females. Exposure of male rats to the female pattern of growth hormone secretion led to an increase in hepatic CYP3A9 mRNA expression and suppressed expression of CYP3A18. These findings indicate that the CYP3A subfamily in rats has both male- and female-specific isoforms which are regulated by growth hormone in a manner similar to some other sexually dimorphic cytochrome P450s.

Growth hormone release: Daily fluctuations have been observed in the hepatic cytochrome P450 monooxygenase activities of rodents. The daily fluctuation of 7-alkoxycoumarin *O*-dealkylase (ACD) activities in female rats was examined by Furukawa *et al*., 1999 [43]. The results indicated the absence of any apparent daily fluctuation in the hepatic ACD activities of the females, so the study confirmed a sex difference in the daily rhythm of the ACD activities. Hypophysectomized (Hpx) rats were investigated whether this sex difference in daily rhythm was attributable to growth hormone (GH) release, an activity which is known to affect the sex difference in the expression of P450 isoforms. It was found that the Hpx rats, given subcutaneous injections of GH twice during the light period to mimic a male pattern of GH secretion, showed obvious light-dark fluctuation in ACD activities with high values in the dark period, and similar results were obtained in sham-operated males. Conversely, the Hpx rats given continuous infusion of GH to mimic a female pattern of GH secretion did not show the light- dark fluctuation in ACD activities, and similar results were obtained in sham-operated females. In conclusion, there is a sex difference in the daily rhythm of the hepatic P450 activities in rats and this difference is apparently influenced by the difference in the patterns of GH secretions.

Cocaine metabolism: Bowman *et al*., 1999 [14] studied whether sex differences in metabolism of cocaine (COC) exist that could contribute to the greater behavioral sensitivity of females to COC administration. To investigate this question, concentrations of COC and its two principle metabolites benzoylecgonine (BE) and ecgonine methyl ester (EME) were measured by gas chromatography/mass spectroscopy in brain and plasma collected from male and female rats that were sacrificed between 5 and 90 mm after injection COC (15 mg/kg i.p.). COC concentrations did not differ in plasma or brain tissue of males and females, but sex-specific patterns of metabolite distribution were detected. BE was 2-fold higher in plasma and brain of males than females, whereas EME was much higher in brain and plasma of females.

Hepatic transport of palmitate: Palmitate is probably the most widely distributed Berberis alkaloid. Hepatic clearance of long-chain fatty acids is substantially faster in females than in males, a fact that may underlie known gender-related differences in lipoprotein metabolism and associated disease states. To further investigate the transport steps responsible for this difference, Luxon *et al*., 1998 [81] used a novel method combining multiple-indicator dilution and steady-state measurements of palmitate extraction from albumin solutions. They found that cytoplasmic transport of palmitate is sufficiently slow (diffusion constants 9.0 and 5.9 x 10^{-9} cm^2/s for male and female liver, respectively) that the steady-state concentration of palmitate

in the center of the cell should be ~ 0.5 of that found in the cytoplasm just beneath the plasma membrane. Female liver had a greater influx rate constant and a larger vascular volume than male liver but had a similar rate of metabolism. Rapid cytoplasmic diffusion enhances movement of palmitate into deeper layers of the cell cytoplasm, thus reducing efflux. The larger sinusoidal volume in females not only permits more dissociation of palmitate from albumin within the sinusoids but also may generate a greater permeability-surface area product. These multiple sex-related differences combine to produce a nearly 2-fold greater steady-state uptake rate by female liver.

Phencyclidine : Shelnutt *et al*., 1999 [110] determined the differences in phencyclidine (PCP) *in vitro* metabolism and pharmacokinetics in female and male Sprague-Dawley (SD) rats. Formation rates of five major PCP metabolites in liver microsomes were significantly higher ($p < 0.05$) in males compared with females. The formation rate for an irreversibly bound PCP metabolite in males was the second highest of the six metabolites measured. The liver microsomes from the females produced essentially no metabolite binding. The PCP's pharmacokinetic profile was determined in female SD rats after administration of a pharmacologically active i.v. dose of PCP (1 mg/kg) and then these data were compared with the pharmacokinetic profile in male SD rats. The value for PCP systemic (and nonrenal) clearance was more than 45% lower ($p < .05$) in female rats. In addition, the terminal elimination $T_{1/2}$ was significantly longer ($p < .05$) in the female rats (5.5 versus 3.4 h, respectively). Because the initial serum concentration, volume of distribution at steady state, and renal clearance were not significantly different between the sexes, the longer half-life was attributed directly to a decreased ability of females to metabolize the drug. Consequently, these pharmacokinetic data suggest pharmacological differences in PCP effects between female and male rats are due primarily to a decreased ability of female rats to metabolize the drug.

Antimalarial compound artemisinin : The pharmacokinetics of artemisinin were compared in the male and female SD rat after single dose i.v. (20 mg x kg ($^{-1}$) or i.p. (50 mg x kg ($^{-1}$) administration of an emulsion formulation [2]. Plasma clearance of artemisinin was 12.0 (95 % confidence interval: 10.4, 13.0) $l.h^{-1}x\ kg^{-1}$ in the male rat and l0.6 (95% CI: 7.5, 15.0) $l.h^{-1}x\ kg^{-1}$ in the female rat suggesting high hepatic extraction in combination with erythrocyte uptake or clearance. Artemisinin half-life was ~ 0.5 h after both routes of administration in both sexes. Values for plasma clearance and half-lives did not statistically differ between the sexes. After i.p. administration artemisinin AUC_S were 2-fold higher in the female compared with male rat ($p<0.001$).

Digitoxin : The cardiac glycoside digitoxin has been used clinically in the treatment of congestive heart failure. In rats, cytochrome P450 3A has been shown to be an important determinant of digitoxin toxicity [34] CYP3A cleaves the two sugar residues of digitoxin to give digitoxigenin-bisdigitoxide and digitoxigenin-monodigitoxide, the latter being conjugated by UDP-g ucuronosyltransferase [49]. Induction of CYP3A by treatment of rats with pregnenolone-16α-carbonitrile and dexamethasone decreased digitoxin toxicity [34]. In addition, the constitutive levels of CYP3A (measured as testosterone 6 β-hydroxylase activity) are greater in male than in female rats, which explains the higher toxicity of digitoxin in female (LD50: 8.9 mg/kg, i.v.) versus male (LD_{50}: 15.4mg/kg i.v.) rats [109].

Acetohexamide an antidiabetic : A significant sex difference was observed by Imamura *et al*., 1999 [55] for the pharmacokinetics of acetohexamide in Wistar-Imamichi (Wistar-IM) rats. However, there was no sex difference of the *in vitro* reductive metabolism of acetohexamide in the liver or kidney of these rats. Testectomy was found to decrease the plasma clearance (*CL*p) of acetohexamide in male rats, whereas ovariectomy had no effect on the *CL*p of acetohexamide in female animals, suggesting that androgens regulate the pharmacokinetics of acetohexamide. The co-administration of sulfamethazine, which is known to be metabolized by a male-specific cytochrome P450 (GYP) isoform (CYP2C11), significantly decreased the *CL*p of acetohexamide in male Wistar-IM rats. Based on these results, it is reasonable to assume that the sex-dependent pharmacokinetics of acetohexamide observed in Wistar-IM rats is associated with the male-specific hydroxylation catalyzed by CYP2C11.

Mexazolam : Mexazolam is metabolised by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors. To identify an appropriate animal model for the study of drug interaction via CYP3A4 inhibition, the inhibition of *in vitro* mexazolam metabolism by various HMG-CoA reductase inhibitors, simvastatin acid, fluvastatin, atorvastatin, cerivastatin and pravastatin in male and female rat liver microsomes was investigated by Ishigami *et al*., 2002 [56]. The metabolism of mexazolam in male and female rat liver microsomes was inhibited by all the HMG-CoA reductase inhibitors examined except pravastatin (acid). The K(i) values in female rats were lower than those in male rats, demonstrating the presence of a sex difference in the inhibition potency of HMG-CoA reductase inhibitors toward mexazolam. Using anti-cytochrome P450 (P450) antisera, the main P450 isozyme responsible for the metabolism of mexazolam was identified as CYP3A in female rats and CYP2C11 in male rats. Based on these results, it has been speculated that the sex difference in the inhibition potency of HMG-CoA reductase inhibitors for mexazolam observed in rats is caused by their different inhibition potencies against CYP2C11 and CYP3A isoforms. For mexazolam metabolism, the results obtained in female rats, rather than those in male rats, seem to be a much better reflection of the results in humans.

Simvastatin a HMG-CoA reductase inhibitor and itraconazole a orally active antimycotic: Ishigami *et al*., 2001 [56] examined the interaction between simvastatin and itraconazole observed in humans and in rats for pharmacokinetic studies. Itraconazole inhibited the *in vitro* metabolism of simvastatin in female rat liver microsomes, but not in male rat liver microsomes. Using anti-P450 antisera, the main P450 isozyme responsible for the metabolism of simvastatin was identified as CYP3A in female rats and CYP2C11 in male rats. Therefore, the sex difference in the inhibition of simvastatin metabolism by itraconazole seems to be caused by a difference in the P450 isozymes responsible for the metabolism of simvastatin in male and female rats and the different ability of itraconazole to inhibit CYP3A and

CYP2C11. The area under the curve value of simvastatin was increased approximately 1.6-fold by the concomitant use of itraconazole (50 mg/kg) in female rats, whereas in male rats, itraconazole had no effect. In conclusion, it was found that the results obtained in male rats did not reflect the results in humans as far as the inhibition of simvastatin metabolism by itraconazole was concerned. The P450 isozymes involved in the metabolism of drugs should be taken into consideration when rats are used as a model animal for humans in the investigation of drug interactions.

Oracin : Oracin,{6-[2-(2-hydoxyethyl) aminoethyl]-5,11-dioxo-5,6-dihydro-11*H*-indeno [1,2-*c*] disoquinoline} is a potential cytostatic agent for oral use. *In vitro* and *in vivo* experiments to investigate possible stereospecific, aspects of oracin reduction in relation to rat gender have been conducted by Wsól *et al*., 1999 [122]. Incubation of oracin with rat microsomes, cytosol, and hepatocytes in the presence of various coenzymes and under aerobic or anaerobic conditions provided evidence for sex differences in the formation of 11-dihydrooracin (DHO) enantiomers. The greatest sex differences were seen in hepatocytes where females showed higher stereospecificity of the reductases than males. While female biotransformation enzymes preferentially generated approximately 82% of (+)-DHO, male enzymes gave only rise to 63% of (+)-DHO. Males displayed higher stereospecificity than females in the microsomal fraction. However, in the cytosolic fraction females exhibited higher stereospecificity than males. Similarly, in *in vivo* studies, the ratio of (+) - and (−) -DHO in feces and urine gave no indication of the significant differences between the male and female rat.

Tamoxifen: Tamoxifen is widely used in adjuvant therapy for breast cancer. Davis *et al*., 2000 [30] indicated that metabolic activation of tamoxifen in rat liver cells involves cytochrome P450-mediated a-hydroxylation, followed by sulfate ester formation, mediated by hydroxysteroid sulfotransferase a (rHSTa), a member of the SULT2A subfamily, which efficiently metabolizes dehydroepiandrosterone. Because it is known that the expression of rHSTa and other SULT2A forms is substantially higher in female rats than in males, it might be predicted that tamoxifen would be a more potent liver carcinogen in females than in males. Yet tamoxifen has been shown to be equipotent in both sexes. To investigate this paradox, primary cultures of hepatocytes were prepared from Fischer (F344) rats and treated with tamoxifen (10 μ M) or a-hydroxytamoxifen (1 μ M). Rats were also treated with tamoxifen daily by gavage (0.12 mmol/kg/day) for up to 14 days. In tamoxifen-treated hepatocytes and after a single dose of tamoxifen *in vivo*, DNA adduct formation in male cells was significantly lower than in female cells, 11- and 6-fold, respectively. However, with increasing daily doses of rats with tamoxifen, the adduct level in males increased to a level 89% of that in females by 14 days. Dehydroepiandrosterone sulfotransferase activity in male rat liver cytosols was only 17% of the activity of female cytosols after one dose of tamoxifen but 64% after 14 days of exposure to the compound. After prolonged exposure to tamoxifen, DNA adduct formation and rHSTa expression in males are significantly closer to the levels in females than they are after initial exposure. These changes explain the similar susceptibility of male and female rats to tamoxifen carcinogenesis.

Donepezil: Donepezil hydrochlonde (Aricept1®) is used tor the treatment of Alzheimer's disease. The Cl_{int} of donepezil in the male rat and female rat liver microsomes were 33.7 and 13.4 d/min/mg microsomal protein respectively, and sex difference in rat was found by Matsui *et al*., 1999 [84]. After a single intravenous administration to the male rat and female rat, total plasma clearance (Cl^{p}_{total}) was 78.6 and 29.5 ml/min/kg respectively, and a sex difference was observed in rat.

2. Induction of Cytochrome P-450

As an underlying cause of serious adverse effects, P450 induction is generally less important than P450 inhibition, because the latter can cause a rapid and pro-found increase in blood levels of a drug, which can cause toxic-effects and symptoms of drug overdose.

Treatment of rats with pregnenolone-16 α -carbonitrile (PCN) causes an induction of CYP3A1 and CYP3A2, which are two independently regulated CYP3A enzymes with very similar structures and substrate specificities. In contrast to CYP3A1, CYP3A2 is present in liver microsomes from untreated rats, although the levels of this enzyme decline markedly after puberty in female rats. Consequently, CYP3A2 is a male-specific protein in mature rats, and it is inducible in mature male but not mature female rats (whereas CYP3A1 is inducible in mature male and female rats). In addition to PCN, inducers of CYP3A enzymes include steroids (e.g., dexamethasone and spironolactone), macrolide antibiotics (e.g., troleandomycin and erythromycin estolate), and imidazole antifungals (e.g., clotrimazole, ketoconazole, and miconazole).

Treatment of rats with isoniazid causes a 2- to 5-fold induction of CYP2E1. In sexually mature rats, the levels of liver microsomal CYP2E1 are slightly greater in female than in male rats. Treatment of male rats with clofibric acid causes a marked induction (up to 40-fold) of CYP4A1, CYP4A2, and CYP4A3, which are three independently regulated CYP4A enzymes with similar substrate specificities [115]. CYP4A1 is expressed in the liver, whereas CYP4A3 is expressed in the liver and kidney. CYP4A2 is expressed in the liver and kidney of male rats, but it is neither expressed nor inducible in female rats.

In mature rats, the levels of certain P450 enzymes are sexually differentiated; that is, they are higher in either male or female rats. Male-specific enzymes include CYP2A2, CYP2C11, CYP2C13, CYP3A2, and CYP4A2. The only known female-specific P450 enzyme is CYP2C12, although the levels of several other P450 enzymes are greater in female than male rats, including CYP2A1, CYP2C7, and CYP2E1. These gender-related differences in P450 enzyme expression are clue in large part to sex differences in the pattern of secretion of growth hormone, which is pulsatile in male rats and more or less continuous in females (Waxman *et al*., 1991). Treatment of mature male rats with various xenobiotics perturbs the pattern of growth hormone secretion and causes a partial "feminization" of P450 enzyme expression, which includes decreased expression of CYP 2C11. Sex differences in the expression of

P450 enzymes occur to a limited extent in mice, but no marked sex differences in P450 expression have been observed in dogs, monkeys, or humans.

3. Renal toxicity, diuretic, urethral pressure response and urinary calculus formation

Kacew *et al*, 1995 [59] noted much of the following: Acute administration of hydroquinone, the reducing agent used in photographic developer formulations, produced renal dysfunction in F344 but not in SD rats, as evidenced by glycosuria, pronounced enzymuria, and an increased number of urinary epithelial cells [9]. It is of interest that female F344 rats were markedly more sensitive to hydroquinone-induced nephrotoxicity compared to male F344 rats. Surprisingly, in a chronic 2-year study, English *et al*., 1994 [35] found that hydroquinone induced an increased incidence of renal tubule adenomas, enzymuria, and tubular degeneration, measured histopathologically, in male but not female F344 rats. SD rats were also resistant to the acute and chronic nephrotoxic actions of hydroquinone. It is not surprising that F344 rats are more susceptible to chemical-induced nephrotoxicity as a similar pattern or renal toxicity was noted in the case of aminoglycosides [104] and acetaminophen [117]. Although it is difficult to ascertain the basis for the reversal in toxicity in F344 rats from acute nephrotoxicity seen in female versus chronic toxicity seen in males, it should be noted that male F344 rats are more susceptible to chemical-induced intestinal carcinoma [116] and chloroform-induced nephrotoxicity [99]. Because the male F344 rat in general is more sensitive than the female to carcinogens [46] and tumorigenesis is seen in male rats chronically administered hydroquinone [35], it would appear that susceptibility to nephrotoxicity is reversible in the female and that sensitivity increases with age in the male F344 rat. It would be of interest to determine the role of testosterone in hydroquinone-induced nephrotoxicity.

Strain and sex also play an important role in hyalin droplet nephropathy. Administration of decalin to male F344, SD, Buffalo, or BN rats increased a2u-globulin content associated with hyalin droplet formation [105]. In contrast, female F344, SD, Buffalo, and BN rats showed no evidence of hyaline droplet formation or accumulation of α 2u-globulin [79], as shown in Fig. 1.

It is of interest that both male and female NC1-Black-Reiter (NBR) rats resembled female responsiveness, as there was no hyalin droplet nephropathy [105]. Clearly, the decalin-induced hyalin droplet nephropathy is strain-related, but the role of male hormones is difficult to decipher due to the lack of response noted in NBR male rats. However, there are marked differences in endogenous circulating testosterone levels among strains [24], and this may account for the differences between NBR and other strains. It is also conceivable that NBR male rats, unlike SD or F344, lack the gene necessary to synthesize α 2u-globulin.

A great number of eosinophilic bodies (large homogenous HE-proofreaded granule) are normally present in the proximal renal tubular epithelium of untreated male rats. Deposition of α2 microglobulin on eosinophilic bodies is now a well known phenomenon. Deposition of minerals in the kidneys occurs spontaneously in aged rats, especially in animals with nephropathy and/or hyperplasia of parathyroids, and occurs more markedly in males.

Azosemide: Azosemide [5-(4-chloro-5-sulphamoyl-2-thenyl-aminophenyl)-tetrazole] is a loop diuretic closely resembling furosemide in its diuretic action. Gender differences in pharmacokinetics and pharmacodynamics of azosemide were evaluated by Lee *et al*., 1999 [75] after intravenous, 10 mg kg^{-1}, and oral, 10 mg kg^{-1}, administration to male and female rats. After intravenous administration to male rats, the percentages of intravenous dose of azosemide recovered from entire gastrointestinal tract at 24 h (13.2 versus 3.93%) was significantly greater than those in female rats. In male rats, the nonrenal clearance of azosemide tended ($p < 0.066$) to be faster and kidney weight tended ($p < 0.068$) to be greater than those in female rats. After oral administration of azosemide to male rats, the 8-h urinary excretion of potassium (0.395 versus 0.766 mmol g^{-1} kidney) and 8-h kaluretic efficiency (55.9 versus 284 mmol mg^{-1}) decreased significantly compared with female rats.

Urethral pressure response: Kontani and Shiraoya 2000 [72] studied pharmacology of transmitters mediated by the response, and the characteristics of the hypogastric nerve (HGN) of female rats. Electrical stimulation of the HGN was found to unexpectedly reduce urethral pressure in female rats. Male and female Wistar rats, 10 weeks and 6 months old, respectively, were used under anesthesia. Fluid was infused from the bladder neck into the urethral lumen at a constant rate (0.5 ml/10 minutes), and infusion pressure signals were measured. Bilateral HGNs were electrically stimulated at 5 and 10 Hz for 30s. Electrical stimulation of the HGN reduced urethral infusion pressure in about 80% of the female rats, and the introduction of N^{ω}-nitro-L-arginine methylester (L-NAME, 30 mg/rat, i.v.) elevated the urethral pressure response from a reduced state. Prazosin α_1-adrenargic antagonist (0.1 mg/kg., i.v.) and hexamethonium (10 mg/kg., i.v.), which inhibited elevation of urethral pressure in male rats, also reversed and inhibited the elevation of urethral pressure in the female rats treated with L-NAME. The HGN in female rats contained nerve endings that released nitric oxide (NO) and norepinephrine (NE). NO released during HGN stimulation inhibited the release of (NE) and reduced urethral infusion pressure in female rats. Nerves with synapses in the pelvic ganglia released NE in both male and female rats, but nerves that released NO did not have synapses in the ganglia. Only NE was released from the HGN nerve endings in male rats.

Bisphenyl: Ohnishi *et al*., 2000 [97] reported about bisphenyl. Bisphenyl is commonly used as a synthetic resin, as a heat transfer medium in manufacturing, and as a fungistatic agent for preserving fresh citrus fruits. To obtain definitive information about the mechanisms of urinary calculus formation and the structural characteristics of the calculi induced by biphenyl administration in rats, with a focus on the sex dependency, the constituents of the urinary calculi were analyzed by HPLC, inductively coupled plasma spectroscopy (ICP), micro Fourier transform infrared spectroscopy (mFT-IR), and ion chromatography (IC), and structural analyses were carried out by microscopy, mFT-IR, and the electron probe microanalyzer (EPMA) method. The methodologies were used to attempt to

account for the appreciably higher incidence of calculi in males than in females. mFT-IR analysis revealed that the biphenyl-induced urinary calculi in male rats are composed mainly of potassium 4-hydroxybiphenyl-o-sulfate (4-HBPOSK), whereas the calculi in female rats are composed mainly of 4-hydroxybiphenyl (4-HBP) and $KHSO_4$ produced by the hydrolysis of 4-HBPOSK. Observations of photomicrographs and the results of mFT-IR analysis indicated that the calculi in males have a multilayer structure consisting of alternating layers of 4-HBPOSK and calcium phosphate, whereas the calculi in females have no multilayer structure, but open holes in which needle-shaped crystals are present in some places.

4. Endocrine disruptors, Dioxin (2,3,7,8-TCDD) and agrochemicals (pesticide, herbicide)

Toxicologists know the difficulties of extrapolating results from rats and mice to humans, which for a general biologist are quite closely related species. In ecotoxicology, where only a few representative species can be tested, the problem of extrapolation to other species and taxonomic categories in the environment appears even more difficult than in toxicology. Nevertheless there is a certain need and a great deal of political pressure for such extrapolations aimed at establishing, for example, safety standards or quality criteria covering all biota, even if the data base is small, such as it is for many "industrial chemicals. Pesticides have a comparatively large environmental data base, therefore data will be presented from a few widely used pesticides.

According to the information of the Japanese Ministry of Health, Labor and Welfare [URL: http://www.env.go.jp/chemi/dioxin/kento/hr_r_02.html], gender differences exist in acute toxicity of dioxin and females tend to be more sensitive to this toxicity than do males. Based on the results of chronic studies, females tend to be more sensitive to lethal and hepatic toxicities than males. In a carcinogenicity study of 2, 3, 7, 8-TCDD in rats [70], the incidence of tumors of the pituitary, uterus, mammary glands, spleen and adrenals decreased and the incidence in the liver tumors increased in females.

An observed increase in late hemorrhagic disease (LHD) in breast fed neonates gave rise to the hypothesis that polychlorinated biphenyl (PCB) and dioxin, P450-inducing contaminants present in human milk, might effect vitamin K-dependent blood coagulation. This hypothesis was studied by Bouwman *et al*., 1999 [13] in rats. Administration of a single oral dose of 0.003 to 30 nmol, 7, 8-tetrachlorodibenzo -p-dioxin (TCDD) /kg bw or 0.75 to 500μmol 2,2',4,4',5,5' -hexachlorobiphenyl/kg bw (HxCB) to female and male rats resulted in dose-related reductions of the vitamin K-dependent coagulation factor VII. The highest factor VII reduction in female rats was 44%, observed after TCDD exposure. The Lowest Observed Adverse Effect Level (LOAEL) of TCDD on female factor VII levels was 0.3 nmo1/kg bw (96 ng/kg). There was a significant inverse correlation between factor VII levels and induction of hepatic ethoxyresorufin *O*-deethylating (EROD) activity, reflecting CYP1A1, and total P450 content. HxCB had no effect on female coagulation factors. In male rats only exposure to HxCB, which induces mainly CYP2B1 and 2D2, decreased both coagulation factors dramatically up to 88%. The LOAEL of HxCB on factor VII in male rats was 100 μmol/kg bw (36 mg/kg). Effects on coagulation factors in male rats exceeded those in females. Sex-dependent differences of TCDD and HxCB were observed on the hepatic vitamin K cycle enzyme activities in female and male rats. Vitamin K-dependent γ -glutamyl carboxylase activity was mainly induced in female rats; 2.3-fold in the highest dose group of TCDD. In male rats only vitamin K 2, 3-epoxide reductase (KO-reductase) activity was induced 1.7-fold by the highest dose of HxCB. KO-reductase activity in female rats was also increased by TCDD, however, less pronounced than the carboxylase activity.

Dichlorodiphenyltrichloroethane (DDT): DDT is an organochlorine pesticide still widely used in various countries for the control of malaria and other vector-transmitted diseases. DDT is a well-known inducer of microsomal monooxygenase systems in rodent liver. Sierra-Santoyo *et al*., 2000 [111] tested sex different effects of rats for CYPs after DDT administration. Single doses of DDT were gavage to Wistar rats (Table 4). The highest dose induced 18-fold the expression of CYP3A2 in female rats without producing significant induction (< 3-fold) in males. The effects on this isozyme, which is not normally expressed in females, suggest that DDT is able to modulate sexual metabolic dimorphism, as 3A2 expression is androgen dependent. DDT did not significantly alter CYP3A1 in males, suggesting that DDT is not a pure phenobarbital (PB) - type inducer. The effects on CYP2B1/2B2 protein (19-fold) and associated enzyme activities indicated that males had a lower response threshold than females, but that the latter were able to reach a higher relative induction. The preferential induction of CYPs 2B and 3A by DDT in a sex-related manner suggest that CYP regulation could play an important role in endocrine disruption.

2, 4-Dichlorophenoxyacetic acid (2, 4-D): 2, 4-D, a widely used broadleaf herbicide, is under investigation in a study of peroxisome proliferators. Laboratory animals were dosed with ^{14}C-2, 4-D orally at 5 and 200 mg/kg and tissue distributions were determined by Griffin *et al*., 1997 [47]. At early time point's tissues from female rats consistently contained higher amounts of radioactivity than did corresponding tissues from males (up to 9 times). Plasma elimination curves were generated in male and female rats after i.v. and oral administration. Kinetic analysis revealed significant differences in elimination and exposure parameters consistent with a greater ability to clear 2, 4-D by male rats relative to females. This suggests that at equivalent doses, female rats are exposed to higher concentrations of 2, 4-D for a longer time than males and may be more susceptible to 2, 4-D-induced toxicity.

5. Cholinergic analgesia

Male and female rats with indwelling intrathecal catheters received injections of neostigmine, bethanechol (muscarinic agonist), or RJR-2403 (neuronal nicotinic agonist) alone or with atropine (muscarinic antagonist), mecamylamine (nicotinic antagonist), or phentolamine (α -adrenergic antagonist) with antinociception determined to a noxious heat stimulus to the hind paw. Time versus subcutaneous paw temperature

relationships were defined for males and females [22]. Neostigmine produced dose-dependent antinociception with five time's greater potency in female than in male rats. Neostigmine-induced antinociception was reversed in male rats by atropine and unaffected by mecamylamine, whereas it was partially reduced by each antagonist alone in females and completely reversed after injection of both. RJR-2403 was more potent in females than in males, whereas there was no sex difference to bethanechol. Phentolamine partially reversed antinociception from RJR-2403 in females. Paw temperature increased more rapidly in females than in males for the same lamp intensity. These data demonstrate a large sex difference in antinociception to intrathecal neostigmine that is primarily the result of a nicotinic component in females. Phentolamine reversal suggests that part of this nicotinic component may rely on spinal norepinephrine release. A better understanding of this sex difference could lead to development of novel paintherapy for women.

Also, male and female rats with indwelling intrathecal catheters received injections of neostigmine, bethanechol (muscarinic agonist), or RJR-2403 (neuronal nicotinic agonist) alone or with atropine (muscarinic antagonist), mecamylamine (nicotinic antagonist), or phentolamine (α-adrenergic antagonist). The effect of these agents was determined on mechanical allodynia produced by either intraplantar injection of capsaicin or ligation of spinal nervers [74]. Neostigmine and RJR-2403 but not bethanechol were more potent in female than in male rats in reducing allodynia after nerve injury, and antaginist studies were also consistent with a nicotinic component to explain ths sex difference. Phentolamine did not reverse neostigmine's effect. In contrast, for capsaicin-induced allodynia, neostigmine plus phentolamine but not neostigmine or RJR-2403 was more potent in female than in male rats. These data demonstrate a sex difference to intrathecal neostigmine after nerve injury-induced allodynia similar to that observed in normal animals that received acute noxious thermal stimulation.

6. Morphine

Morphine tolerance and naloxone a specific opioid antagonist: Several investigators have shown that male rodents are more sensitive than females to morphine's antinociceptive effects : The study on morphine's antinociceptive effects was conducted by Craft *et al*., 1999 [27] to determine whether this sex difference is stable after chronic morphine treatment. Acutely administered morphine produced significantly greater hotplate and tail withdrawal antinociception in males than in females. In contrast, there was no sex differences in morphine's hotplate or tail withdrawal effects under repeated (1-week interval) dosing conditions. In a separate group of rats, after 2 weeks of twice-daily morphine treatment (10-20 mg/kg per injection), the ED_{50} for morphine's antinociceptive effects increased approximately 6.9-fold in males versus only 3.7-fold in females; chronic morphine treatment also disrupted the estrous cycle of females. In a separate group of rats treated with 10 mg/kg morphine twice daily for 5 days, treatment with naloxone (1.0 mg/kg) on day 6 produced greater withdrawal scores in males than in females. These experiments demonstrate sex differences in development of tolerances to and dependence on morphine in the rat.

Cardiovascular responses: Putative gender differences in opiate cardiovascular effects were evaluated by Cruz and Rodriguez-Manzo 2000 [28] in spinal rats. After a 4-h exposure to a single dose of morphine (30 mg/kg, i.v.), abstinence was precipitated by naloxone (0.03 - 3 mg/kg, i.v.). Morphine produced a long-lasting bradycardia and a transient increase in arterial pressure that was similar in both genders. Thereafter, blood pressure decreased both in males and females. Naloxone precipitated a similar dose- dependent heart rate increase in both sexes and a gender-dependent increase in blood pressure. This sex difference appeared in the shape of the response. Prazosin α_1-adrenargic antagonist (0.2 mg/kg), prior to naloxone, reduced the pressor response in all animals, suggesting a similar participation of the noradrenergic system in both genders. The present results extend to acute dependence the notion of a sex-dependent differential effect of morphine. The need to consider gender as a factor when studying the effects of opioids is highlighted.

Morphine antinociception: Mogil *et al*., 2000 [88] examined whether the observation of statistically, significant sex differences in nociception and morphine antinociception might depend on the particular outbred rodent population chosen for study. Rats of both sexes and three common outbred strains were obtained from three suppliers (Long Evans, Simonsen; SD, Harlan; Wistar Kyoto, Taconic) and tested for nociceptive sensitivity on the 49 ℃ tail-withdrawal assay, and antinociception following morphine (1-10 mg/kg, i.p.). These data are discussed with respect to the existing sex difference and pain literature, and also as they pertain to future investigations of these phenomena (Table 5).

Morphine and N-methyl-D-aspartate receptor antagonist dizocilpine (MK-801): Rats show gender differences in responses to morphine and the N-methyl-D-aspartate receptor antagonist dizocilpine (MK-801); the role of sex steroids in mediating these differences is unclear. D'Souza *et al*., 2002 [32] examined the overall hypothesis that circulating gonadal steroids determine the gender differences in morphine- and MK-801-induced behavior and c-Fos expression. Morphine caused a greater expression of c-Fos in the striatum of intact males than that of females, which was independent of sex steroids. MK-801 completely inhibited morphine-induced c-Fos in intact females but only caused partial inhibition in intact males; castrated males showed complete inhibition, which was reversed by testosterone, but gonadal steroids had no effect on this response in females. In thalamus, there was a large sex difference in the response to MK-801 that was independent of gonadal steroids. Behavioral responses to morphine were greater in males, but responses to MK-801 were greater in females; both were sex steroid independent. These findings show significant sex differences in response to morphine and MK-801 that are mediated by sex steroid-dependent and -independent mechanisms, which may be important in treatment outcomes of drug addiction.

7. Cocaine

Cocaine injections: The putative neurotransmitter peptide cocaine- and amphetamine-regulated transcript (CART) has been suggested to be involved in the actions of psychostimulants. Fagergren and Hurd 1999 [37] analyzed the CART mRNA expression in mesolimbic brain areas of male and ovariectomized 17 β -estradiol- (30 μ g) and vehicle-treated female rats. A gender difference was noted in the accumbens shell during basal conditions; male rats expressed higher levels of CART mRNA than both female groups. Following binge cocaine injections (3 x 15mg/kg), elevated levels were found in the central amygdala of male but not female rats. In the medial accumbens shell CART mRNA was elevated after cocaine, but only in the non-treated females.

Self-administered cocaine and heroin: Lynch and Carroll 1999 [82] examined that acquisition of i.v. cocaine and heroin self-administration in Wistar rats. An autoshaping procedure was used to train rats to press a lever that resulted in either a 0.2 mg/kg infusion of cocaine or a 0.015 mg/kg infusion of heroin under a fixed-ratio 1 (FR 1) schedule. Daily sessions consisted of six 1-h autoshaping components followed by a 6-h self-administration component. During each autoshaping component, a retractable lever briefly (15 s) extended into the test chamber on a random interval schedule with a mean of either 90 s (cocaine groups) or 480 s (heroin groups) and either ten (cocaine groups) or five (heroin groups) computer-automated infusions were delivered each hour. During each 6-h self-administration component, the lever remained extended and each response on the lever resulted in an infusion of either cocaine (0.2 mg/kg) or heroin (0.015 mg/kg). Female rats acquired both cocaine and heroin self-administration more rapidly than males. Acquisition of cocaine self-administration occurred in a greater percentage of female rats compared to males. Female rats self-administered more cocaine than males after acquisition criteria had been met. These findings indicate that female rats were more vulnerable than males to the acquisition of cocaine and heroin self-administration under the conditions of this experiment.

Gestational/Prenatal cocaine exposure: The impact of gestational cocaine in conjunction with postnatal handling on schedule-induced polydipsia (SIP) was examined by Katovic *et al*., 1999 [65]. Rat offspring were derived from SD dams injected subcutaneously with 40 mg/kg/3 cc cocaine hydrochloride (C40) on gestational days 8-20, dams injected with vehicle and pair fed 4 (PF4) days to mimic the acute anorexic effects of cocaine administration, and nontreated (NT) control dams. In adulthood, offspring were food deprived and given 13 daily 30-mm SIP sessions, with water intake recorded during the scheduled (fixed time 60 s-FT60) food delivery. For 4 days thereafter, animals received saline, 5 or 10 mg/kg of cocaine in counterbalanced order prior to SIP testing. Acquisition and maintenance of SIP, but not cocaine-induced suppression of SIP performance, were observed to be dependent upon prenatal treatment, handling, and gender. Females acquired SIP faster and exhibited notably higher levels of polydipsia than mates. Early handling increased levels of established SIP in NT offspring, while enhancing SIP acquisition in both PF4 and C40 offspring. In nonhandled animals, NT offspring exhibited less SIP than PF4 and C40 offspring, differences that were attenuated by early handling.

Markowski *et al*, 2000 [83] conducted a longitudinal analysis of rat operant behavior under two different schedules of reinforcement following prenatal exposure to cocaine. Offspring were derived from four maternal exposure groups: 50 mg/kg cocaine, their pair-fed controls, 25 mg/kg cocaine and freely fed controls. Cocaine was administered via gavage from gestation days 6-20. A maternal fostering procedure was used. Pairs of male and female littermates were assigned to a 7-, 14-, or 21-month cohort and at the appropriate age were trained to respond on one lever in a two-lever operant chamber. Reinforcement was delivered with a series of random ratio (RR) schedules where the RR value was increased across sessions. After RR training, animals were examined with a delayed spatial alternation (DSA) procedure in the same chambers. Male offspring responded at higher rates than females during high-probability RR schedules, whereas advancing age was associated with lower response rates during low-probability RR schedules in both males and females.

8. Fungal and mycotoxin toxins

Fungal toxin fumonisin B_1 (FB_1): FBl is a contaminant of corn-based foods and feeds produced by members of the genus *Fusarium*. FB_1 toxicity was examined by Bondy *et al*., 1998 [11] using gavage administration of purified toxin to female SD rats. Each rat received a single daily dose of FB_1 11 days , at the following concentrations: control (saline), 1 to 75 mg FB_1/kg body weight/day. Significantly depressed body weight and food consumption occurred at 35 and 75 mg FB_1/kg/day. By the end of the dosing period there were no significant changes in food consumption. Compared to previous work [10] with male rats gender-related differences in FB_1 responses lacked consistency but indicated that males may be marginally more sensitive than female SD rats.

Mycotoxin Ochratoxin A (OTA): OTA was shown to be a potent kidney carcinogen in rats demonstrating a marked sex difference in the response. Compared to female rats, male rats had a 10-fold higher incidence of kidney carcinomas. The objective of this study was to investigate whether this sex difference in tumor response was due to an exacerbation of effect resulting from the interaction of the male rat specific urinary protein α2u- globulin (α2u) with OTA [102]. Male and female rats were treated by oral gavage with OTA (1 mg/kg per day), D-limonene (dL; 1650 mg/kg per day) as a positive control or corn oil for 7 consecutive days. OTA induced severe renal lesions predominantly in the P_3 region of the proximal tubules. The lesions consisted of necrotic cells and cell exfoliations. No hyaline droplets were found in the P_2 segment following OTA treatment, whereas dL induced the expected accumulation of droplets. The results suggest that OTA induced kidney lesions are in all characteristic points different from the known α 2u-nephropathy induced by dL (Table 6). Based on these experiments the male rat specific protein α 2u does not seem to be involved in the mechanism(s) leading to the high tumor incidence observed in OTA exposed male rats.

9. Behavior, learning and stress

Nitric oxide (NO) and water maze (WM): In a WM, rats employ different and sexually dimorphic behavioral strategies to solve a place-learning task, a test of cognitive/propositional ability. Kanit *et al*., 2000 [63] assessed possible sex differences in cognitive style before and after puberty in a WM place-learning task. Since nitric oxide (NO) is implicated in spatial learning and hippocampal function and since brain NO^-_2 + NO^-_3 levels (stable metabolites of NO) display region-specific sex differences in rat brain, NO^-_2 + NO^-_3 levels were determined after behavioral testing. The sex-related style difference emerged very clearly but only in the adult rats, which suggests that the female behavioral strategy in the WM place-learning task requires the presence of female sex hormones at puberty. Although NO^-_2 + NO^-_3 levels were higher in the adult rats and males compared to prepubertal and female rats, respectively, no significant correlations emerged between brain NO and behavior.

Naloxone on freezing behavior: Klein, *et al*., 1998 [69] examined the effects of naloxone, an opioid antagonist on freezing behavior in male and female rats following stress and no-stress conditions. Wistar rats were exposed to 10 min of mild, unpredictable footshock stress and to a comparable no-stress condition. Immediately following stress or no-stress conditions, subjects were injected with naloxone or saline, and two independent observers measured freezing behavior. In male rats, naloxone potentiated freezing following stress but had no effect on freezing following no-stress. In females, naloxone did not affect freezing regardless of stress conditions. These results reveal a sex difference in effects of naloxone on freezing behavior and suggest that sex differences may exist with respect to the role of endogenous opioids under stress.

Water maze performance: Lukoyanov *et al*., 1999 [80] examined if age-related deterioration of spatial memory and cholinergic innervation of the dentate gyrus is gender-specific. Aging progressively affected the performance of male and female rats in place discrimination version of the water maze task. On repeated acquisition task, only old males, but not old females, were significantly impaired relative to young and adult animals of both sexes. In parallel, they found that the age-associated reduction of the density of cholinergic fibers in the dentate gyrus was significantly more profound in old males than in age-matched females. These results suggest that, although male and female rats have an identical pattern of reference memory decline, impairment of the working memory and deterioration of the hippocampal cholinergic system are slower to develop in females than in males.

Arm maze learning: Bimonte and Denenberg 2000 [8] investigated sex differences in vicarious trial-and error (VTE) behavior in rats during radial arm maze learning. Females made more VTEs than males, although there were no sex differences in learning. Further, VTEs and errors were positively correlated during the latter testing sessions in females, but not in males. This sex difference may be a reflection of differences between the sexes in conflict behavior or cognitive strategy while solving the maze.

Activity driven by anxiety: A principal component solution with an orthogonal rotation of the factor matrix was used, ensuring that the extracted factors are independent of one another, and thus reflect separate processes [39]. In the elevated plus-maze test of anxiety, in male rats factor 1 accounted for 75% of the variance and reflected anxiety, factor 2 represented activity, and accounted for 24% of the variance. This contrasted with the finding in female rats in which factor 1 was activity, accounting for 57% of the variance, with the anxiety factor accounting for only 34% of the variance. When behaviour in both the plus-maze and holeboard were analysed, a similar sex difference was found with anxiety emerging as factor 1 in males and holeboard activity as factor 1 in females. Locomotor activity in the inner portion of the holeboard loaded on the anxiety factor for males, but on activity for females.

Nicotine: Nicotine has been considered as the gateway drug, because many teenagers experience cigarette smoking before seeking out other drugs [113]. By using an animal model system, they assessed the effects of chronic continuous nicotine exposure during periadolescence on ethanol intake during young adulthood. Periadolescent Sprague-Dawley rats (35 days old) were used at the beginning of this study. These animals received subcutaneous implantation of nicotine pellets (15 or 25 mg in 21-day time-release pellets) or placebo pellets (0 mg of nicotine) on postnatal day 35. Beginning on postnatal day 53, the animals received various concentrations of ethanol solution during their active period (5:00 PM to 9:00 AM) starting with 2% (v/v, 4 days), then 5% (5 days), 8% (6 days), and 10% (6 days). Between 9:00 AM and 5:00 PM of the same day, the ethanol solution was replaced by regular tap water. The amounts of ethanol solution and regular water were measured daily. The analyses showed that ethanol intake (g/kg b.w.) in the nicotine 15 and 25 mg groups did not differ from that in the nicotine 0 mg group, and no sex difference was found in ethanol intake. However, ethanol intake was increased as a function of the treatment days.

Nicotine prenatal/postnatal exposure: The purpose of this study was to determine if prenatal/postnatal nicotine exposure results in hyperactive offspring. Rat offspring were exposed to nicotine, through implantation of osmotic minipumps in dams, at levels of 0.75, 1.5 and 3.0 mg/kg/day, for 19 days prenatally and 16 days postnatally [94]. Offspring were measured for gestation length, body weight, litter size, sex difference and locomotor activity. No significant effects were shown for gestation length, litter size or male to female pup ratio. However, higher percentage of pup deaths resulted from nicotine-exposed dams than from control dams. Significantly less litter body weight was shown in nicotine-exposed offspring on postnatal day 1 when compared to controls. However, these offspring surpassed the control groups in litter body weight on postnatal day 14 and 21. Hyperactivity was shown in offspring exposed to prenatal/postnatal nicotine at levels of 0.75 and 3.0 mg/kg/day on postnatal day 14, but not on postnatal day 21 or at the 1.5 mg/kg/day condition.

Context and discretes conditioned stimuli (CS), freezing behavior: Pryce *et al*., 1999 [100] have reported on an analysis of the effect of sex in contextual and discrete CS conditioning to foot shock, assessed via measurement of freezing behavior in a

novel automated paradigm, in three rat strains: Wistar, F344, and Lewis. In Wistar rats, there was a consistent but nonsignificant tendency for males to demonstrate both more contextual and more CS conditioning than females; in F344 rats, males demonstrated both more contextual and more CS conditioning than females; in Lewis rats, a markedly enhanced acquisition of freezing in males did not translate into a sex difference in either context or CS conditioning at expression. Therefore, within each strain the effect of sex was consistent between context and CS conditioning. These findings, taken together with the hippocampal long-term potentiation (LTP) evidence, suggest that the latter mediates both contextual and discrete CS aversive conditioning, and contributes to sex differences in both these forms of conditioning, in those strains where these sex differences exist.

Genistein: The phytoestrogen genistein, the principal isofiavone in soybeans, has adverse effects on animal reproduction. Pregnant rats were fed soy-free diets containing 0, 25, 250, or 1250 ppm genistein (approximately 0, 2, 20, or 100 mg/kg/day) beginning on gestational day 7, and offspring continued on these diets through postnatal day (PND) 77. Male and female offspring were assessed for levels of sexually dimorphic behaviors: open field activity, play behavior, running wheel activity, and consumption of saccharin- and sodium chloride-flavored solutions [41]. Running wheel activity and saccharin solution consumption showed significant sex differences, but no effects of genistein treatment.

Amphetamine: When ovariectomized female rats receive estrogen, the response to the psychomotor stimulants amphetamine is enhanced. Estrous cycle-dependent differences in amphetamine-stimulated behaviors and striatal dopamine release are also noted. Intact female rats exhibit a greater behavioral response to amphetamine on estrus than they do on other days of the cycle. Ovariectomy results in attenuation of amphetamine-induced behavior and the striatal dopamine response to amphetamine. Physiological doses of estrogen given to ovariectomized rats reinstate both of these responses to a level comparable to that in estrous females [7]. Also, a sex difference is noted, in that females tend to exhibit a greater behavioral response to the psychomotor stimulants, and estrogen enhances this sex difference. Repeated treatment with amphetamine produces a progressive increase in behavioral responsiveness with subsequent drug administration, a process known as sensitization. In rats, behavioral sensitization results in increases in both frequency and duration of psychomotor behaviors such as rotational behavior, stereotyped grooming, headbobs, and forelimb movements. Females display greater sensitization of behaviors in response to psychomotor stimulants than do males. Results indicate gender differences in sensitization independent of gonadal hormones, suggesting that the neural systems that undergo sensitization are sexually dimorphic.

Maternal separation (MS) stress: A single 24-h MS in the rat during the stress hyporesponsive period alters adult behavior and neuroendocrine stress response. The age of the animal at MS might be a crucial factor for effects in adulthood. Lehmann *et al*., 1999 [76] reported the adult behavioral effects of MS performed on postnatal day 4 (MS4), 9 (MS9), or 18 (MS18) in male and female Wistar rats. Unrelated subjects were used to avoid confounding litter effects. Subjects were tested on paradigms of unconditioned fear/anxiety, *i*, *e*., open field and elevated plus-maze, and on paradigms involving learning in an aversive situation, *i.e*., conditioned freezing, active avoidance, and water maze (Table 7). In line with their predictions they obtained (a) sex differences that were consistent with enhanced fear/anxiety in males relative to females, (b) evidence that MS4 yielded deficits in active avoidance learning and conditioned freezing (trend level), whereas MS9 yielded enhanced active avoidance and water maze learning, (c) evidence (at trend level) that these effects of MS are greater in males than in females.

8-OH-DPAT a HT_{1A} agonist: Sipos *et al*., 2000 [112] reported that the chronic stress desensitizes serotonergic 5-HT_{1A} receptors and alters behavioral changes following 5-HT_{1A} agonist administration. Eating, acoustic startle response (ASR), and locomotor activity were measured in stressed and non-stressed male and female rats after 8-OH-DPAT administration. Stressed rats were paired and stressed by around-the-clock intermittent foot shock. Controllable stress (CS) rats could avoid/terminate shock for themselves and their yoked partners by pulling a ceiling chain, whereas their partners, the uncontrollable stress (UCS) rats, could not. Rats earned their entire daily ration of food by pressing a lever. 8-OH-DPAT did not alter percent prepulse inhibition (%PPI) at 100 dB, but significantly decreased %PPI in males but not females at 120 dB. Stress did not have a consistent effect on ASR, but reduced %PPI in ma1es, but not females. Neither stress nor 8-OH-DPAT significantly altered locomotor activity.

Exogenous glucocorticoid: To determine whether estrogen contributes to a sex difference, Young *et al*., 2001 [124] examined the effects of the estrogen antagonists tamoxifen and C1628 on the ACTH and corticosterone responses to restraint stress. CI628 increased both the ACTH and corticosterone response to restraint stress, and tamoxifen increased the ACTH response to restraint. Using overiectomized female rats, they also examined the effects of seven days of estradiol and/or progesterone replacement. Low dose estradiol decreased the ACTH but not the corticosterone response to restraint stress while progesterone had no effect on ACTH or corticosterone responses. The combination of estradiol and progesterone decreased the ACTH response to stress, and the magnitude of the effect did not differ from that found with estradiol treatment alone. Results suggest that in the physiological range estradiol is an important inhibitory factor in the hypothalamic-pituitary-adrenal stress response of females.

Neurosteroid allopregnanolone (3 α -hydroxy- α -pregnan-20-one, 3 α , 5 α -THP): The neuroactive steroid allopregnanolone has been shown to be involved in the central nervous system's response to stress. Zimmerberg *et al*., 1999 investigated whether response to the neuroactive steroid allopregnanolone, a positive modulator of the $GABA_A$ receptor, would be altered in neonatal or adult rats previously exposed to a chronic stressor,daily maternal separation during the first week of life. Subjects were then tested either as neonates or adults. In neonates, allopregnanolone decreased the number of ultrasonic vocalizations after brief maternal separation. Previously separated subjects vocalized

less and were less active than controls, but were not more sensitive to allopregnanolone on either measure. In adulthood, subjects with a prior history of maternal separation had a greater grooming response to a novel environment after a 10-min cold water swim test than nonseparated subjects. Allopregnanolone reduced grooming, but, again, there was no difference due to stress history. A significant effect of gender was noted in the adult subjects females were largely responsible for the effects reported. These results suggest that early maternal separation stress can produce and habituation response in neonates and a long-term sensitization response to later novel stress in adults. The results indicated that females was more responsive to allopregnanolone than males.

Melatonin: The effects of melatonin administered chronically to Long-Evans rats that were then forced-swim test were examined by Brottoe *et al*., 2000 [18]. The forced-swim test has been shown to be sensitive to all major classes of antidepressants and evidence indicates that melatonin possesses putative antidepressive properties. Rats received either a regimen of chronic administration of melatonin or the control condition for 14 days via the drinking water. On day 15, each animal was individually introduced into a swim chamber, and was scored for 15 min on the duration of swimming, struggling, and immobility. After 24 h, each animal was again tested in the forced-swim test for 10 min. Results revealed that females consistently showed higher activity levels than males in the forced-swim test. Melatonin significantly increased struggling in males on day 15, but failed to do so in females.

10. Body weight gains and food intake

Body weight gains following amygdaloid lesions: King *et al*., 1999 [67] have reported the lesions of the most posterodorsal aspects of the amygdala resulted in equal weight gains (mean = 58 g) in male and female rats during a 22-day observation period. However, the absolute weight gains in the first 5 days after lesions were greater in females (+41.4 g) than in males (+18.8 g), as were the longer-term gains relative to their respective control groups. In a second study with female rats, it was found that amygdaloid lesions had little effect on the estrous cycle and fat ovariectomy resulted in additional excessive weight gains in both rats with sham lesions and those with amygdaloid lesions. The weight gains produced by amygdaloid lesions and ovariectomy were additive. It is concluded that there is a sex difference in weight gains after amygdaloid lesions, but that the lesion-induced obesity is independent of estrogen levels. Similarities to lesions of the ventromedial hypothalamus are noted, and an amygdaloid-ventromedial hypothalamic pathway for the regulation of feeding behavior is proposed.

Prolactin (PRL): Results of previous studies indicate that there is a sex-specific feeding response to PRL by rats: Only female rats significantly increase their food intake [50]. The possible roles of the activational and/or organizational actions of the gonadal hormones in this sex difference were explored. In the first experiment, activational hormone exposure was manipulated in adult male and female rats. The results suggest that the activational actions of estrogen are not necessary for, and that testosterone does not block, PRL-induced increases in food intake by female rats. In a second experiment, organizational hormone exposure was manipulated in male and female rats during the early postnatal period. Genetic male rats organized as females by postnatal Day-1 castration significantly increased their food intake, while genetic female rats organized as males by postnatal androgen treatment maintained baseline levels of food intake. Overall, these experiments suggest that organizational, but not activational, gonadal hormone exposure plays a critical role in the development of this sex-specific response to PRL.

Body growth and food intake: Orden *et al*., [98] examined using Wistar rats of both sexes. The significance of the three in dependent variables (age, sex, and nutrition) was tested by multiple regressions. Covariation and degree of linearity between relative food intake (RFI) and age were tested by simple correlation and simple regression tests. The intercepts of the regressions were all significant. For both sexes they were greater in controls than in the moderately malnourished group, in the latter being greater than in the severely malnourished group. The slopes were significant and negative in all of the groups. While, in males and females, control and moderately malnourished rats showed little differences, the severely malnourished slopes were, respectively, 3.2 and 2.4 times lower than in controls. In controls there were sexual differences for RFI correlated with age which was not present in the malnourished groups. The age-of-fitness point (AFP) was calculated by extrapolation: 80-90 days of age in males, with RFI of 80mg/g, and 100-110 days of age in females, with RFI of 70 mg/g. In males trend-to-fitness stage (TFS) was 29.5% and 88.6 % in moderate and severe malnutrition, respectively. In females these values were 9.1% and 63.2%, respectively. Sexual dimorphism in TFS was significant in the moderately malnourished animals and nonsignificant in the severely malnourished ones.

11. Hepatic damage

Carbon tetrachloride (CCl4): Moghaddam *et al*., 1998 [87] examined the temporal kinetics of cellular regeneration and tissue repair processes in SD rats following an acute CCl_4 exposure (0.8 ml/kg, i.p.). In female rats, hepatic damage peaked at 24 h following the treatment and was approximately 2.5-fold (AST 2.7-fold, ALT 2.3-fold) greater than the damage observed in male rats. The hepatic damage in male rats appeared to peak by 3 h post-exposure and did not significantly change through the 36-h time-point. The activity of cytochrome P 4502E1 was 20% greater in male rats and did not correlate with the magnitude of hepatic damage. Morphometric analysis of cell cycle indices revealed that cellular regeneration was significantly greater in female rats as compared to male rats at 48 h and corresponded proportionally to the extent of liver damage. This study demonstrated that female rats respond more severely to equivalent doses of acute CCl_4 with greater hepatotoxicity than male rats and greater tissue repair and cellular regeneration. Results suggest that tissue repair is unlikely to account for the different responses exhibited by male and female rats to CCl_4 hepatotoxicity.

Diquat-induced hepatic necrosis: Redox cycling metabolism

of diquat catalyzes generation of reactive oxygen species, and diquat-induced acute hepatic necrosis in male F344 rats has been studied by Gupta *et al*, 2000 [48] as a model of oxidant mechanisms of cell killing *in vivo*. At equal doses of diquat, female F344 rats sustained less hepatic damage than did male rats, as estimated by plasma alanine aminotransferase (ALT) activities after 6 h. Biliary efflux of glutathione disulfide (GSSG) was greater in male than in female rats at each dose of diquat, but even comparable rates of GSSG excretion were associated with less hepatic injury in female rats. Hepatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were similar in the two genders, and activities of glutathione reductase (GR) and glutathione S-transferase- α (GST- α) activities were higher in the male rats.

Alcohol-induced liver injury: Kono *et al*., 2000 [71] reported that early alcohol-induced liver injury (ALI) in females was associated with changes in $CD1_4$ on Kupffer cells, activation of hepatic nuclear factor (NF)- κ B , and expression of tumor necrosis factor (TNF)- α mRNA. Male and female rats were given high-fat control or ethanol-containing diets for 4wk using the intragastric enteral protocol. Physiological parameters were similar in both genders. Ethanol was increased as tolerance developed with higher blood levels than previously observed, resulting in a 4-fold increase in aspartate aminotransferase (males 389 $\pm$ 47 IU/l vs. females 727 $\pm$ 66 lU/l). Hepatic pathology developed more rapidly and was nearly 2-fold greater and endotoxin levels were significantly higher in females after ethanol. Also, expression of $CD1_4$ on Kupffer cells was 1.5-fold greater and binding of transcription factor NF- κ B in hepatic nuclear extracts and TNF- α mRNA expression were 3-fold greater in females. These data are consistent with the hypothesis that elevated endotoxin after ethanol triggers more activation of Kupffer cells via enhanced $CD1_4$ expression in females. NF- κ B is activated in this process, leading to increases in TNF- α mRNA expression in the liver and more severe liver injury in females.

Liver hyperplasia: Sexual dimorphism exists in the response of rats to lead nitrate, with liver hyperplasia occuring earlier and being more pronounced in males. Excess dietary choline in females shifted the growth pattern towards that of males. To determine whether phosphatidylcholine-induced growth modulations could be related to a derangement of cholesterol metabolism, Tessitore *et al*., 2000 [118] investigated esters and plasma lipoprotein patterns after accumulation of cholesterol in the liver. In males, lead-induced liver hyperplasia was associated with increased total cholesterol hepatic content, accumulated cholesterol esters and reduced concentration of plasma High Density Lipoprotein (HDL) cholesterol. Females were less responsive to the liver mitogenic signal of lead nitrate; there was no elevation of cholesterol content or any marked accumulation of cholesterol esters. This is consistent with the lack of change in the plasma levels of HDL cholesterol. Continuous choline feeding displaced the liver cholesterol ester pattern and plasma HDL cholesterol levels in females, and in parallel that of DNA synthesis, towards those of males. Choline was not observed to have any effect in males. These results suggest that the derangement of phosphatidylcholine metabolism induces growth-related changes in cholesterol turnover; a finding consistent with the proposal that the intracellular content of cholesterol esters may have a role in regulating liver growth rates.

12. Gonadotropin, gonadal steroids and growth hormone

Currently, it has been described that gonadal hormones act in the central nervous system (CNS) during an early "organizational" period to promote the development of long-lasting changes in the structure and neurochemistry of sexually dimorphic areas and circuits, and during an "activational" period, at adulthood, these steroids stimulate the already developed circuits.

GABA related compounds: In adult male rats, serum luteinizing hormone (LH) rises within a few hours of castration. By contrast, in adult female rats, serum LH does not increase reliably until 4-6 d after ovariectomy. The release of gonadotropin-releasing hormone (GnRH) declines in female rats postovariectomy, suggesting an increase in inhibition of the release of GnRH. Hood and Schwartz 2000 [51] investigated whether differences in gamma-aminobutyric acid (GABA)-ergic transmission, which inhibits GnRH release, accounts for the sex difference in the response of serum LH to gonadectomy. They examined the effects of GABA-A receptor antagonist bicuculline methiodide (BMI), GABA-B receptor antagonist phaclofen, and transaminase inhibitor aminooxyacetic acid (AOAA), injected subcutaneously, on the postgonadectomy rise in LH. AOAA prevented the postcastration rise in male rats ($p < 0.05$). Female rats treated with BMI, phaclofen, or both BMI and phaclofen ($p < 0.05$) showed a significant increase in LH postovariectomy. BMI had no effect in male rats. GnRH antagonist blocked the BMI-induced increase in serum LH. They conclude that the delay in the rise of serum LH in female rats postovariectomy is at least partly owing to GABAergic inhibition of the release of GnRH.

Exogenous testosterone replacement: Testosterone is the principal gonadal hormone responsible for the masculinization of the rat nervous system. Sex differences in both the ligand and receptor availability may play a role in the process of sexual differentiation [86]. In some brain regions, males express more androgen receptor (AR) messenger RNA (mRNA) than females by postnatal day (PND) 10. Gonadectomy on the day of birth (PND-0) eliminated the sex differences in AR mRNA expression at PND-10, and exogenous testosterone replacement restored this sex difference. Because testosterone can be converted to both androgenic and estrogenic metabolites in the brain, the present experiments were performed to determine whether androgenic or estrogenic metabolites of testosterone are responsible for region-specific regulation of AR mRNA content in the developing rat forebrain. They used a 35S-labeled riboprobe and *in situ* hybridization to assess relative steady-state levels of AR mRNA in animals killed on PND-10. In the principal portion of the bed nucleus of the stria terminalis (BSTpr) and medial preoptic area (MPO), males gonadectomized on PND-0 and treated daily with dihydrotestosterone propionate (DHTP), a nonaromatizable androgen, had low levels of AR mRNA that were not significantly different from AR mRNA levels in intact females. In contrast, males gonadectomized on PND-0 and treated daily with diethylstilbestrol (DES), a synthetic estrogen, maintained high, male-typical levels of AR mRNA in

the BSTpr and the MPO. AR mRNA content in the VMM was not sexually differentiated in PND-10 rats and was unaffected by gonadectomy or hormone replacement. To further assess whether AR mRNA was autologously regulated, neonatal male rats were treated with the androgen receptor antagonist, flutamide. Flutamide at a dose of either 40μg/day or 300μg/day had no effect on AR mRNA expression in any area examined. Thus, AR mRNA is up-regulated by estrogen but is not regulated by androgen during the early postnatal period.

The medial amygdala (MeA) has receptors for gonadal hormones and is a sexually dimorphic area in rats [101]. The aims of this work were (1) to look at sex differences and the effect of gonadal hormone withdrawal in males castrated as offspring or at adulthood on neuronal soma area in the anterior and posterior MeA and (2) to study the dendritic branching and the density of dendritic spines in neurons from the MeA of intact males and females. Stellate and bitufted cells were found in the MeA following Golgi staining. Comparing data among groups, no significant difference in cell body area was found. Dendrites divide sparingly and have very different lengths, and a statistical difference ($p < 0.001$, males higher than females) in the spine density in the anterior MeA, but not in the posterior MeA, was found. These results suggest that castration does not alter the somal area in males submitted to gonadectomy during the early postnatal period or at adulthood. In addition, the already described sex difference in this nucleus may be more related to the neuropil than the neuronal somal area, which may be relevant for the function of the MeA.

Growth hormone-releasing hormone (GHRH): Kamega *et al*., 1999 [60] studied the age and sex dependency of the expression of receptors for two known stimulators of GH release, growth hormone-releasing hormone (GHRH) and the synthetic peptidyl and non-peptidyl GH secretagogues (GHSs). Pituitary GHRH receptor (GHRH-R) and GHS receptor (GHS-R) mRNA levels were measured by reverse transcriptase-polymerase chain reaction (RT-PCR) in male and female rats at postnatal day 1, 10, 30 and 75. They examined the age- and sex-dependent expression of the GHS-R in whole hypothalamic extracts, since the GHS-R is also expressed in a variety of nuclei within the hypothalamus and has been linked to central regulation of the GH-axis. Pituitary GHRH-R mRNA concentrations were age-dependent; the highest levels were observed in d1 pituitaries and then declined with age, reaching a nadir by d30. These results are in concordance with the age-related decline in pituitary GHRH sensitivity. In contrast, the ontogenic pattern of GHS-R expression was bimodal; GHS-R mRNA concentrations in dl and d30 pituitaries were approximately twice those at d10 and d75. These results mirror the transient increase in GHS sensitivity observed around the onset of puberty, suggesting that gonadal steroids mediate GHS-R expression. GHRH-R mRNA levels were comparable in males and females within each age while GHS-R mRNA levels were gender dependent. At d30, male GHS-R mRNA levels were 30% greater than in their female counterparts. This was reversed at d75, when females had 89% more GHS-R mRNA per pituitary and 65% more per somatotrope than did age-matched males.

13. Dopaminergic function

Intact female rats show more intense and prolonged stereotyped behavior after amphetamine (AMPH) or apomorphine administration [3, 53], a greater decrease in activity in response to chlorpromazine or haloperidol [4] and more AMPH-stimulated rotational behavior than do males [107]. Although there are gender differences in drug metabolism, the gender difference in rotational behavior persists even when brain levels of AMPH are equivalent [6]. This suggests that although most reported gender differences in response to AMPH may be greater in magnitude than would be found if brain levels of the test drugs were equalized, there are underlying gender differences in the organization of the striatal dopamine (DA) system. This idea is supported by research on gender differences in the behavioral response to cocaine, where males and females do not differ in cocaine metabolism [52]. Female rats also exhibit greater locomotor activation in response to cocaine than do male rats [119]. In addition to gender differences in the behavioral response to psychomotor stimulants, there are more D_1 DA receptors in the striatum of male rats than in intact female or ovariectomized (OVX) rats, but no gender difference in striatal D_2 DA receptors [52, 77]. In vitro, the AMPH-stimulated increase in striatal DA release is comparable in intact male rats and intact female rats in estrus [5]. Females, however, are found to have a higher density of DA transporter mRNA in striatum than do males [12]. There are also gender differences in basal and AMPH-stimulated striatal DA release in the absence of gonadal hormones. Following OVX, the AMPH-induced increase in striatal DA release is significantly less than the response of tissue from castrated (CAST) [5]. Finally, results from in vivo microdialysis in freely moving rats indicate that the basal extracellular concentrations of DA are twice as high in striatum of CAST males as in OVX females [123]. These sex-related differences in striatal DA release and receptors likely reflect an underlying sexual dimorphism in the organization of the striatum [20].

Quinpirole: Quinpirole is a dopamine D_2 receptor subfamily agonist. Periadolescence in the rat [postnatal day (PND) 35-50] is an important but understudied period of neurobehavioral development. In this experiment, an ongoing survey of the effects of quinpirole in developing rats was completed by the addition of periadolescent rats to the range of ages tested. PND40 or 50 rats were injected subcutaneously with the dopamine D_2/D_3 receptor agonist, quinpirole (0, 0.02, 0.2, or 2.0 mg/kg), and their locomotor activity was recorded [42]. Periadolescent rats showed adult-like locomotor responses to either the 0.2 or 2.0 mg/kg doses of quinpirole, i.e., the responses were biphasic with respect to time: early suppression of locomotion followed by later activation within a single test session. In younger female rats (PND40) but older male rats (PND50), the lowest dose of quinpirole suppressed activity early in the test session but did not increase it later. In male rats, the magnitude of locomotor activation declined with age.

Attention deficit hyperactivity disorder (ADHD): Andersen and Teicher 2000 [1] reported that striatal male D_2 receptor density increases 144±26% between 25 and 40 days (the onset of puberty), while female D_2 receptor density increases only 31 ± 7%. Male receptor density is then sharply eliminated

by 55% by adulthood. Periadolescent females show little overproduction and pruning of striatal D_1 and D_2 receptors, though adult density is similar to males. The rise of male, but not female, striatal dopamine receptors parallels the early developmental appearance of motor symptoms of ADHD and may explain why prevalence rates are 2-4 folds higher in men than women. Pruning of striatal dopamine receptors coincides with the estimated 50-70% remission rate by adulthood. Transient lateralized D_2 dopamine receptors (left > right) in male striatum may increase vulnerability to ADHD. More persistent attentional problems may be associated with the overproduction and delayed pruning of dopamine receptors in prefrontal cortex. Differences in D_1 receptor density in nucleus accumbens may have implications for increased substance abuse in males.

Haloperidol: Field *et al*., 2000 [40] found that when haloperidol-induced cataleptic rats are placed facing downward on an inclined plane, these ratswill brace against the resulting downward force by pushing backwards, and if they lose postural stability, or their position on the inclined plane, they will jump forward. Females, however, jump from the inclined plane at a significantly lower angle than do males. Frame-by-frame analysis of the jumping sequences revealed that females and males use a different combination of postural adjustments to maintain their position on the inclined plane prior to jumping. Furthermore, gonadal hormone manipulations at birth and in adulthood reveal that these sex differences in postural adjustments are dependent on the organizational effects of gonadal hormones in the perinatal period. These results provide evidence for sex differences in postural support mechanisms and suggest that the sex of subjects, or their hormonal state, must be considered when studying the behavioral aspects of neurological disorders such as Parkinson's disease which include a postural component.

14. Brain and CNS

The acoustic startle reflex (ASR) : The acoustic startle reflex (ASR) and pre-pulse inhibition (PPI) of the ASR are used extensively to index drug effects in rodents. Faraday and Grunberg, 2000 [38] reported an experiment that measured the acoustic startle responses with and with-out a pre-pulse of adult SD rats tested individually and in same-sex groups at four time points. Individual testing increased startle responses and PPI of males at time I and altered PPI of females at times 1, 2, and 3 compared with group testing. Responses were indistinguishable in the two testing environments at time 4. Results indicate that testing environment may affect responses when subjects have not been acclimated to the testing situation and that there is sex differences in these effects. Because responses stabilized by the fourth testing point, repeated testing of subjects particularly females, may be an important methodological inclusion when evaluating effects of drugs and other manipulations on ASR and PPI.

The bed nucleus of the stria terminalis (BST): BST in the rat forebrain differs between males and females [23]. To test whether apoptosis may contribute to the development of sex differences in the BST, the incidence of apoptosis was determined in sham-treated males and sham-treated females sacrificed on postnatal days (PN) 2, 4, 6, 8, 10, and 12 (PN 1 being day of birth). More apoptotic nuclei were found in the principal nucleus of the BST (BSTpr) in females than in males, whereas the reverse was true for the lateral division of the BST (BST1). Moreover, the volume of the BSTpr was larger in males than in females, whereas there was no sex difference in the volume of the BST1. Their results also confirmed earlier reports indicating that the incidence of apoptosis in the central part of the medial preoptic nucleus (MPNc) is higher in females than in males. No sex difference in apoptosis was found in the ventromedial hypothalamus (VMM) and paraventricular nucleus (PVN). The volume of the MPNc and VMM was larger in males than in females, whereas the PVN volume did not differ between males and females. To test whether sex differences in neonatal levels of gonadal steroids may cause sex differences in the incidence of apoptosis in the BSTpr, the incidence of apoptosis was compared between castrated males and females that were treated with testosterone propionate or vehicle on the day of birth. In the BSTpr of gonadal steroid-treated animals, the incidence of apoptosis was lower when compared to animals treated with vehicle, which was also true for the MPNc. These results are consistent with the hypothesis that gonadal steroids contribute to the sexually dimorphic differentiation of the BST by controlling the incidence of apoptosis.

Imipramine N-oxidation: Imipramine (IMI) is one of the tricyclic antidepressant agents. IMI N-oxidase activity in brain microsomes from rats of both sexes was determined by high performance liquid chromatography, and compared with the results in rat liver microsomes [93]. Brain and liver microsomal IMI N-oxidation (a pharmacologicaly active) was sensitive to thermal inactivation and had an optimal pH at around 9.0. IMI N-oxidase activity (15.54 pmol/min/mg protein) in brain microsomes was about one-hundredth that of liver microsomes (2.08 nmol/min/mg protein) at a substrate concentration of 5 mM. IMI N-oxidase activities in both brain and liver microsomes displayed biphasic kinetics that associated a low Km-low Vmax element with a high Km-high Vmax component. Furthermore, a significant sex difference was observed in Vmax values (male>female) in both phases, but Km values were similar between male and female rats, resulting in a significant sex difference (male>female) in intrinsic clearance values (Vmax/Km) of the low-Km and the high-Km phases.

Anterior commissure size: Earlier studies have shown that the corpus callosum of rats tends to be larger in males than in females. Noonan *et al*., 1998 [95] report here that the anterior commissure of rats is also larger in males than in females. The sizes of the two commissures were positively correlated in both sexes, but significantly more so in females than in males (Table 8). The anterior commissure size difference in rats reported here is opposite in direction from that reported elsewhere for humans, and they speculate that this may derive from differences in the relative proportions of the constituent fibers that make up the anterior commissure in the two species.

Diazepam (DZ): DZ is an antidepressant. Exposure to DZ during the last week of *in utero* development in rats was examined by Kellogg *et al*., 2000 [66]. Pregnant Long-Evans rats were injected with DZ (2.5) mg/kg over gestation days 14-20, and their male and female offspring were evaluated for

levels of brain-derived neurotrophic factor (BDNF) mRNA and protein in the cerebral cortex and hypothalamus at fetal day 20 and at postnatal ages spanning birth to young adulthood. At fetal day 20 the expression of BDNF was reduced by about 20% in the hypothalamus of males only. The early exposure affected postnatal expression of BDNF in the hypothalamus only modestly, influencing the age-related profile in both sexes. Postnatal development of BDNF in the cerebral cortex was significantly affected by the in utero exposure in males only with mRNA Ievels lower in the exposed group and protein levels higher during juvenile ages. At adulthood, both levels were lower in DZ-exposed males.

L-glutamate: The relationship between N-methyl-D-aspartate receptor (NMDAR) and the sex-specific neurotoxicity of L-glutamate on the preoptic area (POA) of neonatal rats was studied by Hsu *et al*., 1999 [54]. The kinetic change of intracellular calcium and lactate dehydrogenase (LDH) efflux were monitored as rapid and delayed toxic signals, respectively. The results showed that: (1) the NMDAH expression in POA of male rat is higher than that of females; (2) the L-glutamate (500 μ M) induced a more significant elevation of intracellular calcium in neuron derived from male rat than that from female; (3) after glutamate-treatment, the LDH efflux in neuronal culture of male rat is higher than that of females. These results suggest that the quantitative difference in NMDAR between male and female rats may contribute to the sex-specific neurotoxicity of L-glutamate on the POA of neonatal rats.

Neuropeptide: Rugarn *et al*., 1999 [108] have investigated possible sex differences in the regional concentrations of neuropeptides in the rat brain. Immunoreactive neurotensin (NT), neurokinin A (NKA), galanin (GAL), calcitonin generelated peptide (CGRP), Substance P (SP) and neuropeptide Y (NPY) were measured by radioimmunoassay in frontal cortex, occipital cortex, hippocampus, striatum, hypothalamus and pituitary in male and female pre- and postpubertal rats. Sex differences were found for NPY ($p < 0.001$). NT ($p < 0.01$) and GAL ($p < 0.05$), in particular in hippocampus, striatum, hypothalamus and pituitary, but not for CGRP, SP and NKA. The authors concluded that results from analysis of neuropeptides in one sex may not be entirely applicable to the other.

GABA amino acid neurotransmitter: GABA, glutamate and aspartate are the predominant amino acid neurotransmitters in the mammalian brain. Davis *et al*., 1999 [29] have previously reported a developmental sex difference in messenger RNA levels of glutamate decarboxylase, the rate-limiting enzyme in GABA synthesis. Males were found to have significantly higher levels of messenger RNA in many steroid-concentrating regions of the hypothalamus and limbic system on day 1 of life. In this study, they examined levels of amino acid neurotransmitters during early postnatal development in many of the same or related brain areas. They found that levels of all three transmitters change as animal age. While both GABA and aspartate concentrations increase, glutamate levels decrease. In addition, there are sex differences in neurotransmitter levels in several areas examined, including the ventromedial and arcuate nuclei of the hypothalamus, and the CA1 region of the hippocampus. Sex differences for GABA occur only on postnatal days 1 and 5. However, sex differences in aspartate occur later in development (postnatal day 20). The CA1 region of males has a significantly greater concentration of GABA, glutamate and aspartate than females on postnatal day l. In addition, treatment of females with testosterone propionate on the day of birth results in increased GABA levels, suggesting that these sex differences may be the result of hormone exposure during development. The authors hypothesized that these hormonally mediated sex differences in amino acid transmitters early in development contribute to the establishment of sexually dimorphic neuronal architecture in the adult.

Calbindin-D_{28K} and Calretinin Immunoreactivity : The proteins calbindin-D_{28K} and calretinin buffer intracellular calcium and are speculated to be involved in the integration of neuronal signaling [17]. Using Western blot analysis, they compared the levels of calbindin-D_{28K} and calretinin in the developing male and female rat hypothalamus on postnatal days (PN) 0, PN2, PN4, PN6, PN8, and PN10. Analysis of variance (ANOVA) of mean calbindin levels indicated a significant effect of sex ($p \leq .001$) and age ($p \leq .0001$) and a significant interaction ($p \leq .02$). Post-hoc Neuman-Keuls analysis revealed that PN0 and PN2 males had significantly elevated calbindin levels over PN0 and PN2 females ($p \leq .05$). ANOVA of mean calretinin levels from the same animals also indicated a significant effect of sex ($p \leq .002$) and a significant interaction between sex and age ($p \leq .001$). Post-hoc analysis indicated males had significantly elevated calretinin levels over PN0, PN4 ($p \leq .05$) and PN6 ($p \leq .01$) females. Immunocytochemical analyses indicated calbindin-immunopositive staining for cell bodies in the central subdivision of the medial preoptic nucleus, paraventricular nucleus, arcuate nucleus and dorsomedial nucleus, and an area immediately surrounding the ventromedial nucleus (VMN). Calbindin immunoreactivity was absent from the ventrolateral VMN, but lightly stained cell bodies were observed in the dorsomedial VMN. The sex differences observed in calcium binding proteins parallel their previously observed sex differences in excitatory γ -aminobutyric acid and glutamate early in development and may be related to mechanisms of sexual differentiation of the brain.

15. Hypertension

Renin-angiotensin system : Because males develop higher blood pressures than do females, Reckelhoff *et al*., 2000 [103] hypothesize that androgens may affect the renin-angiotensin system to promote the development of hypertersion in male spontaneously hypertensive rat (SHR). This study was performed to determine the effect of converting enzyme inhibition (CEI) on the development of hypertension in SHR. Male, female, castrated male, and ovariectomized (ovx) female SHR received enalapril (250 mg/L) in drinking water for 8 to 10 weeks. Some ovx females were also given testosterone chronically. At 17 to 19 weeks of age, 24-hour protein excretion and mean arterial pressure were measured. By 13 weeks of age, male rats had higher systolic blood pressures by tail plethysmography than did the other rats, and CEI reduced blood pressures to similar levels in all groups. At 17 to 19 weeks, the same trend was found by direct measurement of mean arterial

pressure. The ovx females treated with testosterone had serum testosterone and blood pressure levels similar to those found in males. CEI reduced mean arterial pressure to similar levels in all gender groups. Untreated males and ovx females given testosterone had significantly higher levels of urinary protein excretion than did the other groups, and CEI had no effect on proteinuria in any of the rats. These data suggest that the development of hypertension in SHR regardless of sex steroids is mediated by the renin-angiotensin system. However, the data further suggest that androgens promote the exacerbation of hypertension in male SHR via a mechanism involving the renin-angiotensin system.

Rauwolscine: Male Dahl salt-sensitive hypertensive (S) rats develop hypertension faster than females. Gong *et al*., 1996 [44] measured renal α_2-adrenergic receptor density of inbred Dalil salt-sensitive (SS/JR) and salt-resistant (SR/JR) rats, using [^{3}H]-rauwolscine saturation binding studies. Male and female SS/JR rats were gonadectomized or sham-operated at 6 weeks of age and fed a high salt diet for 4 weeks. Additional intact SS/JR and SR/JR rats of both sexes were fed the high salt diet for a longer period of time (7 weeks instead of 4 weeks). Both blood pressure and renal α_2-adrenergic receptor density were sigatficantly higher in male than female SS/JR rats on high salt diet for 4 weeks. Gonadectomy did not change blood pressure nor did it change renal α_2-adrenergic receptor density measured at the 4th week of high salt feeding in either male or female SS/JR rats. When the SS/JR rats were fed high salt diet for a longer period (for 7 weeks), blood pressure of female SS/JR reached the level of males, but the density of renal α_2-adrenergic receptors was still significantly lower than that of males. Both renal α_2-adrenergic receptor density and blood pressure were higher in male than female SR/SR. They conclude that higher blood pressure in male Dahl SS/JR and SR/JR rats is associated with higher renal α_2-.adrenergic receptor density compared with their female counterparts.

16. Antiviral drug

Ribavirin: The genotoxic and cytotoxic effects of the antiviral drug, ribavirin, was studied by Narayana *et al*., 2002 [92] in rat bone marrow by employing the micronucleus assay. Ribavirin in doses of 10 to 200 mg/kg, and cyclophosphamide (CP) 40 mg/kg (only for sex-difference study) were injected intraperitoneally. Bone marrow was collected at 24 h and 48 h following the injection. To evaluate the recovery, the bone marrow was also sampled at 72 h from 20, 100 and 200 mg/kg treated rats. The micronucleus assay was conducted according to the standard procedure. Ribavirin elevated the incidence of micronuclei (except 10 mg/kg) in erythrocytes ($P<0.01$). The micronucleated polychromatic erythrocytes showed the initial steep increase at 15 and 20 mg/kg dose level, then with the gradual increase, possibly due to the limited metabolism and action of higher doses. The incidence of micronucleated normochromatic erythrocytes was not dose dependent. The effect was greater at 48 h than 24 h due to prolonged toxicity of the drug or its metabolites, and by 72 h, recovery was observed even though the genotoxicity was significant. The PCE% decreased as the dose was increased up to 75 mg/kg, without much difference between the two higher doses. Cytotoxicity was seen due to hindered erythropoiesis or cell destruction. Their findings suggest that ribavirin is genotoxic and cytotoxic agent for rat bone marrow. The 100 mg/kg ribavirin and CP treatment showed more toxicity on male rats.

17. Hypoglycemia

Non-insulin dependent diabetes mellitus: Corsetti *et al*., 2000 [26] studied the effect of dietary fat on development of non-insulin dependent diabetes mellittis (NIDDM), dyslipidemia, and alterations in organ-specific serum panels in obese Zucker diabetic fatty (ZDF) males and females. Results indicated different effects of dietary fat-content on development of diabetes in males and females. Males, even on low fat-content diets, developed diabetes with the process accelerated as a function of dietary fat-content, whereas only the highest fat-content diet induced development of NIDDM in obese ZDF females. Additionally, triglyceride/apolipoprotein B ratios demonstrated gender-specific differences in the nature of circulating lipoprotein particles independent of diabetic state. Female values were approximately twice those of males indicating more highly triglyceride-enriched lipoprotein particles in females. It was concluded that the obese ZDF female rat has the potential to become an important animal model of NIDDM especially for women where few models currently exist.

Hypoglycemia: Drake *et al*., 1998 [31] studied the influence of sex on the adrenal catecholamine response to acute insulin-induced hypoglycemia in SD rats. Eight male and seven female adult rats were anesthetized with pentobarbital, and a microdialysis probe was placed in the left adrenal. Dialyzed epinephrine and norepinephrine levels were measured by high-performance liquid chromatography during a control period and for 1 hour after insulin administration. The blood glucose level was measured every 15 minutes. The same protocol was applied to 23 adult females at various stages of the estrus cycle. The pattern of blood glucose changes during insulin-induced hypoglycemia was similar in both sexes, but males exhibited a significantly greater increase in epinephrine than females (261% *vs* 52%, $P = .001$) in the sex-comparison experiment. A similar trend was observed for norepinephrine (73% *vs* 0%, $P = .075$). The adrenal response in females for both catecholamines was not significantly affected by the estrus cycle phase ($P = .989$ for epinephrine and $P = .424$ for norepinepnrine). They conclude that sex influences the magnitude of the adrenal catecholamine counterregulatory response to hypoglycemia. Males had a significantly greater increase in epinephrine release than females exposed to hypoglycemia. Estrous cyclicity was shown on to influence the response.

18. Immunosuppression

Cyclosporine (CyA): Cyclosporine (CyA) is a potent immunosuppressive drug widely used to inhibit organ rejection in transplant patients and for the treatment of autoimmune disorders. In a study conducted by Molpeceres *et al*., 2000 [89], a single intravenous dose, 10 mg/kg was use to identify the mechanisms behind such differences. Drug distribution was studied by measuring the CyA levels in blood, liver, kidney,

spleen, adipose tissue, skin and muscle at 48 h post-treatment by using a specific fluorescence polarization immunoassay. Drug blood and tissue levels in male rats were significantly higher than the female counterparts except for adipose tissue where the concentrations were 2-fold higher in females. In males, the highest CyA concentrations were observed in the liver, followed in rank order by kidney and spleen, fat, skin, muscle, then blood. Females showed the highest drug levels in fat, followed by liver, kidney, spleen, skin, muscle and blood. Age exerted a significant influence on CyA tissue levels in males but no effect was observed in females. The potential differences in drug metabolism were established by measuring (HPLC) the amounts of CyA and its metabolites accumulated in faeces after hepatic biotransformation and biliary excretion. The amounts of circulating metabolites in blood as well as those accumulated and excreted in the liver and urine were also estimated by using specific (*m*-FPIA) and non-specific fluorescence polarization immunoassay (*p*-FPIA), respectively. The analysis of faeces revealed that AM9 was the major identified metabolite with females excreting lower amounts of unchanged CyA than males. The comparison of the AUC values corresponding to parent CyA and total CyA derivatives suggested that blood concentrations of CyA metabolites were higher in females indicating higher biotransformation rates.

19. Serotonergic system

The serotonergic system plays a role in regulation of anxiety and ethanol withdrawal (EW) [58]. Nevertheless, few studies have assessed sex differences in serotonergic effects on EW. One study examined sex differences in the anxiogenic stimuli induced by a serotonin $(m)_{lb/2}$ agonist, *meta*-chlorophenyl piperazine (mCPP), prior to ethanol and during EW. Gonadectomized or sham-operated adult male and female rats and 17 β -estradiol (2.5 mg, 21-day release, s.c.) -replaced ovariectomized (OVX) rats were trained to disciminate mCPP (1.2 mg/kg, i.p.) from saline in a two-lever choice task for food. Latency to the first lever press and mCPP lever selection was measured following mCPP (0-1.2 mg/kg). Rats then received chronic ethanol-containing liquid diet (6.5%) for 10 days and were tested for mCPP lever selection 12 h and 36 h after removal of ethanol. Fewer shams female and β -estradiol-replaced OVX rats selected the mCPP lever than male or OVX rats, and showed increased initiation latency after mCPP injection. During EW (12 h and 36 h), fewer sham female and β -estradiol-replaced OVX rats responded on the mCPP-lever after saline injection as wel1 as after mCPP challenge than male or OVX rats. Castration did not alter any response of male rats to mCPP. Conclusions: mCPP discrimination is a useful measure of EW in male and female rats; and sham female and β -estradiol-replaced OVX rats are less sensitive to the discriminative stimulus prior to and during EW, but more sensitive to impaired behavioral initiation induced by mCPP than male or OVX rats.

20. Leukemogenic AF-9 gene

Morgan *et al*., 2000 [90] have investigated the biological role of the cellular counterpart of the leukemogenic AF-9 gene by cloning the rat AF-9 (rAF-9) cDNA and defining the regulation of an anterior pituitary-specific rAF-9 transcript that is expressed in a sexually dimorphic manner. Expression of this transcript is down regulated after puberty in females and can be subsequently up regulated in adults by ovariectomy. Hormone replacement studies have provided direct evidence that rAF-9 mRNA expression is suppressed by estrogen. Mapping the 1.9 kb anterior pituitary transcript has shown that it corresponds in size to the rAF-9 CDNA clone, which contains an open reading frame (ORF) that is truncated compared with the human AF-9 ORF, but encodes a previously defined transcriptional activation domain. Thus, the cellular AF-9 gene is alternatively expressed in a manner that reflects the presence of translocated, functionally active (oncogenic) AF-9 sequences in leukemias. Using novel antisera raised against a rAF-9 peptide, they have also demonstrated tissue- and sex-specific expression of a nuclear 41-kDa anterior pituitary protein and have localized this protein to a major population of growth hormone synthesizing cells. By localizing the expression and defining the physiological regulation of rAF-9, their studies have provided novel insights into the AF-9 gene that will facilitate an understanding of both oncogenic and endocrine roles.

21. Cataract induction by transforming growth factor-beta (TCF- β)

Sun *et al*., 2000 [114] utilized a differential display procedure to examine gene up- and down-regulation in male, normal female and ovariectomized female rat lenses exposed to TCF- β . Male and normal female rat lenses were cultured with or without 0-15 ng ml^{-1} TGF- β . Lenses were then harvested, and total RNA was isolated for analysis by reverse-transcriptase differential display. Differentially expressed mRNAs were subcloned, sequenced and identified through GenBank database searches. The same experiment was repeated with the addition of ovariectomized female TGF- β (+/-) conditions, and all differential patterns of gene expression were verified using Northern blot and RT-PCR analysis. Screening of approximately 12% of the mRNA population led to the identification of 27 differentially expressed cDNAs. Notably, strong gender differences were found in expression levels of γ B-crystallin. In addition, proteasome Z subunit was up regulated in TGF- β -treated male and ovariectormized female lenses, but was down regulated in TGF- β -treated normal female lenses. This pattern of expression is consistent with the increased susceptibility of male and ovariectomized lenses to TGF- β -induced cataract. They conclude that differential display is a useful and expedient method for analyzing changes in gene expression in the lens. Structural and functional studies of the genes identified in this study may further elucidate mechanisms underlying the TGF- β -induced cataract formation and differential rates of cataractogenesis in male's versus females. In particular their data suggest that the role of proteasome Z subunit in TGF- β -induced anterior subcapsular cataract warrants further investigation.

22. Pain and inflammation

Dynorphin: Dynorphin is an endogenous kappa-opioid peptide that is upregulated in an animal model of peripheral inflammation and hyperalgesia. The present study compared behavioral responses of male, cycling female, and

gonadectomized SD rats in a model of persistent pain [16]. Cycling female rats were behaviorally tested over a 14-day period, and their estrous cycles were monitored by daily vaginal smears. Thermal hyperalgesia was measured by paw withdrawal latencies taken prior to and 24-72 h after rats received a unilateral hindpaw injection of complete Freund's adjuvant (CFA). Prior to CFA administration, there was no significant difference in paw withdrawal latencies between male rats, cycling female rats, and ovariectomized female rats. Following CFA administration, female rats in proestrus exhibited significantly increased hyperalgesia compared with male rats, ovariectomized female rats, and female rats in other estrous stages ($P<0.05$). Levels of spinal preprodynorphin (PPD) mRNA induction in the L4-L5 segments were assessed by Northern blot analysis. PPD mRNA expression ipsilateral to the injected paw was significantly higher in female rats in diestrus ($P < 0.05$) and proestrus ($P < 0.01$) compared with rats in estrus and intact male rats. Ovariectomized rats had significantly higher levels of PPD mRNA expression compared with intact male rats ($P < 0.05$). However, castrated male rats had significantly lower levels of PPD mRNA expression than intact male rats ($P < 0.05$). PPD mRNA expression was not altered on the contralateral side of the spinal cord in any group. These results suggest a hormonal regulatory influence on the response of spinal cord dynorphin neurons to chronic inflammation and furthermore, that the association of the endocrine and opioid systems have the ability to influence an animal's sensitivity to pain.

Formalin pain: Immediate early genes are crucial intermediates in a cascade linking membrane stimulation to long-term alterations of neuional activity. In the present experiment, Ceccarelli *et al*., 1999 [21] performed immunohistochemistry for *c-Fos* to determine the effects of persistent pain on cells of the hippocampus of male and female rats. Animals were subcutaneously injected with formalin (50 μl, 10%) and perfused: 2 h later, time 2; 24 h later, time 24; 24 h later after 20 mm of the open-field test, time 24/OF. Controls were left undisturbed. In control, *c-For* was higher in females than in males in all hippocampal fields. In males at time 2, formalin increased *c-For* in the dentate gyrus (DO) and CA3 fields; at time 24, *c-Fos* returned to the control level; at time 24/OF, *c-Fos* was higher than in control in the DO, but not in the other fields. In the formalin-treated females at time 2 and at time 24, *c-For* levels were lower, or tended to be lower, than in control in all hippocampal fields; at time 24/OF, *c-For* levels in the DO were higher than in control and in males. In conclusion, persistent pain had different effects on *c-For* in the hippocampal subfields, depending on the time after treatment and the sex of the subject.

Two analgesic paradigms: The anaigesic potency of opiod drugs varies as a function of gender, and can be modified by the intake of palatable sweet-tasting solutions [61]. To determine if gender interacts with diet-induced changes in antinociceptive responses, male and female Long-Evans rats were fed laboratory chow and water alone, or chow, water and either a 32%w/v sucrose solution or a 0.15%w/v saccharin solution, and tested In two analgesic paradigms, the tail-flick test and the hot-plate test. For both tests, antinociceptive responses of male and female' rats were tested following administration of cumulative closes (1, 5, 2.5, 5.0, and 10.0 mg/kg, sc) of morphine sulfate. On the tail-flick test morphine produced dose-related increases in 'antinociceptive responses. In addition, relative to both the chow only and saccharin conditions, chronic intake of the sucrose solution access significantiy augmentecl morphine's antinociceptive proper-ties. On the hot-plate test, when the plate was heated to 51 ℃, morphine led to significant dose-related increases in antinociceptive responses, but diet did not affected antinociceptive responses. When the temperature of the hot plate was increased to 53℃, there was a trend for animals given sucrose to have greater antinociceptive responses than those given either chow alone or saccharin. No differences in baseline pain sensitivity or morphine-induced analgesia were observed as a function or gender.

DISCUSSION AND CONCLUSION

Regulatory toxicology guidelines, require the same number of males and females for all studies. However, OECD Test Guideline 401 (acute toxicity test) was revised to OECD Test Guideline 425 in 2001, and only females are recommended to be used [96]. According to previous articles, it is known that acute toxicity is more evident in females with many compounds. This revision was considered an attempt to reduce the number of animals used in animal testing, a basic concept of animal welfare. However, since rodents give birth to nearly equal numbers of male and female offspring, does the usage of females only actually result in the reduction of the number of animals? In animal experiments of pharmaceuticals for gynecological treatments and hypogonadism in males, it is sufficient to use targeted sexes only? In such cases, studies in both sexes should not be required simply for curiosity.

The IGS Rat Study Group has collected historical data of CD(SD)IGS rats and is currently available. Such efforts are expected to improve sampling and measuring techniques as well as insure the quality maintenance of laboratory rats. CD(SD)IGS rats have been utilized in toxicity studies for 6 years. It is important to shed light on mechanisms when gender differences are evident in responses of rats following administration of the test compounds. The literature reviewed in this paper should guide the researcher in consideration of gender differences that might affect their research.

The evaluation of the toxicity of chemicals is moving more and more towards the use of biomarkers and breaking away from relying solely on traditional blood chemistry, 10% reduction in body weight gain and findings limited to HE stained tissues. Understanding gender differences for many of these biomarkers will be critical in the interpretation of the results from these evaluations.

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Table 1. Spontaneous tumor incidence (combined benign and malignant) in selected sites of the two species, B6C3F1 mice and F344 rats, used in the NCI/NTP bioassay

Site	B6C3F1 Mice		F344 Rats	
	Male	Female	Male	Female
Liver Adenoma	10.3	4.0	3.4	3.0
Liver Carcinoma	21.3	4.1	0.8	0.2
Pituitary	0.7	8.3	24.7	47.5
Adrenal	3.8	1.0	19.4	8.0
Thyroid	1.3	2.1	10.7	9.3
Hematopoetic	12.7	27.2	30.1	18.9
Mammary gland	0	1.9	2.5	26.1
Lung	17.1	7.5	2.4	1.2

Source: From Goodman *et al*. 1979, Chandra and Frith 1992.

Table 2. Survival to 106weeks of age for F344/N rats and prevalence of thyroid C-cell tumors in F344/N rats

	Survival %	Thyroid C-cell tumors %		
Year	Male	Female	Male	Female
1971	85.3*	83.3	8.4	3.4
1972	76.7	77.9	7.4	5.6
1973	81.6	82.3	8.5	8.6
1974	77.0	83.3	7.4	5.5
1975	65.5	81.0	5.1	6.2
1976	74.1	81.8	9.0	6.4
1977	74.5	78.7	8.4	9.9
1978	75.0	78.8	10.5	9.6
1979	75.7	84.4	11.2	9.7
1980	66.4	75.0	15.1	15.3
1981	62.5	70.8	14.9	14.8
11-yr average	74.0	79.8		

*Mean, Rao, G.N., *et al*, 1990:Toxicol. Pathol., 18: 61-70.

Table 3. Effect of sex on biotransformation in the rat

Analysis	Ratio of Male / Female
Cytochrome P-450	1.4
NADPH-cytochrome P-450 reductase	1.3
Benzphetamine N-demethylation	5.6
Aniline hydroxylation	5.5

Table 4. Relative induction of hepatic CYP isoforms after a single dose of DDT in male and female Wistar rats

Dose	CYP1A1		CYP2B1/2B2		CYP2C11	CYP2E1		CYP3A1		CYP3A2	
mg/kg	♂	♀	♂	♀	♂	♂	♀	♂	♀	♂	♀
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.1	0.5	0.7	0.9	0.6	2.2	1.2	0.9	0.7	1.0	1.8	2.2
1	0.3	1.2	6.6	0.7	1.5	1.0	1.0	0.5	1.0	1.3	1.1
5	0.6	0.7	16.8	3.9	1.3	1.3	1.1	1.3	6.9	2.0	2.9
10	1.1	1.3	13.3	11.6	1.2	1.4	1.0	1.2	15.3	2.3	1.6
100	0.8	1.8	19.3	19.4	1.1	1.5	1.9	1.3	31.9	2.8	18.1

Sierra-Santoyo *et al*., 2000. Toxicol. Sci. 54: 81-87.

Table 5. Morphine antinociception dose-response relationships in male and female rats of three outbred populations

Strain	Sex	AD_{50}(95% CI)[a] mg/kg	Potency ratio(95% CI)[b]
LE	♂	4.7(3.5-6.2)	4.7(3.5-6.2)[c]
	♀	7.6(6.0-9.6)	
SD	♂	5.7(4.2-7.8)	2.4(1.4-3.9)[c]
	♀	13.4(9.0-19.9)[d]	
WKY	♂	4.2(3.0-5.0)	1.1(0.7-1.7)[c]
	♀	4.6(3.4-6.2)	

a: Half-maximal antinociceptive doses (AD50s) and corresponding 95% confidence intervals (CI)
b: Calculated as female AD50/male AD50
c: Male significantly more sensitive to morphine than females, $p<0.05$, as determined by potency ratio comparison with unity.
d: Significantly less sensitive to morphine than WKY strain female rats. Mogil *et al*., 2000. Neurosci. Biobehavior. Rev. 24: 375-389.

Table 6. α 2u-Immunostaining intensity and cell proliferation rates (labeling index) of P2 cells in kidney sections of control and treated male and female F344 rats

	α 2u-Immunostaining		Labeling index %	
	Male	Female	Male	Female
Control	1-2	0	7.0±1.2	7.0±0.7
dL	4-5	0	20.0±6.6*	22±5.0*
OTA	1	0	7.0±1.9	6.0±2.0

OTA: mycotoxin ochratoxin A, dL:D-limonene
Note: grade scale for area and intensity of α 2u-Immunostaining:
0: absent, 1: present, 2: mild, 3: moderate, 4: severe and 5: markedly severe.
*Significantly different ($p<0.05$ two-way ANOVA) from controls and OTA group Rasonyi et al., 1999. Toxicol. Letters. 104: 83-92.

Table 7. Summary of the significant and trend ($0.05<p<0.10$) effects of maternal separation and sex

Paradigm	Parameter measured	Factor	p <	Effect/Trend
Bodyweight	Weight at weaning	Sex	0.001	Male heavier than females
		MS	0.001	All MS group lighter than CON
		Sex x MS	0.03	No sex difference in MS9
Open field	Activity	Sex	0.08	Females more active than males, but no such sex effect in MS18 masked an overall sex difference
Freezing: conditioning	CS conditioning	Sex x CS	0.006	Males reached asymptote faster than females
Freezing: context	Expression of context conditioning	Sex x MS	0.10	Male-specific reduction in MS9 and MS18
Freezing: tone	Expression of CS conditioning	Bins x MS	0.08	Reduction in MS4
Plus maze	Activity	Sex	0.02	Females more active than males
Active avoidance	Conditioning to CS	MS	0.06	Deficit in MS4 (males)
		Sex	0.004	Females learn faster than males
		Sex x MS	0.02	Deficit in MS4 males, accelerated learning in MS9 males
Water maze	Spatial learning (latency)	Day x Sex	0.07	Superior learning in males from day 2 onwards (within males, superior learning in MS9)
Water maze	Spatial learning (distance)	Sex	0.02	Superior learning in males
Water maze	Speed	Sex	0.03	Females faster than males
Water maze	Rule reversal	Sex	0.005	Superior learning in males
		Day x MS	0.02	Superior learning in all MS groups on day 2

Lehmann et al., 1999. Pharmacol. Biochem. Behavior. 64: 705-715.

Table 8. Mean commissue area by sex and age, ±S.D.

	Age (days)	Male	Female
The corpus callosum area (mm^2)	69-100	3.81±0.35	3.26±0.22
	101-175	4.03±0.44	3.85±0.37
	All age	3.97±0.42	3.50±0.41
The anterior commissure area (mm^2)	69-100	0.34±0.02	0.30±0.04
	101-175	0.36±0.04	0.34±0.04
	All age	0.36±0.04	0.32±0.04

Noonan *et al*., 1998. Brain Res. Bull.45:101-104.

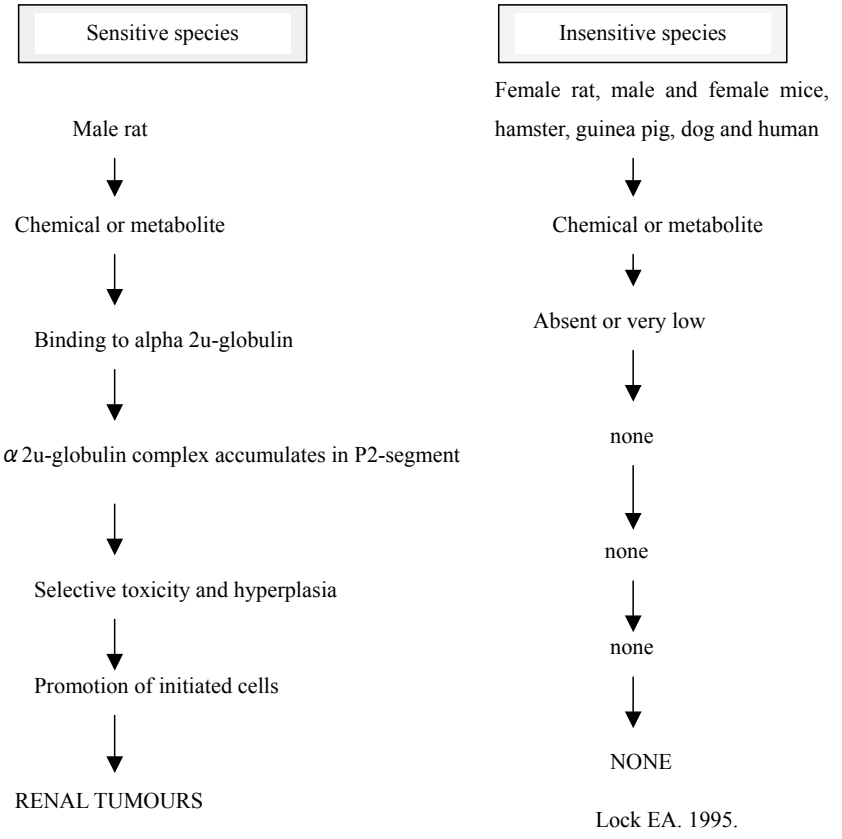

Fig. 1. Proposed basis for the species difference in renal injury and carcinogenicity produced by chemicals that cause α 2u-globulin accumulation

CHAPTER 2

General Toxicology Related To

Background Data of General Toxicological Parameters in Crj:CD(SD)IGS Rats - Body Weight, Food Consumption and Clinical Pathology -

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ABSTRACT This study was conducted to collect background data on body weight and food consumption, and routine clinical pathology and its optional parameters for Crj:CD(SD)IGS rats (both sexes at 7 ~ 11 weeks of age). Under a low-protein feed, the body weights and food consumption were close to those published in the literature. By sampling blood under isoflurane anesthesia, routine clinical pathology revealed white blood cell counts 1.2 ~ 1.3 times higher than those previously reported in conscious rats. Other routine parameters such as hematology, bone marrow examination, urinalysis and blood coagulation were the same or close to those previously reported in several rat strains. The optional parameters, including lactate dehydrogenase isoenzymes, serum total T_3 and T_4, and urinary enzymes, were also unremarkable. D-dimer measurements were all below detectable levels. The present results, therefore, give a baseline for general toxicology studies using Crj:CD(SD)IGS rats. —Key words: general toxicology, background data, isoflurane, low protein diet

CD(SD)IGS-2002/2003 : 27-34

INTRODUCTION

This study was undertaken to obtain baseline of (1) in-life data fed a low-protein feed (LPF), (2) clinical pathology data under appropriate anesthesia, and (3) optional clinical pathological parameters, because of the following reasons.

(1) LPF was developed to prevent overfeeding of the animal which results in shortened life-span. The LPF contains 18% of crude protein which is less than other 20 ~ 25% contents of commercial feed available in Japan [19]. It is also known that the composition of LPF is close to that of a feed used for general toxicology studies in foreign countries (from certificates of the diets). Therefore, the LPF can be used not only to prolong the life span but also to harmonize data with other countries.

(2) Pentobarbital, ether, or carbon dioxide is commonly used as the anesthesia in toxicological study [15, 16]. Isoflurane has been employed as inhalation anesthesia in humans [8], and has a large safety margin [6]. Raper *et al*. [24] reported that isoflurane had less effects on hepatic function than pentobarbital- and ether-anesthesia in rats. It is also important to conduct sampling under anesthesia to minimize pain for the animals. Thus, anesthesia induced with isoflurane would be appropriate for blood collection in toxicological studies.

(3) There are relatively limited data regarding optional clinical pathological parameters in Crj:CD(SD)IGS rats; *e.g*., blood hormone levels and urinary enzymatic activities in the biological reference data on Crj:CD(SD) IGS rats, except for a report by Kojima *et al*. [9] that showed the normal levels of thyroid, gonadotropic and adrenal gland hormones. These optional parameters are highly significant in investigating a target organ or the toxicological mechanism of test substances.

This study was undertaken to collect background data on body weight, food consumption and clinical pathology parameters, including optional parameters, in Crj:CD(SD)IGS rats under feeding LPF and sampling blood with isoflurane anesthesia.

MATERIALS AND METHODS

Animals:

Fifty males and 50 females IGS rats, 4 weeks old, were purchased from Charles River Japan Inc. (Hino farm). One male was removed from the study group due to a decreased body weight caused from malocclusion and overgrowth of teeth during quarantine and acclimatizing period.

Animal husbandry:

All animals were housed in one animal room set the following conditions: temperature at 23±2℃, relative humidity at 55 ± 15%, air change at 10 to 15 times per hour and 12-hour illumination (6:00 to 18:00). The animals were kept individually in metal mesh cages (260×200×180 mm); they had free access to tap water and to a commercially available low-protein pelleted laboratory animal feed (CR-LPF, Oriental Yeast Co., Ltd.; abbreviation thereafter, LPF).

Observations and examinations:

1) Mortality and clinical signs

All animals were observed daily in their cages for clinical signs of viability and any changes in appearance and behavior.

2) Body weight

Each animal was weighed weekly using an electronic balance (LC-4201, Sartorius).

3) Food consumption

The weight of animal diet given and that of the diet remaining were measured weekly for all animals using an electrical balance (LC-4201, Sartorius). Food consumption (g) per day was calculated.

4) Clinical pathology

(1) Hematology

Blood samples for hematology were collected from the jugular vein in the fasted state (15 hours) at 9 and 11 weeks of age. Blood sampling was carried out under inhalation of

isoflurane (Forane™, Abbott) with the anesthetizer (Model TK-5, Bio Machinery) at 9 and 11 weeks of age; regarding sampling at 11 weeks (at terminal necropsy), blood was collected through a small skin incision. EDTA-2K was used as the anticoagulant. The following parameters were measured with ADVIA 120 (Bayer-Medical Co., Ltd.); red blood cell counts (RBC), hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte counts, platelet counts, mean platelet volume (MPV), white blood cell counts (WBC), and the differential of WBC [neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells].

(2) Blood coagulation

Blood samples for blood coagulation were collected from the abdominal vein at a ratio of 1 vol. of 3.8% sodium citrate and 9 vol. of blood in the fasted state (15 hours) under inhalation of isoflurane at 11 weeks of age (at terminal necropsy). Plasma was separated from blood by centrifugation (approximately 1500 × g, 10 min, 4 ℃). Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were determined using a full automated coagulometer (STA Compact, Roche Diagnostics). D-dimer was determined using human clinical latex agglutination reagents (D-Di Test, Roche Diagnostics) [12].

(3) Blood chemistry 1 (Serum chemistry)

Blood samples for serum chemistry were collected in the same way as for hematology. Serum was separated from blood by centrifugation (approximately 1500×g, 10 min, 4℃). The following assays were performed using an automatic analyzer (7070, Hitachi); alanine aminotransferase (ALT) (UV-rate method), aspartate aminotransferase (AST) (UV-rate method), alkaline phosphatase (ALP) (p-nitrophenyl phosphate substrate method), γ -glutamyl transferase (GGT) (γ -glutamyl-3-carboxy-4-nitroanilid substrate method), total protein (Biuret method), albumin (bromocresol green method), total bilirubin (enzymatic assay), total cholesterol (enzymatic assay), glucose (glucose dehydrogenase method), blood urea nitrogen (BUN) (urease UV method), creatinine (enzymatic assay) and calcium (Ca) (o-cresolphthalein complexone method), sodium (Na), potassium (K) and chloride (Cl) (ion-selective electrode method). Globulin and the albumin/globulin (A/G) ratio were calculated from total protein and albumin values. A standard made from rat serum (Wako Pure Chemical Co.) was used to calibrate measurement for total protein and albumin. Another standard used for human clinical examination (Qualitrol HS P, Merck) was also employed to compare the results obtained with the rat serum standard.

Total T_3 and total T_4 were measured using the automated chemiluminescent immunoassay method and an automated system (Immulyze, Diagnostic Products Corporation).

Fractional analysis of serum protein was carried out manually by cellulose acetate electrophoresis and an accompanying reagent system (Titan III and Ponceau S, Helena Laboratories).

(4) Blood chemistry 2 (Plasma chemistry)

Blood samples for measuring lactate dehydrogenase (LDH) and creatine kinase (CK) were collected from the abdominal vein with heparin in the fasted state (15 hours) at 11 weeks of age (at terminal necropsy). Plasma was separated from blood by centrifugation (approximately 1500 × g, 10 min, 4℃). These assays were performed using the UV-rate method using an automatic analyzer (7070, Hitachi). Analysis for LDH isoenzymes was carried out manually by agarose gel electrophoresis using the accompanying reagent system (Titan Gel S-LD Plate and S-LD Reagents, Helena Laboratories).

(5) Urinalysis

Urinalysis was carried out at 9 and 11 weeks of age. Urine was collected individually in a metabolic cage for 15 hours at room temperature. The rats were fasted during the urine collection, but were allowed free access to water. The volume of urine was measured using a cylinder. Urinary pH was measured using a reagent strip (Multistix test paper, Miles-Sankyo Co. Ltd.) and an automatic urine analyzer (Clinitek 500, Bayer-Medical Co., Ltd.). Specific gravity was measured with a refractometer (TS-SE, Atago Co., Ltd). Creatinine, Na, K, Cl, LDH and GGT were determined with the methods same as serum-chemistry. N-acetyl-β-D-glucosaminidase (NAG) was measured using the m-cresolslfonphthaleinyl-N-acetyl-β -D-glucosaminide substrate method using the automatic analyzer (7070, Hitachi). Alpha-glutathione S-transferase (GST) was measured using an enzyme immunoassay kit (Rat alpha GST EIA, Biotrin).

(6) Bone marrow examination

Bone marrow examination was conducted at 11 weeks of age. Before necropsy, all animals were fasted overnight (15 hours) and were exsanguinated by bleeding under isoflurane inhalation. The bone marrow cells were collected from the femur. Bone marrow smears prepared by the Cytospin-4 (Thermo Shandon) were stained with May-Grünwald-Giemsa. The results were expressed as a percentage of each cell type, according to the descriptions of Hulse [7], Matsumoto and Shirai [13], and Moore [18].

RESULTS AND DISCUSSION

The results regarding body weight and food consumption are shown in Tables 1 and 2. Body weight and food consumption in this study were close to those found in the previous studies using the same feed [19, 20, 30]. There were no mortality and no abnormal clinical signs.

Table 3 shows the data on hematological parameters. The results on hematology were well coincident with our previous results on Crj:CD(SD)IGS rats under conscious state [10], except only for a slight increase in WBC (1.2 ~ 1.3-times vs. conscious state) due to increases in neutrophils and lymphocytes. This might be pseudoneutrophilia caused by excitement [26], endogenous hormones [23] and/or opiates [4]. As supporting this, differential cell ratio in bone marrow (Table 4) showed normal values in rats; for example, the ME ratio was 1.2 in this study which was within the range (ME: 1.16 ~ 1.36) previously published in inbred albino strain [7].

The Advia 120 can automatically measure mean platelet volume (MPV). Although the significance of MPV has not been fully established in toxicological evaluation, the larger platelets are assumed to be younger and more active [11], thus expecting that a change in MPV reflects a functional change caused by test substances.

D-dimer, a parameter of fibrin degradation, is a marker of thrombosis and hemorrhage, since D-dimer appears only after fibrin generation (*i.e*., at the end of the blood coagulation cascade). Laudes *et al*. [12] measured rat D-dimer using a reagent system employed in human clinical practice (D-Di Test, Roche Diagnostics). We recently confirmed that this reagent system detects the fibrinolytic substance in rat plasma [29]. As shown in Table 5, the fibrinolytic substance was under the detectable level in all animals tested.

Table 6 shows the results in blood chemistry. Matsuzawa *et al*. [15] have reported a wide variation in rat total protein, albumin and A/G ratio among the laboratories, due to differences in control standards and reagents. Takano *et al*. [27] pointed out a marked high level in rat A/G ratio when a human clinical standard is used for calibration. When calibrated with the rat serum standard, the A/G ratio was approximately 0.8. This was relatively similar to that obtained by electrophoresis (1.1 ~ 1.4 of A/G ratio), but considerably different from that calibrated with a human clinical standard (approximately 2.5 of A/G ratio in the footnote of Table 6). Our results again emphasize the importance to use a suitable standard in measuring rat total protein, albumin and A/G ratio.

ALP activity was lower at 11 weeks than that at 9 weeks. This supports the age-dependent decrease in ALP in rats [28] and our previous finding in Crj:CD(SD)IGS rats [10].

When determining LDH activity, care must be taken in pre-analytical treatment; *e.g*., anticoagulant, centrifugation and storage [14]. The changes in LDH activity and its isozyme, however, are helpful in detecting target organs, particularly heart [1, 2, 22]. Preus *et al*. [22] reported marked increases in LDH1 and LDH2 isoenzymes in rats with heart failure. As shown in Table 6, LDH5 was dominant (approximately 90%), while LDH1 and LDH2 were considerably low (<3%) in Crj:CD(SD)IGS rats. Obvious changes in LDH1 and LDH2 isoezymes are expected in case of heart damage in Crj:CD(SD)IGS rats.

It is generally known that hepatic microsomal enzyme induction leads to thyroid follicular cell hyperplasia and neoplasia via perturbation of T_3 and T_4 in rats [3]. Total T_4 level was 10 ~ 20 times higher than total T_3 in Crj:CD(SD)IGS rats (Table 6). These levels measured by chemiluminescent immunoassay were well coincident with those obtained by enzyme immunoassay in Crj:CD(SD)IGS rats [9].

The results of urinalysis are shown in Table 7. Males had approximately 1.6-times larger urinary volume than females. This difference would simply be reflected by a difference in body size between sexes (Table 1). Almost all gender differences found in the parameter tested were explained by this body-size difference, except alpha-GST in which males had considerably higher levels than females. The reasons of the gender difference in alpha-GST were uncertain in this study.

To measure urinary enzymes is of great importance to detect renal injury in toxicological studies [17, 21]. The levels of urinary enzymes in urine samples collected for 15 hours under room temperature were similar to those previously reported with the same methods; *e,g*., urinary LDH in the male Wistar rats [25], urinary NAG in Crj:CD(SD)IGS rats [10], urinary GGT in Sprague-Dawley rats [5]. Although discussion on ideal sampling methods for urine is ongoing [17], the present data indicate a baseline in evaluating renal toxicity of test substances.

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Table 1. Body weight in Crj:CD(SD)IGS rats

Age (Weeks)	Body weight (g)					
	Male			Female		
	Mean	SD	n	Mean	SD	n
7	258.0	±13.3	(49)	182.5	±16.7	(50)
8	312.3	±17.5	(49)	206.8	±19.4	(50)
9	352.1	±20.8	(49)	223.0	±20.9	(50)
10	384.5	±24.7	(49)	237.4	±23.2	(50)
11	411.6	±28.0	(49)	248.0	±24.8	(50)

Table 2. Food consumption in Crj:CD(SD)IGS rats

Age (Weeks)	Food consumption (g/day)					
	Male			Female		
	Mean	SD	n	Mean	SD	n
7 - 8	28.01±1.79		(49)	19.77±2.41		(50)
8 - 9	29.16±2.14		(49)	20.29±2.58		(50)
9 - 10	30.36±2.36		(49)	21.73±2.27		(50)
10 - 11	29.56±2.38		(49)	20.88±2.51		(50)

Table 3. Hematological parameters in Crj:CD(SD)IGS rats

Parameters	Male						Female					
	9 weeks			11 weeks			9 weeks			11 weeks		
	Mean	SD	(n)	Mean	SD	(n)	Mean	SD	(n)	Mean	SD	(n)
WBC ($x10^3/mm^3$)	12.336±2.924		(48)	11.460±2.887		(49)	9.961±2.596		(49)	9.478±2.857		(50)
Neutrophils (Count) ($/mm^3$)	2032.9±786.5		(48)	1776.4±678.2		(49)	1475.6±637.1		(49)	1343.6±583.7		(50)
Lymphocytes (Count) ($/mm^3$)	9678.0±2230.9		(48)	8975.1±2356.2		(49)	7996.3±2213.4		(49)	7575.2±2395.0		(50)
Monocytes (Count) ($/mm^3$)	384.0±133.8		(48)	431.6±143.7		(49)	275.4±88.6		(49)	305.6±109.3		(50)
Eosinophils (Count) ($/mm^3$)	96.2±42.2		(48)	126.8±48.5		(49)	108.2±43.2		(49)	129.6±41.3		(50)
Basophils (Count) ($/mm^3$)	49.0±18.7		(48)	45.5±18.3		(49)	37.9±18.4		(49)	35.6±17.4		(50)
Large Unstained Cells (Count) ($/mm^3$)	94.8±60.6		(48)	104.2±85.6		(49)	69.3±25.6		(49)	88.7±52.8		(50)
Neutrophils (%)	16.31±3.95		(48)	15.61±4.77		(49)	14.85±4.73		(49)	14.26±4.70		(50)
Lymphocytes (%)	78.65±4.14		(48)	78.23±4.92		(49)	80.12±5.15		(49)	79.75±4.81		(50)
Monocytes (%)	3.11±0.71		(48)	3.79±0.93		(49)	2.86±0.99		(49)	3.28±0.97		(50)
Eosinophils (%)	0.79±0.29		(48)	1.11±0.32		(49)	1.12±0.43		(49)	1.42±0.42		(50)
Basophils (%)	0.39±0.11		(48)	0.39±0.10		(49)	0.38±0.13		(49)	0.36±0.10		(50)
Large Unstained Cells (%)	0.74±0.31		(48)	0.86±0.55		(49)	0.69±0.19		(49)	0.93±0.52		(50)
RBC ($x10^6/mm^3$)	7.955±0.360		(48)	8.086±0.397		(49)	7.864±0.306		(49)	7.772±0.243		(50)
Hemoglobin (g/dL)	15.58±0.63		(48)	15.21±0.54		(49)	15.36±0.48		(49)	14.99±0.41		(50)
Hematocrit (%)	45.24±1.74		(48)	43.74±1.65		(49)	43.23±1.41		(49)	41.80±1.22		(50)
MCV (fL)	56.89±1.37		(48)	54.15±1.44		(49)	55.03±1.56		(49)	53.80±1.32		(50)
MCH (pg)	19.59±0.50		(48)	18.84±0.49		(49)	19.54±0.56		(49)	19.29±0.52		(50)
MCHC (g/dL)	34.43±0.46		(48)	34.79±0.50		(49)	35.52±0.48		(49)	35.86±0.50		(50)
Reticulocyte ($x10^3/mm^3$)	293.56±36.98		(48)	230.76±40.65		(49)	187.48±29.08		(49)	190.13±34.27		(50)
Reticulocyte (%)	3.70±0.52		(48)	2.87±0.63		(49)	2.39±0.39		(49)	2.45±0.44		(50)
Platelets ($x10^3/mm^3$)	1244.2±116.0		(45)	1200.9±117.4		(49)	1251.7±129.8		(48)	1198.0±126.5		(50)
MPV (fL)	7.35±0.40		(45)	7.30±0.72		(49)	7.08±0.36		(48)	6.66±0.41		(50)

Table 4. Differential cell counts of femoral bone marrow in Crj:CD(SD)IGS rats

Bone marrow cells	Differential cell counts (%)	
	Male	Female
	Mean SD	Mean SD
Proerythroblast	1.2±0.7	1.1±0.6
Basophilic erythroblast	4.0±1.8	4.1±1.4
Polychromatic erythrobalst	29.7±4.2	30.1±5.2
Myeloblast	0.8±0.5	0.5±0.4
Promyelocyte	2.4±1.0	2.6±1.0
Myelocyte	6.5±1.9	6.5±1.0
Metamyelocyte	4.9±1.4	4.0±1.6
Band, Neutrophil	3.8±2.2	3.8±1.8
Segmented, Neutrophil	19.4±6.0	19.3±3.3
Eosinophil（Immature)	2.0±1.1	3.0±1.2
Eosinophil	0.4±0.4	0.8±1.1
Basophil (Immature)	0.7±0.3	0.4±0.5
Basophil	0.5±0.4	0.4±0.4
ME ratio	1.2±0.3	1.2±0.2
Monocyte	1.5±0.8	1.4±0.7
Macrophage	0.7±0.5	1.0±0.6
Lymphocyte	20.1±2.5	22.1±3.9
Plasma cell	0.4±0.4	0.5±0.7
Megakaryocyte	0.4±0.3	0.6±0.4
Mast cell	0.3±0.4	0.1±0.2
Reticulum cell	0.4±0.5	0.3±0.5

The bone marrow smears were prepared from the last 10 animal number in each sex (n=10).

Table 5. Blood cogulation data in Crj:CD(SD)IGS rats

Parameters	Male		Female	
	11 weeks		11 weeks	
	Mean SD	(n)	Mean SD	(n)
PT (sec)	17.21±0.91	(49)	16.70±0.56	(50)
APTT (sec)	18.28±1.47	(49)	17.41±1.11	(50)
Fibrinogen (mg/dL)	285.1±21.0	(49)	219.9±18.7	(50)
D-Dimer[a] (μg/mL)	< 0.5 (49/49)		< 0.5 (50/50)	

a: Reaction with antibody against human D-dimer (D-Di Test, Roche Diagnostics)

Table 6. Blood chemistry data in Crj:CD(SD)IGS rats

Parameters	Male				Female			
	9 weeks		11 weeks		9 weeks		11 weeks	
	Mean SD	(n)	Mean SD	(n)	Mean SD	(n)	Mean SD	(n)
ALT (IU/L)	32.0±4.1	(49)	29.3±5.2	(49)	27.3±5.3	(50)	25.3±4.5	(50)
AST (IU/L)	96.4±12.9	(49)	76.8±9.6	(49)	99.3±19.3	(50)	76.5±8.7	(50)
ALP (IU/L)	578.2±109.2	(49)	405.3±76.0	(49)	343.1±75.4	(50)	247.2±56.2	(50)
GGT (IU/L)	1.0±0.0	(49)	1.0±0.0	(49)	1.0±0.1	(49)	1.0±0.3	(50)
CK (IU/L)	NT		159.2±23.9	(49)	NT		126.8±29.8	(50)
LDH (IU/L)	NT		320.3±90.2	(49)	NT		187.9±74.7	(50)
			Electrophoresis					
LDH 1 (%)	NT		2.2±0.9	(15)	NT		3.1±1.3	(15)
LDH 2 (%)	NT		1.0±0.3	(15)	NT		1.6±1.6	(15)
LDH3 (%)	NT		1.4±0.6	(15)	NT		1.5±0.7	(15)
LDH4 (%)	NT		6.5±3.5	(15)	NT		6.7±3.3	(15)
LDH 5 (%)	NT		88.9±4.1	(15)	NT		87.1±4.3	(15)
Total Protein[a] (g/dL)	6.23±0.22	(49)	6.19±0.26	(49)	6.65±0.29	(48)	6.67±0.37	(50)
Albumin[b] (g/dL)	2.79±0.09	(49)	2.67±0.10	(49)	3.08±0.13	(47)	3.06±0.19	(50)
Globulin (g/dL)	3.43±0.19	(49)	3.52±0.20	(49)	3.57±0.19	(47)	3.61±0.23	(50)
A/G Ratio[c]	0.817±0.048	(49)	0.758±0.042	(49)	0.865±0.037	(47)	0.847±0.045	(50)
			Electrophoresis					
Albumin (%)	NT		52.0±2.7	(15)	NT		57.8±1.3	(15)
α 1-Globulin (%)	NT		18.0±1.6	(15)	NT		14.7±1.3	(15)
α 2-Globulin (%)	NT		6.5±0.7	(15)	NT		5.7±1.0	(15)
β -Globulin (%)	NT		17.2±1.0	(15)	NT		15.3±1.1	(15)
γ -Globulin (%)	NT		6.3±1.7	(15)	NT		6.5±0.7	(15)
Total Bilirubin (mg/dL)	0.10±0.00	(49)	0.10±0.00	(49)	0.10±0.02	(49)	0.10±0.01	(50)
Cholesterol (mg/dL)	80.0±14.5	(49)	68.9±13.3	(49)	88.7±17.2	(47)	87.1±16.9	(50)
Glucose (mg/dL)	99.5±9.4	(49)	116.8±12.5	(49)	97.1±9.4	(49)	115.2±15.0	(50)
BUN (mg/dL)	12.6±1.5	(49)	12.8±1.7	(49)	13.7±1.8	(49)	13.7±2.3	(50)
Creatinine (mg/dL)	0.22±0.04	(49)	0.24±0.05	(49)	0.25±0.06	(49)	0.31±0.05	(50)
Ca (mg/dL)	10.83±0.31	(49)	10.58±0.29	(49)	10.76±0.32	(49)	10.64±0.33	(50)
Na (mmol/L)	143.9±1.2	(49)	144.7±1.1	(49)	143.1±1.1	(49)	144.1±1.2	(50)
K (mmol/L)	4.83±0.25	(49)	4.53±0.24	(49)	4.57±0.20	(49)	4.02±0.37	(50)
Cl (mmol/L)	100.4±1.2	(49)	101.1±1.1	(49)	101.6±1.2	(49)	102.8±1.2	(50)
Total T3 (ng/dL)	NT		73.8±14.9	(30)	NT		83.7±15.7	(30)
Total T4 (ug/dL)	NT		6.8±1.3	(22)	NT		4.0±1.2	(22)

LDH and CK were measured in plasma, and others are in serum.
NT: Data were not taken.
a: Total protein concentration (g/dL) with a human standard were 6.01 ± 0.25 (n=48) in male at 11 weeks of age and 6.75 ± 0.13 (n=25) in female at 9 weeks of age.
b: Albumin concentration (g/dL) with a human standard were 4.33 ± 0.16 (n=48) in male at 11 weeks of age and 4.82 ± 0.11 (n=25) in female at 9 weeks of age.
c: A/G ratio with a human standard were 2.574 ± 0.176 (n=48) in male at 11 weeks of age and 2.501 ± 0.166 (n=25) in female at 9 weeks of age.
LDH isozyme, protein electrophoresis, total T_3 and total T_4 were measured in animals basically with the first 22 ~ 30 number in each sex.

Table7. Urinalysis data in Crj:CD(SD)IGS rats

Parameters	Male				Female			
	9 weeks		11 weeks		9 weeks		11 weeks	
	Mean SD	(n)	Mean SD	(n)	Mean SD	(n)	Mean SD	(n)
Volume (mL)	18.00±12.08	(46)	17.62±9.20	(49)	11.42±6.19	(50)	11.60±9.64	(50)
Specific gravity	1.0214±0.014	(46)	1.0231±0.0115	(49)	1.0231±0.0127	(50)	1.0329±0.0226	(50)
pH	6.79±0.36	(46)	6.77±0.27	(49)	6.30±0.38	(50)	6.44±0.33	(50)
Creatinine (mg/dL)	52.31±35.99	(46)	62.51±34.02	(49)	48.66±27.62	(50)	72.73±55.21	(50)
Creatinine (15 hr excretion) (mg)	6.345±0.908	(46)	8.399±0.711	(49)	4.146±0.685	(50)	4.778±0.800	(50)
Na (mmol/L)	23.8±16.4	(46)	23.9±14.0	(49)	25.6±14.7	(50)	30.0±21.3	(50)
Na (15 hr excretion) (mmol)	0.313±0.139	(46)	0.348±0.159	(49)	0.229±0.100	(50)	0.231±0.107	(50)
K (mmol/L)	112.27±76.16	(46)	106.73±54.82	(49)	80.45±46.02	(50)	118.18±89.88	(50)
K (15 hr excretion) (mmol)	1.364±0.243	(46)	1.455±0.264	(49)	0.688±0.174	(50)	0.789±0.195	(50)
Cl (mmol/L)	27.8±24.1	(46)	23.0±14.8	(49)	20.2±15.0	(50)	27.9±29.2	(50)
Cl (15 hr excretion) (mmol)	0.326±0.113	(46)	0.309±0.123	(49)	0.169±0.089	(50)	0.186±0.095	(50)
N-acetyl-β-D-glucosaminidase (NAG) (U/L)	11.70±8.35	(46)	12.23±8.55	(49)	13.47±7.72	(50)	17.42±13.86	(50)
NAG (15 hr ecretion) (U)	0.1419±0.0438	(46)	0.1542±0.1199	(49)	0.1168±0.0391	(50)	0.1201±0.0365	(50)
Aplha-GST (μg/L)	179.9±46.3	(20)	188.6±52.9	(19)	55.7±42.9	(20)	38.2±30.6	(20)
Alpha-GST (15 hr excretion) (μg)	2.21±1.22	(20)	3.22±1.16	(19)	0.56±0.54	(20)	0.42±0.39	(20)
LDH (IU/L)	16.2±7.5	(20)	NT		13.1±6.1	(20)	NT	
LDH (15 hr excretion) (IU)	0.167±0.050	(20)	NT		0.107±0.027	(20)	NT	
GGT (IU/L)	207.5±223.6	(20)	NT		369.0±343.0	(20)	NT	
GGT (15 hr excretion) (IU)	1.841±1.448	(20)	NT		2.501±1.298	(20)	NT	

NT: Data were not taken.
Urine enzymes, except NAG, were measured in animals with the first 20 number in each sex.

Urinary Protein Profiles in Crj:CD(SD)IGS Rats Determined using ProteinChip Technology

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ABSTRACT. Urinary protein profiles in IGS rats aged from 5 to 60 weeks were examined using the ProteinChip system. Results showed that protein excretion in young-adult and adult male IGS rats consisted mainly of low molecular weight species, with the major protein identified as $\alpha_{2\mu}$-globulin (18.7kD). $\alpha_{2\mu}$-Globulin levels in the urine of male IGS rats increased rapidly at puberty and then gradually between 10 and 60 weeks of age. Female rats, which excrete little or no $\alpha_{2\mu}$-globulin, exhibited a much lower level of proteinuria than males up until age 60 weeks. —Key words: urinary protein; $\alpha_{2\mu}$-globulin; ProteinChip

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INTRODUCTION

The recent development of surface-enhanced laser desorption/ionization (SELDI)-time of flight (TOF) technology, based on improvements in methods for the chemical preparation of absorptive surfaces and their use for solid-state mass spectrometry, allows high-throughput protein analysis of crude biologic samples. The potential advantages of SELDI ProteinChip technology include rapidity and reproducibility in the screening of protein expression profiles known as "phenomic fingerprints." In general, this technology can be used to provide phenomic fingerprints of complex protein mixtures. The utility (accuracy, reproducibility, and sensitivity) of this technique in studies of urinary protein composition have been well established[4]. However, there are few published data on the use of this technique in studies of urinary protein profiles in rats[3,4].

Urinary protein is a sensitive indicator of renal toxicity and an important parameter in toxicity evaluation. $\alpha_{2\mu}$-Globulin nephropathy is a well-known renal syndrome that occurs exclusively in male rats [5,7,8,12]. $\alpha_{2\mu}$-Globulin is the major urinary protein excreted by adult male rats [1,2,5,6], and is not synthesized by any other species. Although the biological function of this protein is not well defined, it is clear that $\alpha_{2\mu}$-globulin contributes to the development of a male rat-specific nephrotoxicity which manifestes acutely as the excessive accumulation of protein in renal phagolysosomes [5,7]. The prerequisite step in the development of this renal toxicity is the ability of a chemical to bind to $\alpha_{2\mu}$-globulin [7]. The inter-action between these chemicals and $\alpha_{2\mu}$-globulin is very specific, and there is convincing evidence that $\alpha_{2\mu}$-globulin is the only protein involved in this syndrome. Specifically, no other protein accumulates within the droplets in the male rat kidney [3]. Moreover, male rats that do not synthesize $\alpha_{2\mu}$-globulin do not develop this nephropathy.

In the present study, using SELDI-ProteinChip technology, age-related urinary protein profiles were investigated in IGS male and female rats at 5-60 weeks of age, mainly based on the $\alpha_{2\mu}$-globulin.

MATERIALS AND METHODS

Animals and animal care

Six male and 6 female Crj:CD(SD) IGS rats were purchased from Charles River Japan Inc (Atsugi, Japan) at the age of four weeks. Groups of three animals were maintained in suspended-metal cages(42^{L}x25^{W}x 22^{H}cm) with a commercial pellet diet (CRF-1, Oriental Yeast Industries,Tokyo) and tap water available ad libitum. The animal room was air-conditioned and maintained at 23±3℃ with a 13-hr fluorescent light cycle (0800-2100 hr). Clinical observations and mortality checks were performed on all animals daily. Body weight was measured monthly. Rats were used at age of 5('young'), 10('young-adult'), 20, 30 and 60 ('adult') weeks for a longitudinal study of proteinuria pattern.

Collection and preparation of urine samples

For urine collection, rats aged 5-60 weeks of age were placed in individual metabolic cages. One-hour urine samples (0800-0900 hr) were collected on ice without preservative and centrifuged for 5 min at 3000 x g. The supernatants obtained were stored at -80℃ until use, at which time they were slowly thawed on ice. Samples collected at the same time points from 3 animals were pooled so that equal volumes were obtained for males and females, and supplied for analysis. Total urinary protein concentration was estimated using the micro-TP test Wako (Wako Pure Chemical Industries, Ltd., Osaka).

SELDI-TOF Protein Analysis

SELDI analysis was performed using weak cation-exchange (WCX2) ProteinChip arrays (Ciphergen Biosystems, Fremont, CA, USA). The ProteinChip arrays were fitted into a bioprocessor (Ciphergen Biosystems), a well-forming device, to facilitate the loading of urine sample volumes up to 50 μL onto each activated spot. Prior to sample application, the spots on each ProteinChip array were conditioned initially equilibrated twice using 50 mM sodium acetate (pH 4.5) at ambient temperature and nonbound proteins and other contaminants were washed from the ProteinChip array with deionized distilled water, an energy- absorbing molecule (Sinapinic acid, Ciphergen) was applied in solvent containing acetonitrile and 0.5% trifluoroacetic acid, and the sample was allowed to dry. After insertion of the ProteinChip array into the ProteinChip reader, a laser beam was focused on the sample *in vacuo*. This caused the proteins absorbed to the matrix to become ionized and, simultaneously, to be desorbed from the ProteinChip array surface. The ionized proteins were detected and their molecular masses were determined using TOF analysis. TOF

mass spectra were collected with a ProteinChip System (PBS II series; Ciphergen) using Ciphergen Peaks (versions 2.0) software. Spectra were collected in the positive-ion mode. Real-time signal averages of 70 laser shots were used to generate each spectrum. The ProteinChip system was calibrated with horse Cytochrome C (12.4 kD), horse myoglobin (17.0 kD), rabbit GAPDH (35.7 kD), bovine albumin (66.4 kD) and β -galactosidase (116.4 kD) (all from Ciphergen Biosystems, Fremont, CA, USA).

RESULTS

On comparison, levels of urine protein were 10 times higher in males than in females at any time, and increased with advancing age (data not shown).

The representative SELDI-TOF protein profiles of urine of male rats of different ages are shown in Figure 1.

Under 5 kD, a greater number of components were observed in both males and females.

In the 5-30 kD range, the protein profiles of urine from male rats indicated that the most prominent peaks occurred at 8.6, 11.6 and 18.7 kD. The major protein identified were of the α_2-microglobilin family (18.7 kD). Levels of $\alpha_{2\mu}$-globulin in the urine of male rats increased rapidly after age 5 weeks, and then gradually between 10 and 60 weeks. This protein was absent in female and immature rats. Other low-molecular weight proteins were estimated as rat urinary protein 1 (8.6 kD) and β_2-microglobulin (11.6 kD). In young-adult and adult rats protein excretion consisted mainly of low molecular weight species.

Over 30 kD, the major proteins was albumin (66 kD) in both male and female(data not shown).

DISCUSSION

The purpose of this study was to investigate variations in the urinary protein profile in IGS rats using the ProteinChip. A striking difference was observed in the pattern of urine from males and females. The former was characterised by large amounts of proteins from the $\alpha_{2\mu}$-globulin multigene family (approximately18 kDa), whereas the latter contained a greater number of components under 10 kDa and albumin. The present urinary data by the ProteinChip technology are in accordance with previously reported data using electrophoresis [1,11] or HPLC [6].

$\alpha_{2\mu}$-Globulin are the major urinary proteins excreted by adult male albino rats [2,9,12], and it has been suggested that, because of their abundance, they also serve important physiological functions. $\alpha_{2\mu}$-Globulin are synthesized and secreted by the hepatic parenchyma cells and is rapidly filtered through the kidneys into the urine. Because of its rapid filtration rate, $\alpha_{2\mu}$-globulin represents only 0.5% of the serum proteins. $\alpha_{2\mu}$-Globulin actually consists of a family of low-molecular-weight proteins. They are resolved into two distinct molecular forms (Mr 18,800 and 18,100) by SDS polyacrylamide gel electrophoresis [13]. The ratio of the two molecular forms of $\alpha_{2\mu}$-globulin varies in different strains of rats. Five major and two minor isoelectric variants of $\alpha_{2\mu}$-globulin within the total hepatic proteins also have been identified by 2-dimensional polyacrylamide gel electrophoresis [10]. Given the ligand binding function of the $\alpha_{2\mu}$-globulin superfamily, it is thought that $\alpha_{2\mu}$-globulin contributes to the transport of pheromones into the urine. Although the physiological function of $\alpha_{2\mu}$-globulin is not well established, it is clear that this protein is central to the devel-opment of chemically induced hyaline droplet nephropathy in male rats [5,7]. $\alpha_{2\mu}$-Globulin is an androgen-dependent protein, which appears at puberty (approximately 6 weeks of age). It is absent in female and immature rats [13]. Hepatic synthesis of $\alpha_{2\mu}$-globulin in the male rat begins at puberty (about 40 days), reaches a peak level at about 80 days, and ceases at about 750-800 days of age. Because the levels and synthesis of $\alpha_{2\mu}$-globulin decreased dramatically with increasing age in F344 rats [13], it is also considered a senescence biomarker. However, in the present study in IGS rats, analysis of urine in aged rats older than 60 weeks was not conducted, and further investigation is considered necessary.

Reagents for analytical quantitation of the protein are not commercially available at this time. Our results confirm the existence of an age- and sex-dependent pattern of proteinuria in IGS rat. The present method should be useful in studying the renal handling of urinary proteins under normal and nephrotoxic conditions.

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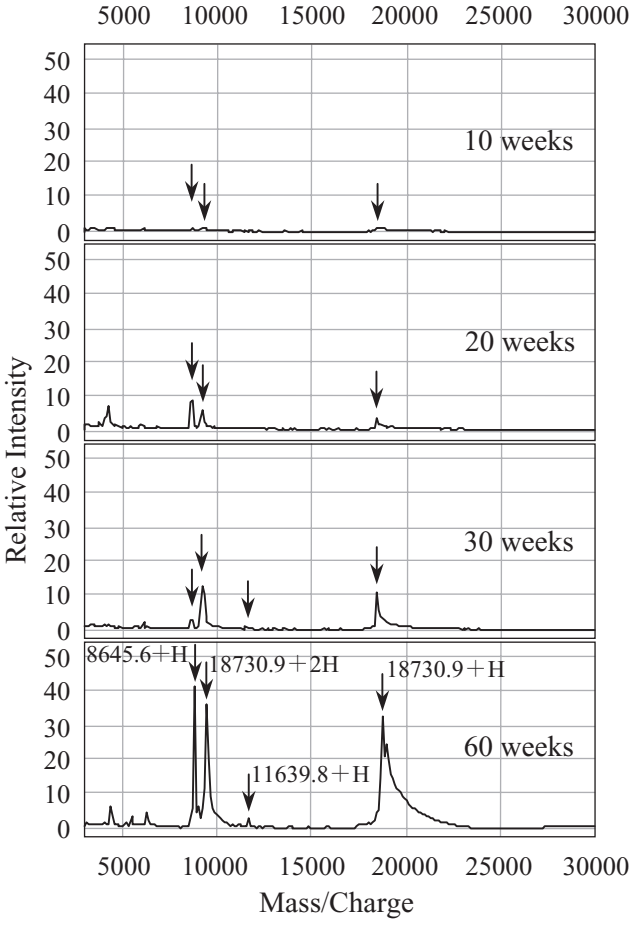

Figure.1 SELDI-TOF protein profiles of urine samples from male IGS rats on WCX-2 Proteinchip array. The peak at 9365 D (18730.9+2H) represents doubly charged $\alpha_{2\mu}$-globulin (18730.9).

Alteration of Thyroid Hormone in Crj: CD (SD) IGS Rats by Repeated Administration of Erythrosine

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ABSTRACT. Thyrotoxicity of erythrosine (ERY) was evaluated by gavage administration to male Crj:CD (SD) IGS rats at 150 and 450 mg/kg. Six dose groups were used. Each three groups, blood sample collected from the orbital sinus (OS groups) and the abdominal aorta (AA groups), were used for serial houmone assay in two different blood sampling ways. Body weight, food consumption and serum levels of triiodothyronine (T3), thyroxine(T4), free T3(fT3), free T4(fT4) and thyroid-stimulating hormone (TSH) were measured on the 3rd day prior to administration and the 1st, 4th, 8th, 11th and 14th day of administration period. The liver, kidney, thyroid gland and pituitary gland of animals administered ERY were weighed and examined histopathologically. The hormone levels of animals in OS and AA groups had some differences, but ERY treated hormonal changes in OS and AA groups were similar. Relatively, T3 level tended to be decreased and fT3 level was decreased but not statistically significant in OS groups. T4 and fT4 levels in ERY treated groups were significantly increased in both OS and AA groups. TSH level was increased but not statistically significant in ERY treated animals. In addition, mean body weight, food consumption, water consumption, urinary volume and urinary calcium concentration were increased significantly in ERY treated animals in OS groups in a dose-dependent mammer but not in AA groups. Organ weights of ERY treated OS and AA groups showed no significant differences when compared to their respective control values, and no obvious histopathological changes were found in any organs.

Subsequently, an in vitro rat gastric fundus test was performed to examine the direct effect of ERY on the gastric tract. The gastric fundus strip contracted in the presence of ERY at concentrations of 2 mg/mL or more.

The present results indicated that ERY disturbed pituitary-thyroid axisin Crj: CD(SD)IGS male rats as well as promoted rat's appetite. The appetizing effect was induced by facilitated motility of the stomach. — Key words: erythrosine B, thyroid hormone, thyroid-stimulating hormone, appetite, gastric fundus

CD(SD)IGS-2002/2003 : 38-43

INTRODUCTION

Erythrosine B, also known as FD & C red No. 3, or 2'4'5' 7'-tetraiodofluorescein (ERY), is one of the seven coloring food additives permitted under the Food and Drug Act of 30 June 1906 [1]. As its high iodine content, approximately 60% by weight, previous studies had concerned about its effects on endocrine functions, and found that its oral administration caused elevations of serum protein bound iodine (PBI) concentration in animals [2, 3]. Subsequent studies showed that there were statistically significant increases in the incidence of benign thyroid tumors after chronic ERY exposure [4, 5]. Some studies of ERY on thyroid function indicated that ERY disturbed rat thyroid functions and increased TSH, T4 and rT3 (reverse T3), and decreased T3. These findings were consistent with an inhibition of the deionization in the 5'-position of T4 and rT3 by ERY, resulting in a decreased production of T3 from T4 and a decreased deiodination of rT3 respectively [6-9].

The present study was conducted to confirm the disturbing effects by ERY of thyroid functions in Crj CD(SD) IGS rats.

MATERIALS AND METHODS

Chemicals

Erythrosine (ERY, disodium salt, Lot. No.03626MU) was purchased from Aldrich Chem. Co. Inc. ERY was dissolved in water for injection (Hikari Pharmacol. Indust. Co.Inc.)

Animals

Male Sprague-Dawley [Crj:CD (SD) IGS] rats, 8 to 9 weeks of age, 240 to 330 g, were obtained from Chlarles River Japan, Inc. The animals were housed individually in stainless steel hanging cages with free access to food (CE-2, pellet food from CLEA Japan Inc.) and tap water in a room with an automatic light cycle set to 12 hr of light (7:00am to 7:00pm) and 12 hr of dark. Temperature (21 to 25℃) and relative humidity (40.0 to 75.0 %) were monitored by hygrothermographs.

In vivo experiment

Experimental procedures.

After 7 days of the acclimation period, rats were divided into 6 groups randomly based on the body weight of the last day of acclimation period, 3 groups included 8 rats each and other 3 groups included 12 rats each. Three 8-animal groups were used as OS groups which included the control group, 150 mg/kg and 450 mg/kg ERY groups. Blood collection for hormone assay was performed on the 1st, 4th, 8th and 14th day in all the animals of OS groups. Blood samples of the animals in OS groups were collected from the orbital sinus in every animal in every sampling point. Three other groups consisted of the same dose groups as OS groups were used as AA groups. Blood samples of the animals of AA group were collected from 3 animals of each dosage groups from the abdominal aorta.

From the 4th day after the first blood collection of OS groups, administration of ERY was started in all the animals. ERY at 150 mg/kg or 450 mg/kg was given by gavage. The control groups were given water for injection at a volume of 10 mL/kg. On the 1st, 4th, 8th, 11th and 14th day of administration, body weight and food consumption were measured. The measurement of water consumption, urinary volume, urinary calcium (Uca) and urinary inorganic phosphorous (UIP)

concentration was performed on the 8th and 14th day of administration. At terminal, animals of OS groups are sacrificed under ether anesthesia afer 16 hours of fasting and subjected to necropsy. The liver, kidneys, thyroid gland and pituitary gland were weighed and relative organ weights were calculated. Histopathological evaluation was conducted on the thyroid and pituitary gland for all animals.

T3, T4, fT3, fT4 and TSH of all animals were determined. T3, T4 and TSH were measured using EIA kits (Diagnostic Systems Laboratories Inc., Diagnostic Systems Laboratories Inc. and Amersham Pharmacia Biotech, respectively). fT3 and fT4 were measured using RIA kits (Diagnostic Products Corporation and DiaSorin lnc., respectively).

Data analysis.

For TSH, T3, T4, fT3, fT4, food consumption, body weight, organ weight, UCa and UIP, comparisons based on the F-test for homogeneity of variance followed by Student's t-test were made following analysis of variance to compare the potential differences between the ERY groups and the control group.

In vitro Experiment

Tissue Preparation

Rats weight between 250 g to 350 g were fasted for 24 hours with free access to water. The animal were anaesthetized with pentobarbital sodium (60 mg/kg). A laparotomy was performed and the stomach was removed and its large curvature was opened up. The mucosa was removed and longitudinal muscle strips of 10 mm long and 3 mm wide was prepared. The muscle strips were mounted under 1 g tension in organ baths (20mL) that were filled with Klebs-Ringer solution. The solution was maintained at 37 ℃ and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording

One end of the muscle was anchored to a glass rod, and the other end was connected to a strain gauge transducer for continuous recording of isotonic tension. The strips were allowed to equilibrate for at least 60 minutes before experimentation, and the solution was exchanged every 15 minutes during the equilibration period. After the equilibration, 100 μ L of the solution of ERY (45mg/mL) was added cumulatively at a 3 to 5 minute interval.

RESULTS

In vivo experiment

Body weight gains of animals in OS group were inhibited when compared to the animals of AA group. However, the loss of body weight became unclear by ERY in a dose-dependent manner. Mean body weight of animals in 450 mg/kg ERY group was significantly ($P<0.05$) higher on the 4th, 8th and 11th day than that in the OS control group. In contrast, mean body weights of ERY treated animals in AA groups were not obviously different from those of animals in AA control group.

Mean food consumption of animals in ERY treated OS groups was increased dose-dependently through the test, and significantly ($P<0.05$) increased on the 4th and 14th day when compared with OS control. The food consumption of ERY treated AA groups was not significantly different from that of the control group. The water consumption of ERY treated OS groups was dose-dependently increased on the 8th and 14th day, and the increased consumption was statistically significant on the 8th day for 450 mg/kg, and no obvious difference was found in ERY treated AA groups when compared with its control group.

Urinary volume was changed in parallel with water intake for ERY treated groups. Uca in 24 hour's urine were significantly increased in OS 450 mg/kg group on the 8th day than those in the control group (Table 1).

TSH, T3, T4 and fT4 levels in OS control group were not different respective levels in AA control group, but fT3 level of AA control group was lower than that of OS control group. ERY treatment produced hormonal changes both in animals of OS and AA groups. TSH levels of 150 mg/kg OS group and 450 mg/kg AA group tended to be increased. T3 and fT3 levels in both AA and OS groups decreased dose-dependently. In both OS and AA groups, increase of T4 and fT4 levels were dose-dependent and time-dependent. These increases were statistically significant. (Table 2.1, 2.2).

Neither absolute weight nor relative weight for organs of ERY treated animals in both OS and AA groups showed significant difference compared to their respective controls. No adverse gross pathological change was observed, and no microscopic alteration related to ERY administration was also observed.

In vitro experiment, ERY potentiated of contraction amplitude of rat gastric fundus preparations, but it was obvious only at

Table 1. Urinary calcium concentration for rats given erythrosine by gavage in 14 day toxicological study

dose	group	OS		AA	
(mg/kg/day)	day	8	14	8	14
Control		1.1±0.4	1.3±0.4	1.3±0.4	1.7±0.4
150		1.6±0.9	1.6±0.7	1.1±0.5	1.3±0.5
450		2.0±0.5**	1.4±0.3	1.2±0.3	1.4±0.2

Values(mg/24hr) are expressed as mean ± S.D.
day : The day for urine collection
OS : Blood samples were collected from orbital sinus
AA : Blood samples were collected from abdominal aorta
Number of animals : 8 for OS on each day, 6 on 8th day and 3 on 14th day for AA
** : Significantly different from the value of Control on the same day ($p<0.01$)

Table 2.1. TSH,T3,FT3,T4 and FT4 concentrations for OS rats given erythrosine by gavage 14 day toxicological study

Parameter (Unit)	dose (mg/kg/day)	Days of administration				
		-3	1	4	8	14
TSH (ng/mL)	Control	11.1±2.79	12.8±2.12	16.5±6.75	15.3±2.00$^{\#}$	13.1±2.98
	150	12.3±2.25	15.8±1.66$^{*\#}$	19.6±4.48$^{\#}$	23.0±10.6	17.6±1.42$^{*\#\#}$
	450	13.6±5.62	14.3±4.22	14.3±3.59	14.5±1.95	13.7±2.05
T3 (ng/mL)	Control	1.27±0.22	1.27±0.10	1.08±0.23	1.11±0.38	1.15±0.20
	150	1.15±0.13	1.11±0.13	1.08±0.19	0.96±0.06$^{\#\#}$	1.03±0.09
	450	1.15±0.26	0.92±0.23**	0.99±0.20	0.98±0.24	0.98±0.17
FT3 (pg/mL)	Control	0.85±0.21	0.93±0.31	1.08±0.29	0.99±0.22	1.20±0.38
	150	0.82±0.27	ND	0.66±0.02	0.81±0.18	0.79±0.07
	450	0.94±0.48	0.62±0.10	0.80±0.37	0.83±0.20	0.55±0.04
T4 (ng/mL)	Control	124±21.4	139±40.4	150±35.3	154±44.2	159±45.8
	150	140±33.9	153±40.5	180±47.3	197±44.8$^{\#}$	192±53.3$^{\#}$
	450	132±34.5	126±33.6	179±28.2$^{\#}$	190±44.4$^{\#}$	182±34.3$^{\#}$
FT4 (ng/mL)	Control	16.6±2.21	16.2±3.47	11.9±7.13	15.9±3.89	18.3±4.39
	150	15.2±2.33	13.9±1.92	16.9±2.98	18.3±1.55	19.6±3.05
	450	18.3±3.05	16.6±3.15	21.7±2.29*	23.8±1.56$^{**\#}$	24.3±3.14$^{\#}$

Values are expressed as mean ± S.D. of 8 rats
OS : Blood samples were collected from orbital sinus
* : Significantly different from the value of Control on the same day ($p<0.05$)
** : Significantly different from the value of Control on the same day ($p<0.01$)
# : Significantly different from the value of -3 day's in each day ($p<0.05$)
: Significantly different from the value of -3 day's in each day ($p<0.01$)
ND : Lower than detection limit (FT3<0.48 pg/mL)

Table 2.2. TSH,T3,FT3,T4 and FT4 concentrations for AA rats given erythrosine by gavage 14 day toxicological study

Parameter (Unit)	dose (mg/kg/day)	Days of administration			
		1	4	8	14
TSH (ng/mL)	Control	12.1±1.58	20.1±4.75	16.5±3.68	18.6±6.18
	150	13.4±2.44	19.7±4.74	15.0±2.14	17.6±1.78
	450	14.4±4.79	21.4±1.98	22.0±4.25	23.8±7.73
T3 (ng/mL)	Control	0.91±0.10	1.07±0.35	1.06±0.21	0.97±0.15
	150	0.87±0.22	0.90±0.04	0.87±0.09	0.88±0.14
	450	0.81±0.11	0.84±0.02	0.83±0.06	0.81±0.07
FT3 (pg/mL)	Control	ND	0.69±0.24	ND	ND
	150	ND	0.64±0.05	ND	ND
	450	ND	0.62±0.07	ND	ND
T4 (ng/mL)	Control	126±32.0	115±9.55	97±22.5	127±23.3
	150	123±24.4	123±24.4	133±6.03	134±4.36
	450	119±7.90	197±63.0	169±29.6*	231±114
FT4 (pg/mL)	Control	15.2±3.55	16.3±2.87	13.7±0.91	14.9±1.48
	150	15.6±1.37	17.4±2.66	13.7±1.23	16.6±2.00
	450	14.6±1.11	19.8±0.97$^{\#\#}$	19.4±3.82	19.9±0.87$^{**\#\#}$

Values are expressed as mean ± S.D. of 3 rats
AA : Blood samples were collected from abdominal aorta
* : Significantly different from the value of Control on the same day ($p<0.05$)
** : Significantly different from the value of Control on the same day ($p<0.01$)
: Significantly different from the value of -3 day's in each day ($p<0.01$)
ND : Lower than detection limit (FT3<0.48 pg/mL)

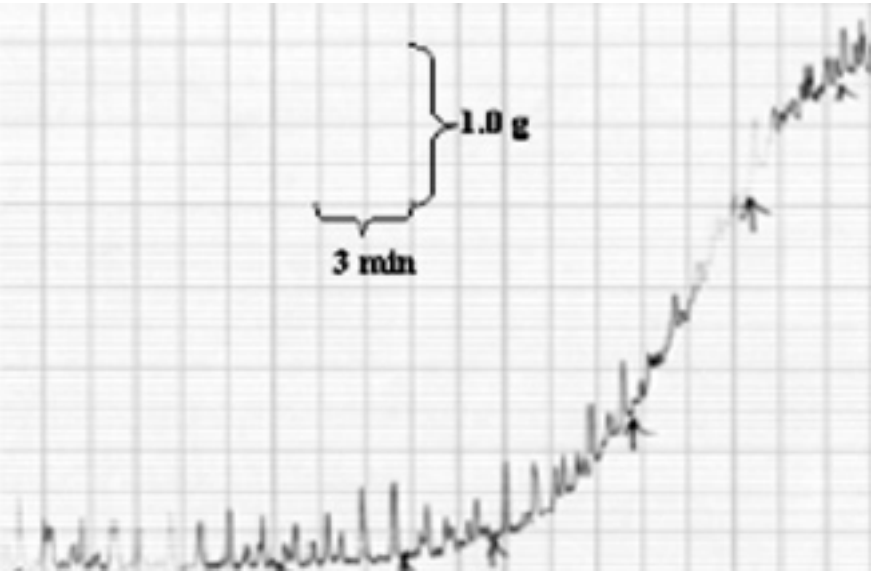

Fig 1. The contractile of ERY on rat gastric fundus

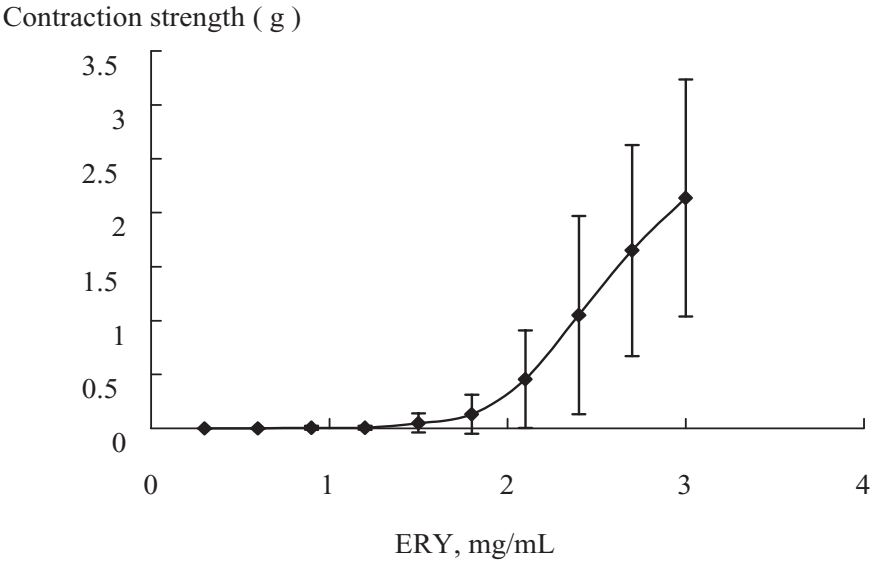

Fig 2. Concentratin-response curve for the contractile of rat gastric fundus by ERY. Each point represents the mean and standard deviation of the mean of 8 strip tests.

concentrations at 2 mg/mL or more and reached the maximum contraction at bout 3.5 mg/mL in most of the preparations (Fig. 1, Fig. 2).

DISCUSSION

Body weight, food consumption, water intake and urinary volume were significantly increased only in ERY treated animals in OS groups. These findings are similar to those in previous reports from many laboratories. However, such changes were not found in ERY-treated animals in AA groups. Vorhees et al. reported increases in body weight and food consumption in their reproductive toxicity studies for ERY in rats [10]. Statistically significant increase of body weight was found at pre-breeding and lactation period (both $p<0.01$) in their first study, and statically significant increase in food consumption of parental phases was found in their second one. In his study, the highest dosage of ERY in diet was 1.0% w/w. In a series of toxicological studies of ERY, Collins T.F.X. et al. noted an increase in food consumption by ERY in rats[11, 12].

Studies in the mouse also showed such an increase in food consumption by ERY. In their lifetime toxicity/carcinogenicity study of ERY in mice, Borzelleca and Hallagan reported that food consumption in the 1% and 3% of ERY groups was statistically higher than that in the control group[13]. Both 1% and 3% of ERY groups showed an increase in mean body weight of animals as compared to that in the control. Previous studies showed that significant inhibition of body weight gain occurred in groups given food containing ERY at 1% or more, but food consumption in these dosages was significantly increased or tended to be increased [3, 13-16]. In fact, increase of food consumption by ERY administration was more obvious in a restricted conditions than on "normal" conditions. Focusing on individual body weight of OS animals in the present study, body weight of some animals was decreased due to urinary collection on the 8th and 14th day. However, body weight in most of the animals decreased but such temporary decrease appeared to be antagonized dose-dependently by ERY (Fig. 3). This difference might be induced with ERY administration but reason of which was not clear.

The animals given ERY appeared to show increased urination compared with the control animals. Both water consumption and urinary volume were significantly increased in ERY treated OS animals on the 8th day, and this might be related to increased food consumption.

ERY treatment changed T3 and T4 levels in both OS and AA groups. These change has been known to be due to the inhibition of 5'-monodeiodination of T4 to T3 by ERY.

Though previous studies demonstrated that ERY altered acetylcholine release [17, 18], dopamine uptake [19] or dopamine receptor interaction [20]. Kaplita et al. demonstrated that ERY contracted myenteric plexus-longitudinal muscle preparations of guinea-pig ileum at about 10^{-6}M[19], but reduced the amplitude of the mechanical response to acetylcholine and electrical stimulation, and the effects were not concentration- and time-dependent. Responses of myenteric plexus-longitudinal muscle preparation of guinea-pig ileum to ERY might be different from that of the rat gastric fundus strip preparations, and the concentration range used in the present study was about $5.7\sim39.7\times10^{-4}$M. Though the concentrations in our studies were higher, but it might be enough concentration to induce local effect in stomach when compared to estimated concentrations in the stomach of animals (supposed maximum rat gastric volume is 20 mL) when given ERY more than 150 mg/kg by gavage method or 1% mixture levels in food.

On the whole, it is a better understanding of food promoted effects of ERY on rat by a direct contraction on gastric tract. Couch et al. pointed out that feeding ERY at 4% in diet produced changes in clinical chemistry parameters[22], body weight, and food consumption were indicative of hyperthyroidism. In this study TSH and T4 levels were elevated and T3 level was depressed.

Our results confirmed the disturbing effects on thyroid functions and proved the appetite promoting effects on Crj CD(SD) IGS rats. However, it might be have any additional study to prove any conclusion whether the effects were related or not.

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External Abnormalities Observed during the Quarantine Period in Crj:CD(SD)IGS Rats

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ABSTRACT. To provide healthy animals for toxicological research, we have to carefully examine all animals delivered to us. We here report the abnormalities observed in the 17,474 Crj:CD(SD)IGS rats that we received from 1997 to 2001. We examined them externally at the time of delivery and at the end of a one-week quarantine period. Of 17,474 rats, 78 (0.45 % of total) showed apparent abnormalities, such as external wounds or depilation (53.8 % of the observed abnormalities), dental abnormalities (26.9 %), ocular abnormalities (10.3 %), and others (9.0 %). These findings indicate that the quality of IGS rats can be further improved by more careful observation of the state of animals, and that careful observation of animals plays an important role in minimizing the use of unsuitable animals for toxicological research. — Key words: external abnormality, Crj: CD(SD)IGS rat, quarantine

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The quality of animals has been greatly improved due to the technical popularization of the maintenance of specific-pathogen-free (SPF) conditions. However, we occasionally encounter animals with apparent anomalies that are unsuitable for toxicological research. Thus, we have been checking whether animals have any abnormality, so as to use only healthy animals for experiments. In this report, we describe several apparent anomalies in the Crj:CD(SD)IGS rats delivered to our facility.

In our facility, IGS rats have been accepted and used for toxicological research since 1997. Up to 2001, we have received 17,474 IGS rats (11,837 males and 5,637 females) from Charles River Japan Inc. as shown in Table 1. They were from 1 to 14 weeks of age (6.0 weeks of age on average), except for 10 age-unknown females that were delivered as foster mothers. After delivery, the animals were individually housed in stainless-steel wire mesh cages in an animal room of our facility maintained at 23±2℃ and 55±10 % relative humidity, and given a commercial palletized food (CRF-1, Charles River Japan) and tap water *ad libitum*. All the animals delivered to our facility were examined for any external abnormalities at the time of delivery and during a quarantine period of one week.

No animals with signs of any infectious disease were observed at the time of acceptance nor were any deaths observed. External abnormalities in the animals received were observed in only 0.45 % of the total (Table 1). These frequencies were stable for each year, and did not differ between two breeding sources, Atsugi farm and Tsukuba farm. The details of the abnormal findings are shown in Table 2.

External wounds including incrustations formed on the skin were found most frequently, and were observed in 26 rats (33.3 % of the total anomalies). Of these 26 rats, 13 had external wounds upon arrival at our facility. The wounded rats were 10.4 weeks of age on average, and were older than the mean age of the entire group of animals delivered. The wounds were likely related to fighting among animals in the transport cage or to stress due to transportation and environmental changes, except for one male rat that was wounded by a technical error in animal handling during its quarantine period.

Depilation or hair loss was found in 16 rats (20.5 % of the total anomalies). Some of these abnormalities might have resulted from self-biting behavior related to the stress from transportation.

As for dental abnormalities, we encountered rats with a chalklike change of incisors (Figure 1A), loss of incisors, uneven incisors (Figure 1B), and malocclusion. Dental abnormalities accounted for 26.9 % of the entire abnormalities observed. Of five rats with chalklike incisors (Figure 1A), three females were delivered from Atsugi farm on March 28, 2001. These abnormal incisors lacked hardness and were easily broken by feeding on a pellet diet, resembling those of mutant rats with amelogenesis imperfecta reported by Ishibashi *et al*. [1]. Interestingly, Katsuda *et al*. [2] have recently reported that a spontaneous mutant with amelogenesis imperfecta had been found in female IGS rats of Atsugi farm origin, which are of the same stock as our rats. Malocclusion was also observed in three of the 72 males delivered on August 2, 2000, and in two females delivered on another date. These two traits, namely, a chalklike change of incisor and malocclusion, should be considered to be hereditary, because these changes were found successively within a short period.

Ocular abnormalities such as eye discharge, lens opacity (Figure 1C) and macrophthalmia (enlargement of eyeball, Figure 1D) were observed in 8 rats (10.3 % of the total anomalies). Eye discharge, which implied a stress-related change, was seen in 6 rats. Lens opacity was observed in one female rat at 5 weeks of age (Figure 1C), and was due to the posterior rupture of the lens as revealed by histological examination. Spontaneous rupture of the lens has been rarely found in aged IGS rats [3].

Behavioral abnormality was also observed in one male rat with abnormal walk involving staggering of hindlimbs. This behavioral abnormality was not detected during the initial check of the animals received because this abnormality was noticeable only by careful observation of the animal when walking. Anemia was observed in one male rat that had gross findings such as discoloration in the eyeballs and palate, and

pale skin without apparent bleeding. This rat had several macroscopic abnormalities including enlargement of the spleen and pancreatic edema. As other abnormalities, defect of a digit on the left hind leg (Figure 1E), split lips (Figure 1F) and anomalous form of the external urethral orifice were each observed in one rat, respectively.

At the time of the shipment of the animals, the supplier, Charles River Japan, carries out appropriate inspections of the animals. However we, as a user of animals, unfortunately encountered animals with some abnormalities at arrival with an incidence of approximately 0.45 %, as shown by results of the present investigation. We have to consider that some 'abnormalities' may be partially related to stress from the transportation that animals are subjected to. In fact, the wounds and hair loss of animals we encountered could be partially attributed to stress. In contrast to this, other distinct abnormalities such as spontaneous mutation that passed many barriers were also observed in the animals delivered to us with an incidence of approximately 0.2 % of the total. This indicates that the quality of IGS rats could still be further improved by more careful observation of the state of animals, and that careful observation of animals plays an important role in minimizing the use of unsuitable animals for toxicological research. We also believe that our data will contribute to the qualitative improvement of IGS rats in all fields handling them.

Table 1. Total number and the incidence of the apparent abnormalities of Crj:CD(SD)IGS rats where were delivered.

Year		Number of animals received			Animals with abnormality			
					total		encountered timing	
							recept	quarantine
		total	male	female	n.	(%)	n.	n.
1997		**160**	**80**	**80**	**1**	**(0.63)**	**1**	**0**
	A*	160	80	80	1	(0.63)	1	0
	T	0	0	0	0	(0.00)	0	0
1998		**1,914**	**1,022**	**892**	**5**	**(0.26)**	**1**	**4**
	A	1,884	1,022	862	4	(0.21)	0	4
	T	30	0	30	1	(3.33)	1	0
1999		**4,742**	**2,959**	**1,783**	**27**	**(0.57)**	**16**	**11**
	A	3,933	2,426	1,507	19	(0.48)	9	10
	T	809	533	276	8	(0.99)	7	1
2000		**5,557**	**3,991**	**1,566**	**24**	**(0.43)**	**11**	**13**
	A	4,904	3,649	1,255	24	(0.49)	11	13
	T	653	342	311	0	(0.00)	0	0
2001		**5,101**	**3,785**	**1,316**	**21**	**(0.41)**	**12**	**9**
	A	4,876	3,630	1,246	21	(0.43)	12	9
	T	225	155	70	0	(0.00)	0	0
total		**17,474**	**11,837**	**5,637**	**78**	**(0.45)**	**41**	**37**
	A	15,757	10,807	4,950	69	(0.44)	33	36
	T	1,717	1,030	687	9	(0.52)	8	1

* Atsugi farm-origin (A) and Tsukuba farm-origin (T) of Charls River Japan Inc.

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Table 2. Gross findings of external abnormality encountered in Crj:CD(SD)IGS rats

Findings		Number of animals				
		total	male	female	age (wk)[1)]	details
External wounds		26	25	1	10.4±3.9	
	(%)[2)]	*33.3*				
Depilation, Hair loss		16	11	5	7.3±3.7	
	(%)	*20.5*				
Dental abnormalities		21	10	11	6.0±1.9	malocclusion (3 males and 2 females)
	(%)	*26.9*				chalklike change of incisors (1 male and 2 females)
						short incisors, uneven incisors
Ocular abnormalities		8	4	4	6.6±3.5	discharge (3 males and 3 females)
	(%)	*10.3*				lens opacity (rupture of lens, 1 female)
						enlargement of eyeball (macropthalomus, 1 male)
Others		7	5	2	5.4±1.6	split lips (2 males)
	(%)	*9.0*				defictive digit of left-hind leg (1 male)
						anomalous form of the external urethral orifice (1 female)
						abnormal walking by staggering of hindlimbs (1 male)
						anemia (1 male)

1) Mean age in weeks of animals having each abnormality, mean±standard deviation (S.D.)
2) Percentage to total number of animals with abnormality

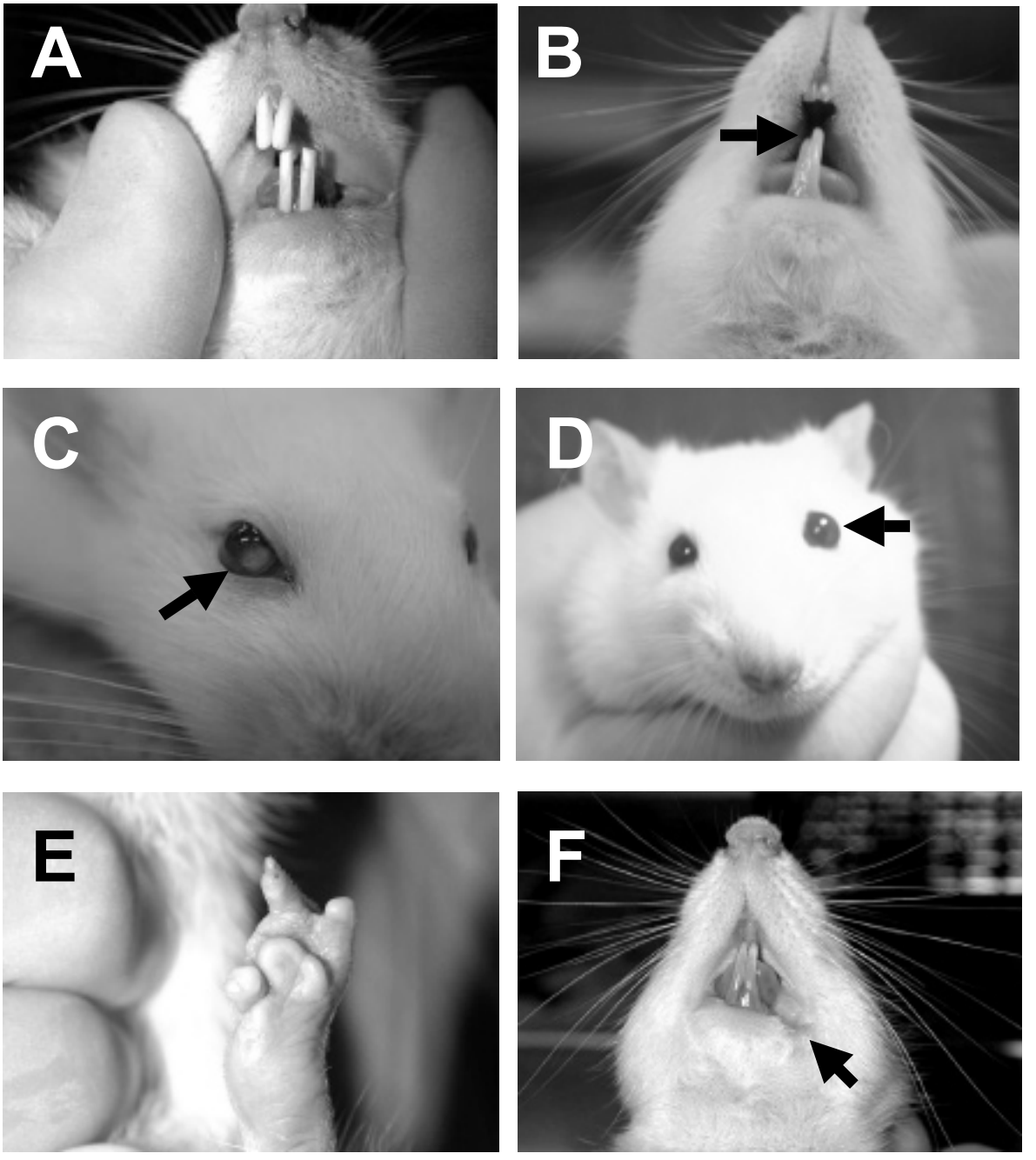

Figure 1. Photographs of the abnormalities in the Crj:CD(SD)IGS rats. A: Chalklike change of incisors. B: Uneven incisors. C: Lens opacity (the posterior rupture of lens). D: Macrophthalmia (enlargement of eyeball). E: Defective digit on the left hind leg. F: Split lips.

Comparison of Hematology and Blood Chemistry Parameters between Crj:CD(SD)IGS Rats and BrlHan:WIST@Jcl(GALAS) Rats

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ABSTRACT. The purpose of this study was to compare Crj: CD(SD)IGS rats with BrlHan:WIST@Jcl(GALAS) rats in terms of hematology and blood chemistry parameters. There were differences between the strains at three different ages in the following hematology and blood chemistry parameters; platelets in both sexes, cholinesterase (CHE), blood urea nitrogen (BUN), leucine aminopeptidase (LAP), potassium (K), and triglyceride (TG) in males, leucine aminopeptidase (LAP), total cholesterol (TCHO), free cholesterol (FCHO), magnesium (Mg), and triglyceride (TG) in females. Particularly, it was clear that there were different trends in lipid parameters (TCHO, FCHO, and TG) between two strains. —Key words: blood chemistry, BrlHan:WIST@Jcl(GALAS), Crj:CD(SD)IGS, hematology,

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INTRODUCTION

Crj:CD(SD)IGS rat (IGS) was produced by International Genetic Standard system and supplied by Charles River Inc. In contrast, BrlHan:WIST@Jcl(GALAS) rat (GALAS), which was a worldwide standardized Wistar strain for minimizing genetic divergence since 1999, was produced by Global Alliance for Laboratory Animal Standardization and supplied by CLEA JAPAN, Inc., M & B A/S., and Taconic Farms, Inc. Recently, these two strains were supplied to various toxicity studies. However, there are few reports compared IGS with GALAS in terms of hematology and blood chemistry parameters. In this study, we compared IGS with GALAS in terms of above parameters at three different ages.

MATERIALS AND METHODS

Animals and housing: IGS and GALAS at 4.5 weeks of age were purchased from Charles River Japan Inc. and CLEA JAPAN, Inc., respectively. The animals were single-housed in wire-mesh cages and in an air conditioned room (temperature, 23±2℃; humidity, 55±15%; light cycle, 12 hr/day). The animals were allowed free access to standard laboratory food (CRF-1, Oriental Yeast Co., Tokyo Japan) and tap water. Blood samples, which were derived from control groups supplied to 2, 4, or 13 weeks subacute toxicity studies in our laboratory, were collected the abdominal aorta under ethyl ether anesthesia following depriving of food overnight (16 hours or more).

Hematology: The following hematological examinations were performed after the treatment of EDTA-2K anticoagulant using an automated hematology analyzer E-4000 (Sysmex Co., LTD); white blood cell counts (WBC), red blood cell counts (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets. Reticulocytes were analyzed by an automated analyzer E-1000 (Sysmex Co., LTD).

Blood chemistry: The following blood chemical examinations were performed after the treatment of heparin anticoagulant and centrifugation using an automated blood chemistry analyzer 736-10 (Hitachi, LTD); glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH), cholinesterase (CHE), amylase (AMY), creatine phosphokinase (CPK), aldolase (ALD), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ GTP), leucine amino-peptidase (LAP), phospholipid (PL), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), albumin globulin ratio (A/G), total bilirubin (Tbil), creatinine (CRE), total cholesterol (TCHO), free cholesterol (FCHO), ester ratio (ESTER.R), triglyceride (TG), non esterified fatty acid (NEFA), calcium (CA), inorganic phosphorous (IP), iron (Fe), unsaturated iron binding capacity (UIBC), magnesium (Mg), glucose (GLU), sodium (Na), potassium (K), and chloride (Cl). A/G was calculated from the total protein and albumin. ESTER.R was calculated from TCHO and FCHO.

Statistical analysis: Statistical significance of differences between IGS and GALAS was examined at 5, 1 and 0.1% probability levels. The Student's t-test was used for those data with homogeneous variance between the strains at each age as determined by F-test. The Aspin-Welch test was used for those data without homogeneous variance between the strains at each age as determined by F-test.

RESULTS

Hematology: The results of hematology were shown in Table 1. In comparison with GALAS, statistical differences were observed in IGS as follows: in males; increases in WBC and platelet, and a decrease in RBC at 7.5 weeks of age, an increase in platelet, and a decrease in reticulocyte at 9.5 weeks of age, increases in WBC and platelet at 18.5 weeks of age, and in females; a decrease in RBC, and an increase in platelet at 7.5 weeks of age, increases in WBC and platelet at 9.5 weeks of age, increases in WBC and platelet, and a decrease in reticulocyte at 18.5 weeks of age, respectively.

Blood chemistry: The results of blood chemistry were shown

in Table 2. In comparison with GALAS, statistical differences were observed in IGS as follows: in males; increases in GOT, ALD, ALP, LAP, TCHO, FCHO, NEFA, IP, K, and Cl, and decreases in CHE, BUN, CA, and Mg at 7.5 weeks of ages, increases in ALD, ALP, LAP, TCHO, FCHO, TG, and IP, and decreases in γ GTP, TP, ALB, and Mg at 9.5 weeks of age, increases in LAP, TCHO, FCHO, TG, NEFA, GLU, and K, and decreases in LDH, CHE, CPK, BUN, Fe, Mg, and Na at 18.5 weeks of age, and in females; increases in GOT, GPT, LDH, ALD, ALP, γ GTP, LAP, NEFA, K, and Cl, and decreases in CHE and BUN at 7.5 weeks of age, increases in GPT, LAP, and K, and decreases in LDH, CHE, PL, BUN, TP, ALB, and Mg at 9.5 weeks of age, increases in ALD, LAP, TG, GLU, K, and Cl, and decreases in CHE, CPK, BUN, Fe, and Mg at 18.5 weeks of age, respectively.

DISCUSSION

The values of TG in males and TCHO, FCHO and TG in females of IGS were higher than those of GALAS. It was clear that the values of lipid parameters (TCHO, FCHO and TG) of IGS tended to increase with aging. This tendency was consistent with other reports [1-8]. Rao *et al*. [9] reported that the decrease in life span of laboratory rats has coincided with a trend to obesity of the affected rats, and Marie-Françoise *et al*. [10] reported that survival rate was improved by restricted diet, with decreasing the values of lipid parameters. In addition, it is known that survival rate of CD strain is lower than that of Wistar strain [9, 11]. Consequently, our results might indicate that the increase in lipids is one of contributing factors of decreasing survival rate. In contrast, we have not established the biological meanings for the differences in other parameters.

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Table 1-1. Hematology values of Crj:CD(SD)IGS rats and BrlHan:WIST@Jcl(GALAS) rats -male

	Age in weeks					
	7.5W		9.5W		18.5W	
MALE	IGS(13)	GALAS(10)	IGS(10)	GALAS(4)	IGS(10)	GALAS(10)
WBC($\times 10^2$/mm^3)	57.2±11.6	34.2±2.6 $$$	67.1±10.6	59.0±13.4	66.3±11.7	38.9±6.8 ***
RBC($\times 10^4$/mm^3)	664.8±34.8	710.0±50.1 ***	780.1±28.7	779.4±26.3	878.4±35.0	882.6±34.1
Hb(g/dl)	13.3±0.5	14.02±0.8	15.0±0.4	14.9±0.4	15.0±0.4	15.2±0.6
Ht(%)	40.5±1.6	42.28±2.3	44.5±1.5	44.5±0.9	45.1±1.4	44.8±1.7
MCV(fl)	61.0±1.9	59.66±2.7	57.1±2.1	57.1±1.5	51.4±1.7	50.8±1.8
MCH(pg)	20.1±1.0	19.78±0.6	19.2±0.7	19.1±0.6	17.1±0.6	17.3±0.7
MCHC(g/dl)	32.9±0.8	33.16±0.6	33.7±0.5	33.4±0.3	33.3±0.7	34.0±0.6
Platelet($\times 10^4$/mm^3)	141.0±17.6	98.18±10.9 ***	116.6±19.7	96.4±4.8 $	107.1±11.3	83.2±9.7 ***
Reticulocyte(‰)	51.5±7.0	47.54±4.0	26.0±2.2	30.2±2.5 *	22.2±4.3	22.4±2.3

*, ***:p<0.05, p<0.001(t-test)
$, $$$:p<0.05, p<0.001(Aspin-Welch test)
Values represent mean±S.D.
( ):Number of animals

Table 1-2. Hematology values of Crj:CD(SD)IGS rats and BrlHan:WIST@Jcl(GALAS) rats -female

	Age in weeks					
	7.5W		9.5W		18.5W	
FEMALE	IGS(13)	GALAS(10)	IGS(10)	GALAS(4)	IGS(10)	GALAS(10)
WBC($\times 10^2$/mm^3)	50.2±22.6	38.8±22.1	53.5±10.8	36.3±10.5 *	44.4±14.1	25.2±7.9 $$
RBC($\times 10^4$/mm^3)	715.5±38.8	761.1±28.4 **	787.6±49.4	779.8±28.3	823.1±18.4	840.8±32.2
Hb(g/dl)	14.0±0.4	14.7±0.6	14.7±0.5	14.7±0.6	14.8±0.5	15.3±0.4
Ht(%)	41.6±1.4	43.7±1.5	43.2±1.6	43.9±2.0	44.0±0.8	45.0±0.5
MCV(fl)	58.2±1.9	57.4±1.5	55.0±1.9	56.3±0.7	53.4±1.2	53.5±2.1
MCH(pg)	19.6±0.8	19.3±0.6	18.7±0.8	18.9±0.4	18.0±0.5	18.2±0.7
MCHC(g/dl)	33.6±0.5	33.6±0.4	34.0±0.6	33.5±0.6	33.8±0.9	33.9±0.5
Platelet($\times 10^4$/mm^3)	136.3±11.5	98.4±14.3 ***	114.0±12.9	95.0±8.6 *	105.5±13.0	81.7±6.8 $$$
Reticulocyte(‰)	31.3±6.0	30.6±6.8	20.7±5.0	26.8±6.5	17.1±2.7	24.6±4.1 ***

*, **, ***:p<0.05, p<0.01,p<0.001(t-test)
$$, $$$:p<0.01, p<0.001(Aspin-Welch test)
Values represent mean±S.D.
( ):Number of animals

Table 2-1. Blood chemistry values of Crj:CD(SD)IGS rats and BrlHan:WIST@Jcl(GALAS) rats -male

	Age in weeks					
	7.5W		9.5W		18.5W	
MALE	IGS(13)	GALAS(5)	IGS(10)	GALAS(5)	IGS(10)	GALAS(10)
GOT(IU/l)	60.8±7.0	53.0±4.1 *	61.4±5.4	58.6±6.5	53.9±6.6	51.0±4.5
GPT(IU/l)	21.5±2.7	17.0±1.0 ***	24.7±3.7	20.2±2.4 *	22.2±2.9	23.3±4.2
LDH(IU/l)	158.8±20.9	126.8±26.4 *	147.6±24.2	186.2±31.1 *	133.2±32.6	142.7±27.4
CHE(IU/l)	36.4±11.0	50.4±6.1 *	34.5±4.2	61.6±9.6 **	37.8±9.2	63.0±20.1 $$$
AMY(IU/l)	2209.5±359.9	2259.0±366.3	2446.6±271.9	2917.8±690.4	2610.7±365.4	2846.2±487.6
CPK(IU/l)	166.1±21.4	167.0±20.0	142.5±16.4	162.2±28.0	74.7±9.3	94.5±17.7 $$
ALD(IU/l)	24.6±1.8	18.9±2.5 ***	19.7±2.5	17.3±2.3	15.5±3.3	12.9±1.5 $
ALP(IU/l)	648.8±123.9	397.4±57.4 ***	458.6±50.0	403.8±139.5	178.0±29.6	190.4±42.1
γ GTP(IU/l)	0.8±0.4	0.2±0.4 *	2.6±1.3	3.4±0.5	2.2±1.1	2.4±0.5
LAP(IU/l)	192.9±13.0	63.6±6.0 ***	192.7±15.0	56.8±2.9 ***	169.2±17.0	51.6±3.9 $$$
PL(mg/dl)	104.0±15.5	105.2±12.1	89.4±7.4	118.2±14.0 ***	112.0±14.5	115.6±19.4
BUN(mg/dl)	12.3±2.8	19.2±3.9 ***	11.5±4.4	20.4±1.8 $$$	12.3±1.8	18.6±1.3 ***
TP(g/dl)	5.3±0.2	5.7±0.2	5.6±0.3	6.0±0.2 *	6.0±0.2	6.2±0.2
ALB(g/dl)	2.1±0.1	2.4±0.1	2.2±0.2	2.4±0.1 *	2.2±0.1	2.4±0.2
A/G	0.7±0.1	0.7±0.1	0.7±0.0	0.7±0.0	0.6±0.0	0.6±0.0
Tbil(mg/dl)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0
CRE(mg/dl)	0.3±0.0	0.4±0.1	0.4±0.0	0.4±0.0	0.4±0.1	0.5±0.1
TCHO(mg/dl)	67.1±13.7	57.4±8.6	50.5±8.3	54.6±5.7	71.6±13.7	62.1±8.8
FCHO(mg/dl)	12.0±3.2	11.8±2.4	9.9±1.6	9.0±1.9	15.5±4.6	13.7±2.7
ESTER.R	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0
TG(mg/dl)	32.4±22.1	26.2±12.5	39.2±14.3	26.8±12.8	50.4±14.0	26.9±16.9 ***
NEFA(uEq/l)	872.5±176.9	605.4±120.2 **	778.1±99.5	734.8±168.7	739.3±127.6	645.4±95.7
CA(mg/dl)	10.0±0.2	10.3±0.2	10.2±0.5	10.5±0.1	10.0±0.2	10.1±0.3
IP(mg/dl)	8.2±0.6	7.0±2.1	6.9±0.9	6.7±0.4	5.3±0.6	4.7±0.9
Fe(mg/dl)	154.6±103.6	231.0±98.0	143.6±51.2	210.4±106.5	133.4±35.6	171.9±23.0 *
UIBC(mg/dl)	242.1±104.1	196.6±98.0	319.0±86.2	277.2±93.6	344.3±60.4	330.2±33.6
Mg(mg/dl)	1.7±0.1	1.8±0.2	1.7±0.2	2.0±0.2 *	1.6±0.1	2.0±0.2 ***
GLU(mg/dl)	131.8±18.7	130.6±9.0	153.7±10.3	167.8±21.9	166.3±15.6	152.3±10.8 *
Na(mEq/l)	142.7±8.3	142.2±1.1	141.6±8.0	142.2±1.6	142.0±2.2	142.7±1.5
K(mEq/l)	4.0±0.3	3.3±0.2 ***	3.6±0.2	3.4±0.1 *	4.1±0.2	2.8±0.5 ***
Cl(mEq/l)	111.2±3.8	102.6±1.8 ***	103.8±2.0	104.0±1.7	105.1±1.9	102.9±2.4 *

*, **, ***:p<0.05, p<0.01, p<0.001(t-test)
$, $$, $$$:p<0.05, p<0.01, p<0.001(Aspin-Welch test)
Values represent mean±S.D.
( ):Number of animals

Table 2-2. Blood chemistry values of Crj:CD(SD)IGS rats and BrlHan:WIST@Jcl(GALAS) rats -female

	Age in weeks					
	7.5W		9.5W		18.5W	
FEMALE	IGS(13)	GALAS(10)	IGS(10)	GALAS(4)	IGS(10)	GALAS(10)
GOT(IU/l)	57.4±4.8	51.7±4.7 **	64.7±9.4	60.3±3.8	57.1±10.5	63.2±14.4
GPT(IU/l)	17.2±4.8	16.0±3.7	22.3±5.5	22.7±4.9	23.7±6.4	29.3±9.8
LDH(IU/l)	180.2±43.4	157.8±44.8	182.5±72.6	152.7±10.6	124.4±29.1	168.2±50.9 *
CHE(IU/l)	145.0±41.8	234.9±95.4 *	232.9±61.1	423.0±153.9	334.9±55.2	597.5±103.8 $$$
AMY(IU/l)	1093.2±127.3	1393.0±461.2	1231.1±263.4	1351.3±115.7	1684.4±413.5	1485.5±272.9
CPK(IU/l)	121.5±13.8	142.8±35.5	101.0±23.7	122.3±5.9	59.0±10.6	79.4±11.8 ***
ALD(IU/l)	18.7±1.8	14.9±1.9 **	16.7±2.5	13.1±2.1 *	9.6±1.4	9.6±1.7
ALP(IU/l)	394.6±99.0	279.4±102.2 *	281.1±29.1	140.3±38.4 ***	90.5±21.5	71.8±22.7
γ GTP(IU/l)	0.9±0.5	1.6±1.4	2.1±0.7	3.3±0.5 *	2.3±0.8	2.7±0.5
LAP(IU/l)	183.2±13.7	54.7±3.8 $$$	171.6±24.4	45.8±5.4 $$$	140.3±16.2	48.5±5.6 $$$
PL(mg/dl)	105.5±22.0	103.4±19.3	109.2±26.3	104.3±7.9	142.8±35.8	120.0±21.1
BUN(mg/dl)	17.1±4.1	20.6±1.9 $	15.3±4.9	19.2±3.4	15.8±1.8	20.8±2.9 ***
TP(g/dl)	5.6±0.2	5.9±0.3	5.7±0.3	6.2±0.1 ***	6.4±0.4	6.7±0.2
ALB(g/dl)	2.3±0.1	2.6±0.3	2.4±0.1	2.7±0.1 **	2.6±0.2	2.8±0.2
A/G	0.7±0.0	0.8±0.1	0.7±0.1	0.8±0.1	0.7±0.0	0.7±0.0
Tbil(mg/dl)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.1	0.1±0.1	0.0±0.0
CRE(mg/dl)	0.4±0.1	0.4±0.0	0.4±0.1	0.4±0.0	0.5±0.1	0.6±0.0
TCHO(mg/dl)	65.0±16.1	45.8±12.7 **	57.2±18.8	42.0±5.2 $	72.6±19.0	48.1±8.5 $$$
FCHO(mg/dl)	10.8±4.4	7.4±1.5 $	12.6±4.9	5.0±1.4 $$	16.6±6.0	11.3±3.2 *
ESTER.R	0.8±0.0	0.8±0.0	0.8±0.0	0.9±0.0	0.8±0.0	0.8±0.0
TG(mg/dl)	7.7±3.2	7.5±5.3	8.3±5.1	2.3±1.7 $$	19.6±10.8	4.0±2.6 $$
NEFA(uEq/l)	890.5±134.7	696.1±106.5 **	723.8±112.2	727.5±138.9	993.7±177.5	713.9±117.1 ***
CA(mg/dl)	10.0±0.2	10.3±0.3 *	10.2±0.4	10.4±0.3	10.2±0.4	10.1±0.3
IP(mg/dl)	7.2±0.6	6.5±0.7 *	7.0±0.9	5.3±0.6 **	4.0±1.0	4.2±1.2
Fe(mg/dl)	191.8±66.7	241.8±85.1	297.0±117.6	258.5±63.5	309.5±60.5	364.5±45.4 *
UIBC(mg/dl)	187.3±60.4	148.6±93.0	135.1±75.8	171.3±71.1	150.4±73.9	98.5±45.5
Mg(mg/dl)	1.7±0.1	1.9±0.1 **	1.8±0.2	2.1±0.2 *	1.6±0.1	2.1±0.1 ***
GLU(mg/dl)	119.4±10.0	130.8±17.3	130.1±14.5	131.0±16.0	147.1±22.6	119.4±23.0 *
Na(mEq/l)	143.2±7.6	141.0±2.1	141.6±6.1	142.8±0.5	140.8±1.5	142.5±0.8 **
K(mEq/l)	3.8±0.4	2.9±0.4 ***	3.8±0.4	3.4±0.2	3.6±0.4	2.4±0.7 ***
Cl(mEq/l)	115.2±3.0	103.8±2.6 ***	106.6±5.4	106.0±0.0	105.9±2.1	105.4±2.0

*, **, ***:p<0.05, p<0.01, p<0.001(t-test)
$, $$, $$$:p<0.05, p<0.01, p<0.001(Aspin-Welch test)
Values represent mean±S.D.
( ):Number of animals

Background Data of Blood Chemistry Parameters in Toxicity Studies Using Crj:CD(SD)IGS Rats at 10, 19 and 32 Weeks of age

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ABSTRACT. Recently, Crj:CD(SD)IGS rat has become one of the strains used most frequently in toxicity studies. Historical control data is useful for evaluating toxicity of the test substances. In the present study, historical control data on blood chemistry parameters (20 items) were collected from the toxicity studies (6 to 10 studies each at 10, 19 and 32 weeks of age) that were conducted in our laboratories during 2001 to 2002, and age-related changes were examined. Age-related changes were: 1) occurrence of animals with extraordinarily high values for ALT, AST and/or LDH activities, especially in females, 2) increase in total cholesterol, triglyceride, phospholipid and total protein in both sexes and 3) decrease in CPK and ALP activities and inorganic phosphorus in both sexes. These findings should be taken into consideration when evaluating the toxicity of the test substances. —Key words: background data, Crj:CD(SD)IGS rats, blood chemistry, age-related change

CD(SD)IGS-2002/2003 : 53-60

INTRODUCTION

Crj:CD(SD)IGS rats were produced by the genetic standard system, which is an animal breeding system developed for providing experimental animals having worldwide uniform quality. Charles River Inc. developed it for internationalization of scientific research and development of new drugs. In 1998, prior to introducing this strain into toxicity studies as an experimental animal, we had reported the data of IGS rats relating to toxicological parameters and compared them to those of the ordinarily used CD rats [1]. In the present study, we collected historical control data on blood chemistry parameters in this strain at three different age levels, and age-related changes were examined.

MATERIALS AND METHODS

Animals and Husbandry: Data were collected from ten 4-week studies, six 13-week studies and six 26-week studies using Crj:CD(SD)IGS rats conducted in our laboratories during 2001 to 2002. In all studies, IGS rats were obtained from Charles River Japan Inc. (Atsugi or Hino Breeding Center, Japan) and administration route was oral by gavage or dietary. The animals were housed individually in hanging stainless-steel wire mesh cages in an animal room under the following conditions: temperature at 23 ± 3 ℃, relative humidity at 50 ± 20%, air ventilation at 10 to 15 times per hour and 12-hour illumination (07:00 to 19:00). The animals were supplied commercial diet CRF-1 or CR-LPF *ad libitum* throughout the course of the study.

Examinations and methods: The blood chemistry parameters determined and the methods used are shown in Table l. Blood samples were collected from the abdominal aorta under ether anesthesia and the sera were used for determination; however, AST, ALT, LDH and CPK activities were determined on the plasma obtained from blood samples treated with heparin. The animals were deprived of food overnight (approximately 16 to 20 hours) prior to the blood collection.

RESULTS

The results of blood chemistry parameters are shown in Tables 2-1 (male) and 2-2 (female). Extraordinarily high values for ALT, AST and/or LDH activities were observed in some females at 19 weeks of age and in some males and females at 32 weeks of age; and the incidence and severity were apparent in females (Fig. 1). The number of animals with higher values (>100 IU/L) for ALT, AST and LDH activities is shown in the following.

Total cholesterol, triglyceride and phospholipid increased with aging in both sexes (Fig. 2). Also total protein increased slightly with aging in both sexes. On the other hand, CPK and ALP activities and inorganic phosphorus decreased in both sexes with aging (Fig. 3 to 5).

DISCUSSION

Historical control data on blood chemistry in Crj:CD(SD)IGS rats were collected from the toxicity studies conducted in our laboratories during 2001 to 2002. The age of rats was 10, 19 and 32 weeks and the number of studies was 6 or 10 at each age.

Extraordinarily high values for ALT, AST and/or LDH were observed in some males and females at 19 and 32 weeks of

Sex		Male			Female		
Age (weeks)		10	19	32	10	19	32
ALT	>100 IU/L	0/67	0/72	2/75	0/61	0/72	11/72
	>300 IU/L	0/67	0/72	0/75	0/61	0/72	1/72
AST	>100 IU/L	0/67	0/72	5/75	0/61	4/72	21/72
	>300 IU/L	0/67	0/72	1/75	0/61	0/72	6/72
LDH	>100 IU/L	0/67	0/72	3/75	0/61	1/72	14/72
	>300 IU/L	0/67	0/72	1/75	0/61	0/72	4/72

age, especially in females. However, hepatic lesions were rarely observed in the corresponding animals (data not shown). Similar changes in IGS rats were reported in other reports [2, 3], and were thought to be a common finding of aged Sprague-Dawley rats [3]. We rarely encountered such cases in F344 rats, so it is likely that occurrence of animals with the extraordinarily high values for AST, ALT and LDH activities with aging is one of the characteristics of SD rats. Total cholesterol, triglyceride and phospholipid increased with aging in both sexes, in agreement with the other report on IGS rats [4]. Also total protein slightly increased with aging in both sexes, in agreement with the other reports on IGS rats [2, 4]. On the other hand, CPK, ALP and inorganic phosphorus decreased in both sexes with aging. Decreases in the above parameters in aging IGS rats were also in agreement with the other reports [2-6].

As stated above, age-related changes noted in the present study were similar to the preceding reports. It is thought that occurrence of the animals with extraordinarily high values for AST, ALT and LDH activities is one of the characteristics of SD rat aged 19 weeks and more. Since increases in the above enzyme activities indicate liver damage, their toxicological significance is large. Therefore, these findings should be taken into consideration when evaluating the toxicity of the test substances, especially in a long-term toxicity study using IGS rats.

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Table 1. Parameters and method for blood chemistry

Parameters	Abbreviation	Method
Aspartate aminotransferase	AST	UV-rate method [a)]
Alanine aminotransferase	ALT	UV-rate method [a)]
Lactate dehydrogenase	LDH	UV-rate method [a)]
Creatine phosphokinase	CPK	UV-rate method [a)]
Alkaline phosphatase	AlP	Bessey-Lowry method [a)]
Total cholesterol	T.cho	CEH-COD-POD method [a)]
Triglyceride	TG	LPL-GK-GPO-POD method [a)]
Phospholipid	PL	PLD-ChOD-POD method [a)]
Total bilirubin	T.bilirubin	Bilirubin oxidase method [a)]
Glucose		Glucose dehydrogenase method [a)]
Blood urea nitrogen	BUN	Urease-LEDH method [a)]
Creatinine		Creatininase-creatinase-sarcosine- oxidase-POD method [a)]
Sodium	Na	Ion selective electrode method [a)]
Potassium	K	Ion selective electrode method [a)]
Chloride	Cl	Ion selective electrode method [a)]
Calcium	Ca	OCPC method [a)]
Inorganic phoshphorus	IP	Molybdic acid method [a)]
Total protein	TP	Biuret method [a)]
Albumin		BCG method [a)]
Proteins fraction		Cellulose acetate membrane electrophoresis [b)]

a) : Toshiba Biochemical Analyzer Model TBA-120FR (Toshiba Corporation)
b) : Automated Electrophoresis CLINISCAN SA-V (Helena Laboratory Inc.)

Table 2-1. Blood chemistry in male Crj:CD(SD)IGS Rats

	Age in weeks					
Parameters	10		19		32	
AST (IU/L)	68 ± 7	(67) [a)]	65 ± 9	(72)	68 ± 35	(75)
ALT (IU/L)	30 ± 4	(67)	32 ± 7	(72)	36 ± 19	(75)
LDH (IU/L)	50 ± 12	(67)	50 ± 12	(60)	59 ± 41	(75)
CK (IU/L)	115 ± 16	(10)	74 ± 13	(40)	65 ± 12	(60)
ALP (IU/L)	708 ± 146	(67)	314 ± 61	(72)	261 ± 71	(75)
T.cho (mg/dL)	53 ± 12	(67)	71 ± 15	(72)	88 ± 22	(75)
TG (mg/dL)	35 ± 18	(67)	52 ± 21	(62)	77 ± 35	(75)
PL (mg/dL)	86 ± 13	(57)	99 ± 16	(10)	127 ± 25	(60)
T.bililubin (mg/dL)	0.1 ± 0.0	(67)	0.1 ± 0.0	(72)	0.1 ± 0.0	(75)
Glucose (mg/dL)	134 ± 14	(67)	143 ± 15	(72)	140 ± 14	(75)
BUN (mg/dL)	13 ± 2	(67)	13 ± 1	(72)	13 ± 2	(75)
Creatinine (mg/dL)	0.29 ± 0.04	(67)	0.30 ± 0.03	(72)	0.31 ± 0.05	(75)
Na (mmol/L)	143 ± 1	(67)	143 ± 2	(62)	144 ± 2	(75)
K (mmol/L)	4.6 ± 0.3	(67)	4.7 ± 0.3	(62)	4.6 ± 0.3	(75)
Cl (mmol/L)	109 ± 2	(67)	108 ± 1	(62)	109 ± 2	(75)
Ca (mg/dL)	9.9 ± 0.3	(67)	10.1 ± 0.4	(62)	10.2 ± 0.3	(75)
IP (mg/dL)	7.7 ± 0.6	(67)	6.5 ± 0.8	(62)	6.0 ± 0.9	(75)
TP (g/dL)	5.8 ± 0.3	(67)	6.2 ± 0.3	(72)	6.5 ± 0.3	(75)
Albumin (g/dL)	2.7 ± 0.1	(51)	2.7 ± 0.1	(60)	2.9 ± 0.1	(15)
Proteins fraction						
Albumin (%)	46.3 ± 1.9	(16)	42.7 ± 1.5	(22)	41.3 ± 2.0	(60)
Globulin						
Alpha1 (%)	21.3 ± 2.4	(16)	22.5 ± 2.4	(22)	22.3 ± 2.1	(60)
Alpha2 (%)	10.5 ± 0.7	(16)	9.6 ± 0.6	(22)	10.2 ± 0.8	(60)
Beta (%)	17.1 ± 1.1	(16)	18.8 ± 1.2	(22)	19.7 ± 1.1	(60)
Gamma(%)	4.8 ± 0.7	(16)	6.4 ± 1.4	(22)	6.4 ± 1.0	(60)

a): Mean±S.D. (Number of animals)

Table 2-2. Blood chemistry in female Crj:CD(SD)IGS Rats

	Age in weeks					
Parameters	10		19		32	
AST (IU/L)	64 ± 7	(61) [a)]	66 ± 22	(72)	126 ± 185	(72)
ALT (IU/L)	24 ± 3	(61)	33 ± 15	(72)	64 ± 88	(72)
LDH (IU/L)	46 ± 13	(61)	49 ± 18	(60)	93 ± 137	(72)
CK (IU/L)	90 ± 10	(10)	60 ± 10	(40)	62 ± 50	(45)
ALP (IU/L)	403 ± 91	(61)	156 ± 48	(72)	112 ± 43	(72)
T.cho (mg/dL)	62 ± 14	(61)	83 ± 16	(72)	101 ± 23	(72)
TG (mg/dL)	13 ± 7	(61)	18 ± 13	(62)	36 ± 31	(72)
PL (mg/dL)	103 ± 17	(51)	143 ± 25	(10)	177 ± 35	(57)
T.bililubin (mg/dL)	0.1 ± 0.0	(61)	0.1 ± 0.0	(72)	0.1 ± 0.0	(72)
Glucose (mg/dL)	116 ± 13	(61)	126 ± 16	(72)	128 ± 14	(72)
BUN (mg/dL)	16 ± 3	(61)	15 ± 2	(72)	15 ± 2	(72)
Creatinine (mg/dL)	0.33 ± 0.05	(61)	0.35 ± 0.04	(72)	0.37 ± 0.04	(72)
Na (mmol/L)	142 ± 1	(61)	142 ± 2	(62)	142 ± 1	(72)
K (mmol/L)	4.7 ± 0.3	(61)	4.6 ± 0.3	(62)	4.4 ± 0.4	(72)
Cl (mmol/L)	111 ± 2	(61)	110 ± 2	(62)	111 ± 2	(72)
Ca (mg/dL)	10.0 ± 0.3	(61)	10.3 ± 0.3	(62)	10.3 ± 0.4	(72)
IP (mg/dL)	7.5 ± 0.6	(61)	5.5 ± 1.2	(62)	4.6 ± 1.1	(72)
TP (g/dL)	6.1 ± 0.3	(61)	6.7 ± 0.4	(72)	7.1 ± 0.5	(72)
Albumin (g/dL)	2.9 ± 0.2	(51)	3.0 ± 0.2	(60)	3.1 ± 0.3	(15)
Proteins fraction						
Albumin (%)	47.8 ± 1.9	(10)	48.9 ± 2.8	(22)	48.9 ± 2.5	(57)
Globulin						
Alpha1 (%)	19.1 ± 2.2	(10)	19.0 ± 2.0	(22)	18.4 ± 2.1	(57)
Alpha2 (%)	9.4 ± 0.7	(10)	8.5 ± 0.9	(22)	8.8 ± 0.9	(57)
Beta (%)	17.7 ± 1.2	(10)	16.6 ± 1.7	(22)	16.2 ± 1.3	(57)
Gamma(%)	5.9 ± 1.0	(10)	7.0 ± 1.1	(22)	7.6 ± 1.3	(57)

a): Mean±S.D. (Number of animals)

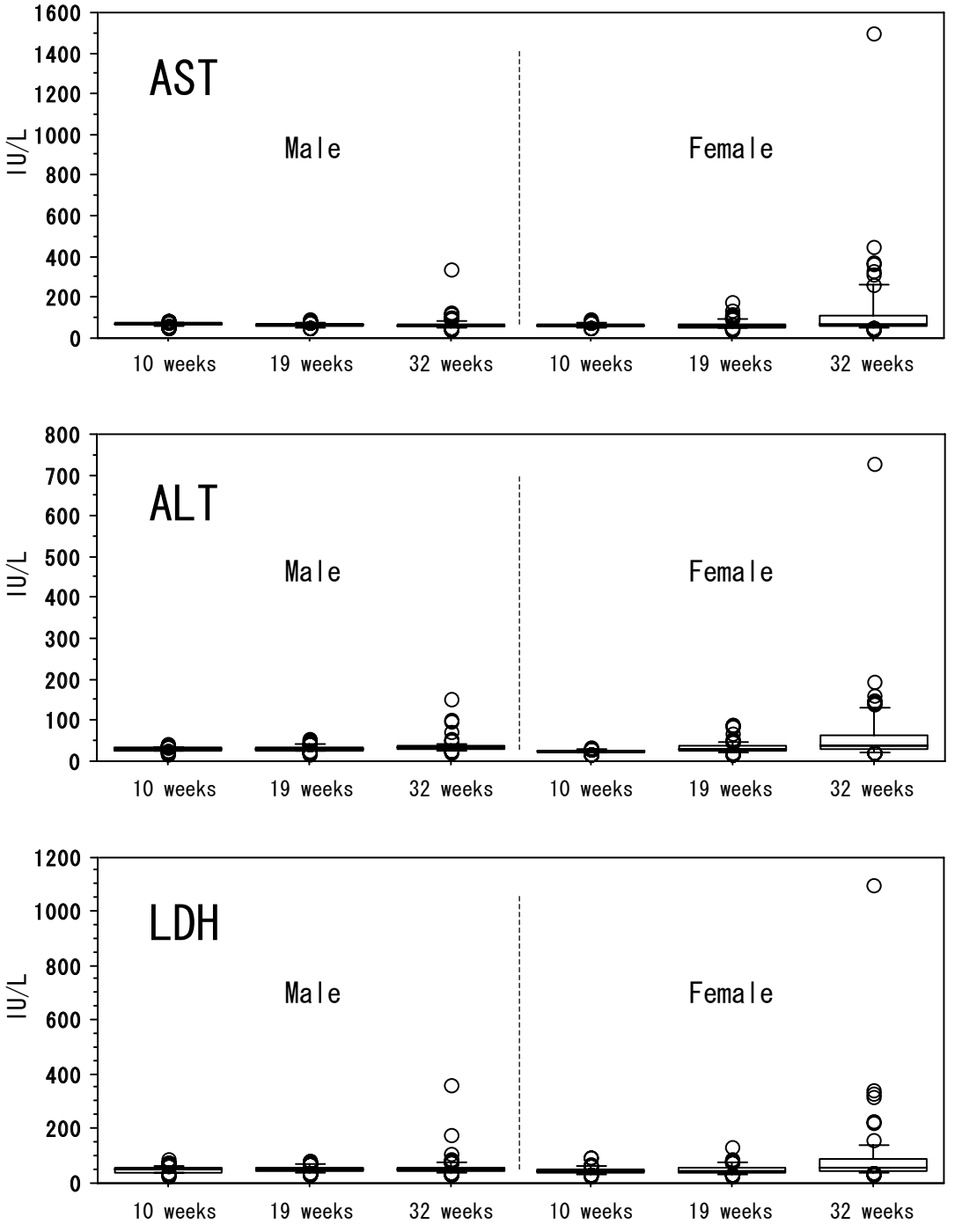

Fig. 1. AST, ALT and LDH in Crj:CD(SD)IGS Rats

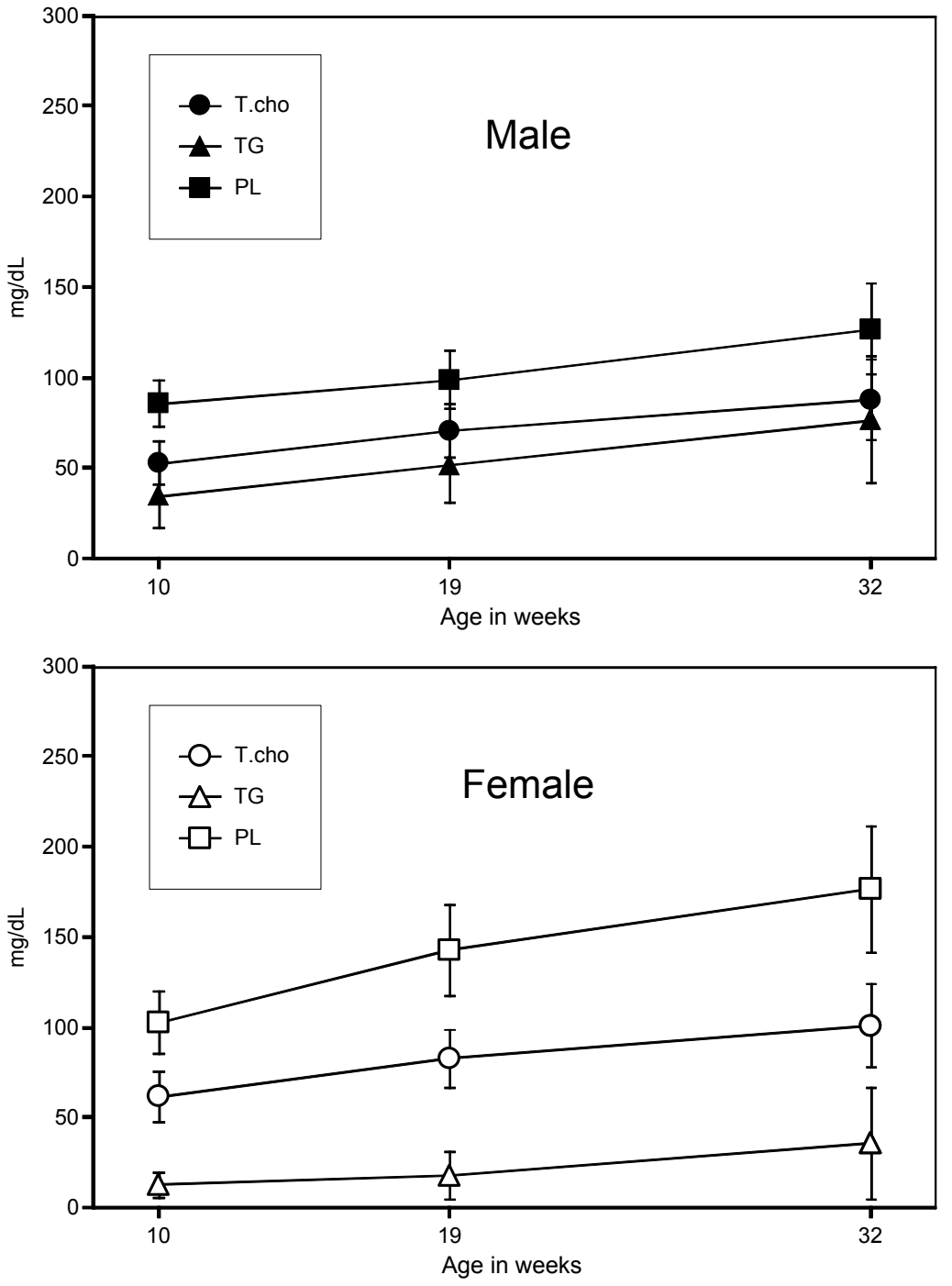

Fig. 2. Total cholesterol, triglyceride and phospholipid in Crj:CD(SD)IGS Rats

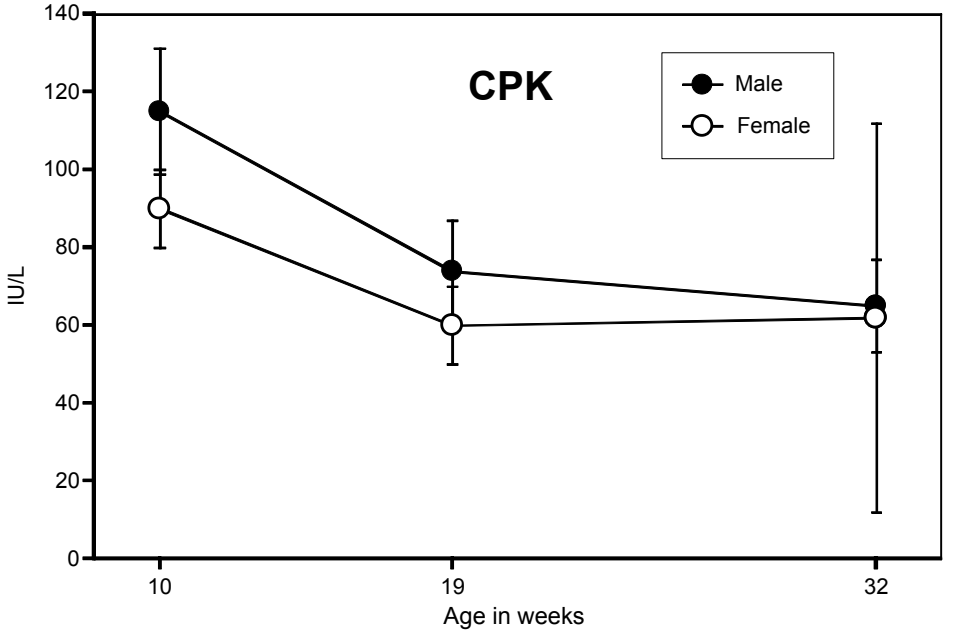

Fig. 3. CPK in Crj:CD(SD)IGS Rats

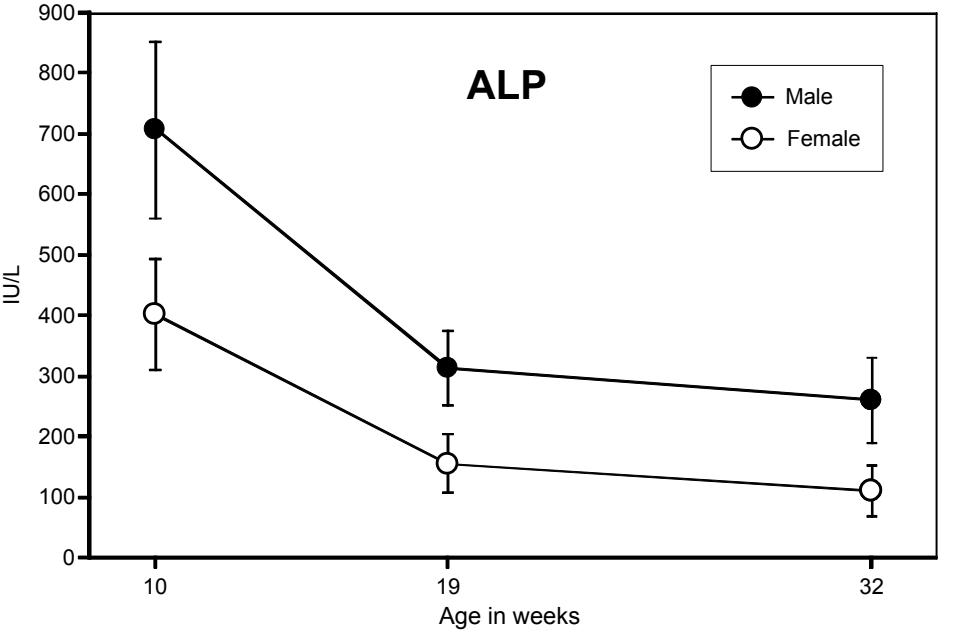

Fig. 4. ALP in Crj:CD(SD)IGS Rats

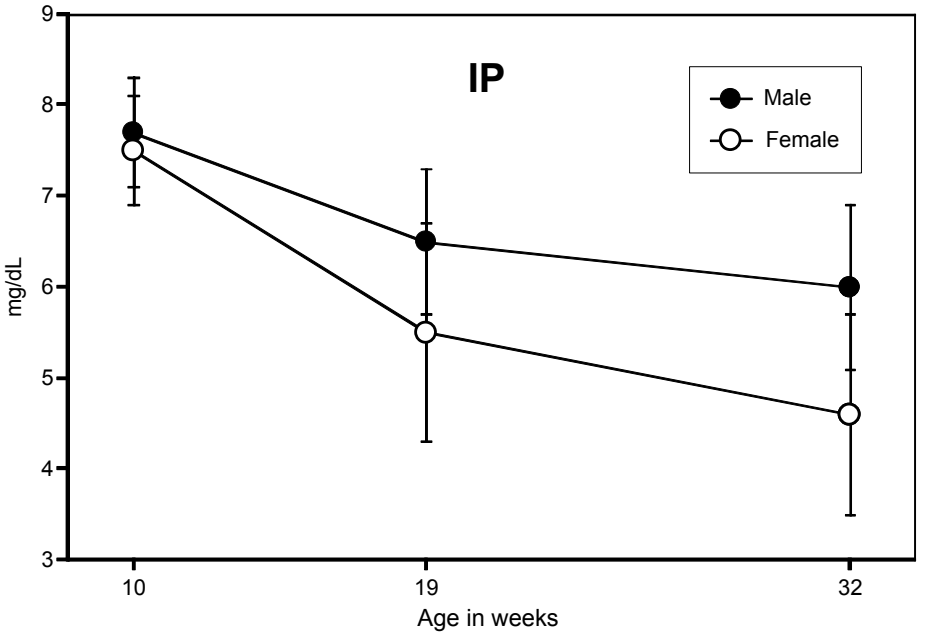

Fig. 5. Inorganic phosphorus in Crj:CD(SD)IGS Rats

Background Data of General Toxicological Parameters in Crj:CD(SD) IGS Rats at 10, 19 and 32 weeks of Age

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ABSTRACT. Several years have passed since Crj:CD(SD)IGS rats were introduced in the toxicity studies. The present study was performed to examine whether changes in the general toxicological parameters have taken place or not in the course of years. It was found that body weight and food consumption in male and female IGS rats had gradually increased when the data were compared with those obtained in 1996 and seemed to reach plateau in 2001. No apparent differences were noted in the hematology, blood biochemistry or organ weights. — Key words: Crj:CD(SD)IGS rat, general toxicological parameter, historical control data

CD(SD)IGS-2002/2003 : 61-72

INTRODUCTION

Crj:CD(SD) IGS rats, which are produced by International Genetic Standard system, became one of the most familiar ones in the toxicity studies. Several years have passed since Crj:CD(SD)IGS rats were introduced in the toxicity studies. The present study was performed to examine whether changes in the general toxicological parameters have taken place or not in the course of years. For the above objective, body weight, food consumption, hematology, blood chemistry and organ weights in Crj:CD(SD) IGS rats obtained in the last three years (2000-2002) were compared with those in 1996.

MATERIALS AND METHODS

Data used: Data were collected from control groups of 4, 13 and 26-week oral gavage toxicity studies (3, 9 and 9 studies, respectively) conducted in our laboratory during the past 3 years. These data were grouped into three groups according to the year obtained (Groups 1, 2 and 3 are 2000, 2001 and 2002, respectively) and were compared to the background data obtained in 1996 [1].

Animals: Male and Female Crj:CD(SD)IGS rats, at 4 weeks of age, were obtained from Charles River Japan Inc. (Atsugi Breeding Center, Japan). The animals were acclimatized for 2 weeks and healthy animals were used at 6 weeks of age.

Housing conditions: The animals were housed in an animal room under the following conditions: temperature at 23 ± 3℃, relative humidity at 50 ± 20%, air ventilation at 10 to 15 times per hour and 12-hour illumination (07:00 to 19:00). They were kept individually in hanging stainless-steel wire mesh cages and supplied commercial low protein feed, CR-LPF (Oriental Yeast Co., Ltd., Japan), and tap water *ad libitum*.

Negative control article and dosing method: The animals were given 0.5 w/v% methylcellulose (MC) or hydroxypropylmethylcellulose (HPMC) solution or water for injection by daily oral gavage for 4, 13 or 26 weeks, at the dose volume of 5 or 10 ml/kg body weight.

Body weight and food consumption: The body weight was recorded once weekly. One day's food consumption was calculated based on the 7 day's cumulative consumption determined weekly.

Hematology: The determined parameters and the methods used are shown in the Table 1. Blood samples were collected from the abdominal aorta into blood collection tubes containing EDTA-2K. However, for determining coagulation parameters, blood samples treated with 3.8 w/v% sodium citrate water were used. The animals were deprived of food for approximately 16 hours prior to blood sample collection.

Blood chemistry: The determined parameters and the methods used are shown in the Table 1. Blood samples were collected from the abdominal aorta and the sera were used for determination; however, GOT(AST), GPT(ALT), LDH and CPK were determined on the plasma obtained from the blood samples treated with heparin. Since the instrument for blood chemistry was changed in 2001, the data from groups 2 and 3 (experiments conducted in 2001 and 2002) were not compared with those obtained before 2000.

Necropsy and organ weights: After collecting the blood samples, all animals were sacrificed and examined macroscopically, and then the following organs were weighed and their relative weights were calculated; brain, thymus, heart, lungs, liver, spleen, kidneys, adrenals, ovaries, uterus, testes and prostate.

RESULTS

1) Body weight (Figs. 1, 2 and Table 2)

In males, body weight gradually increased according to the course of years. However, body weights from groups 2 and 3 were almost the same, and it seems that the increase in body weight reached plateau in 2001. The weights at 6 and 32 weeks of age from group 3 were 16 and 10% higher than the background data obtained in 1996[1].

In females, body weights from groups 1, 2 and 3 were almost the same, but they were higher than the background data obtained in 1996. The body weights at 6 and 32 weeks of age from group 3 were 14 and 9% higher than the background data obtained in 1996.

2) Food consumption (Figs. 3, 4 and Table 3)

Food consumption from groups 1, 2 and 3 was higher than the background data obtained in 1996. However, the degree of increase was slight.

3) Hematology (Tables 4-1 and 4-2)

There were no apparent differences among years in any parameter at the respective age of rats.

4) Blood chemistry (Tables 5-1 and 5-2)

Blood chemistry parameters were compared between group 1 and background data obtained in 1996 and between groups 2 and 3. There were no apparent differences in any parameter among groups at the respective age of rats.

5) Organ weights (Tables 6-1 and 6-2)

There were no apparent differences in any organ among groups or from the background data obtained in 1996.

DISCUSSION

It was found that body weight and food consumption in male and female IGS rats had gradually increased in the course of years when the data were compared with those obtained in 1996. The above phenomenon is noteworthy since obesity is a life-threatening risk, especially in a long-term toxicity study. However, it is likely that biological characteristics of IGS rats are in a steady state at present because the body weight seems to have reached plateau in 2001. No apparent changes were noted in hematology, blood chemistry or organ weights.

REFERENCE

1. Okazaki, S., Suwa, K., Okazaki, K., Hatayama, K., Ichikawa, Y., Komatsu, M., Ogawa, T., and Tamura, K. 1998. Comparison of general toxicological parameters between Crj:CD(SD) Rats fed normal commercial diet and Crj:CD(SD) IGS rats fed normal or low protein commercial diet. pp 71-81. In: Biological Reference Data on CD(SD) IGS Rats- 1998 (Matsuzawa, T. and Inoue, H. eds.) CD(SD) IGS Study Group, Yokohama, Japan.

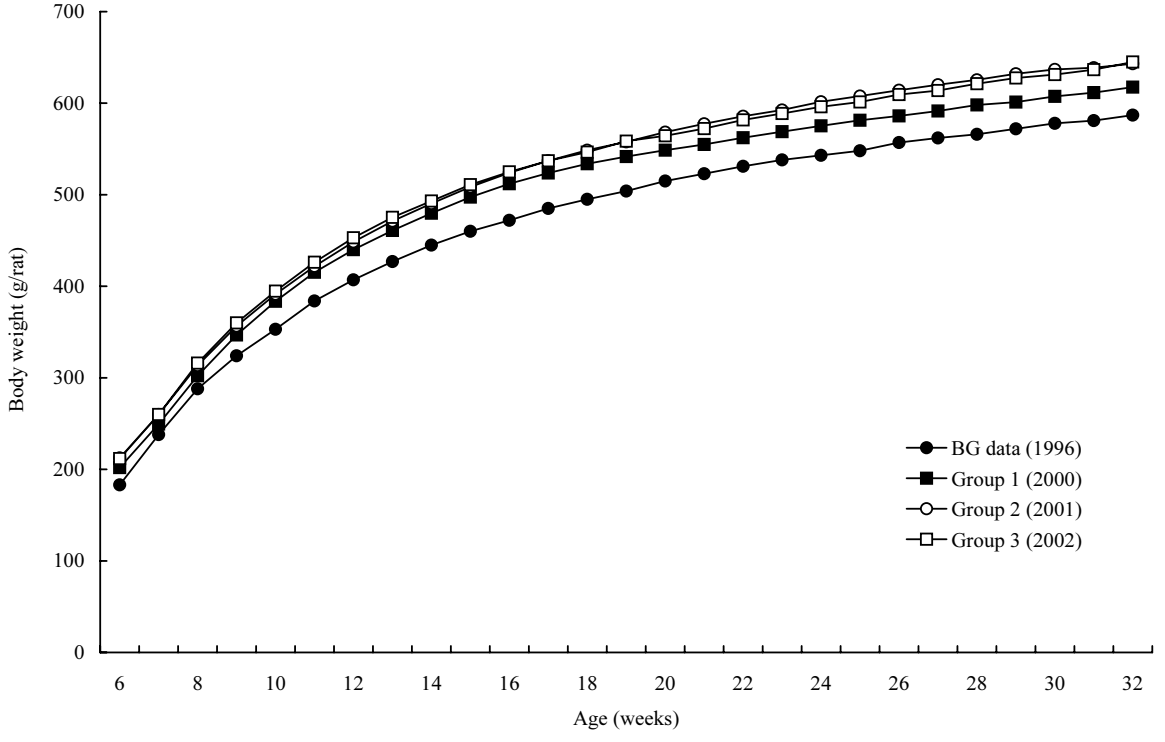

Figure 1. Growth Curve in Crj:CD(SD)IGS Male Rats Fed CR-LPF

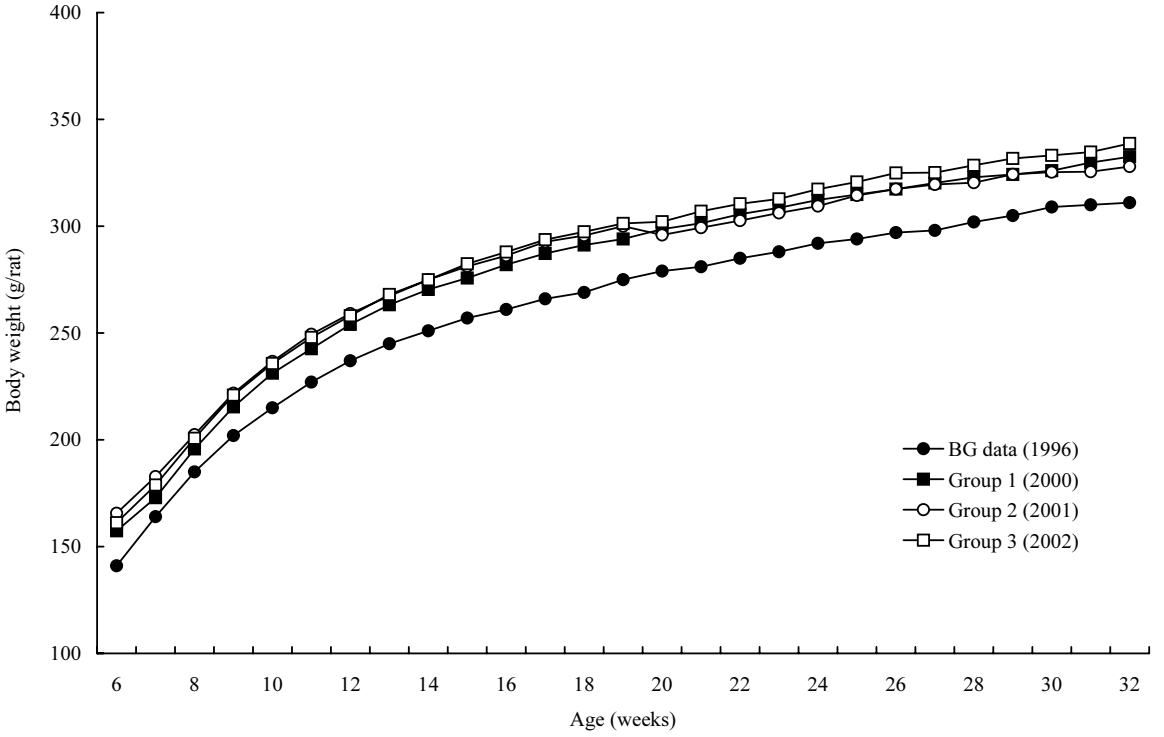

Figure 2. Growth Curve in Crj:CD(SD)IGS Female Rats Fed CR-LPF

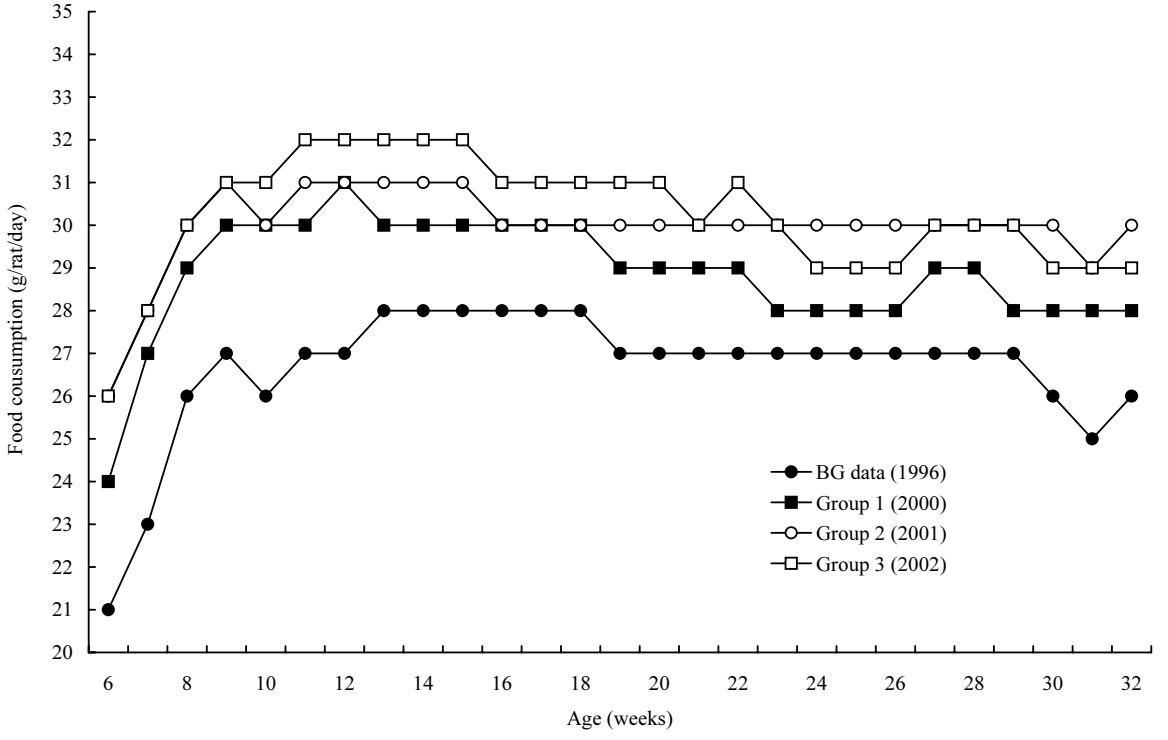

Figure 3. Food cousumption in Crj:CD(SD)IGS Male Rats Fed CR-LPF

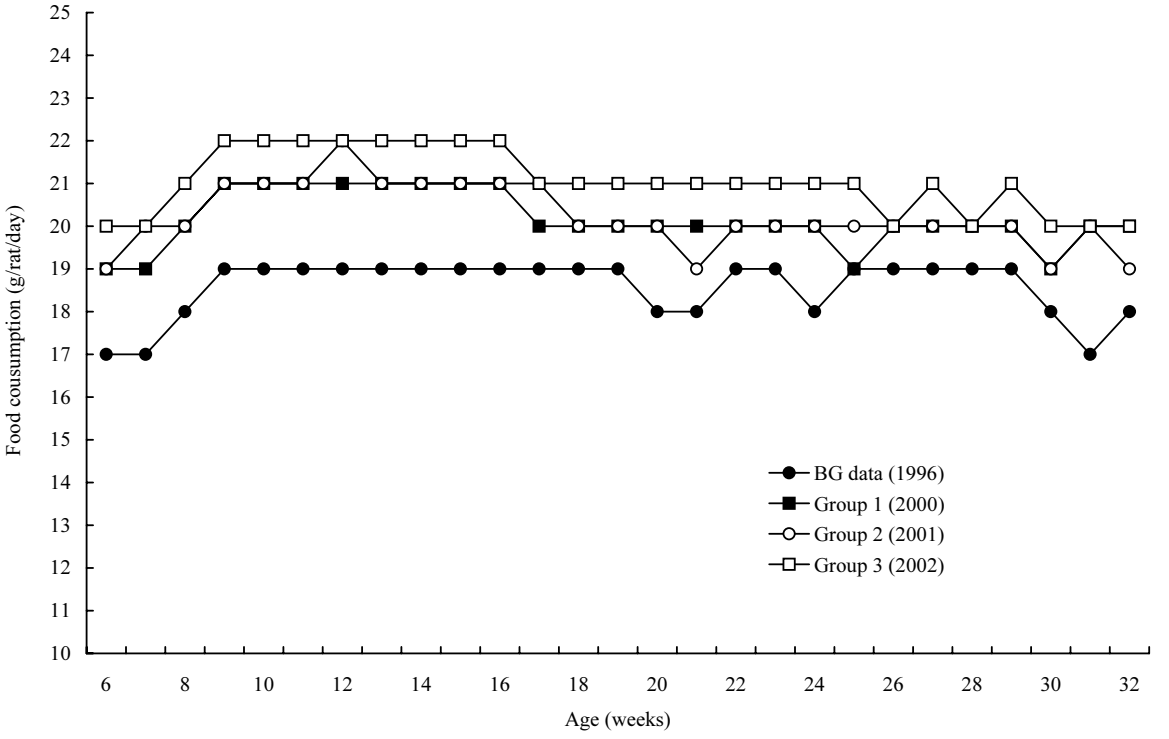

Figure 4. Food cousumption in Crj:CD(SD)IGS Female Rats Fed CR-LPF

Table 1. Item and method for laboratory examinations

Examination	Item	Abbreviation	Method
Hematology	Red blood cell	RBC	Electronic counting method [a)]
	White blood cell	WBC	Electronic counting method [a)]
	Platelet		Electronic counting method [a)]
	Differential leukocyte count		Microscopic examination using May-Giemsa staining
	Hemoglobin	Hb	Cyanmethemoglobin method [a)]
	Hematocrit	Ht	Calculated from MCV and RBC [a)]
	Reticulocyte ratio		Brecher method [a)]
	Mean corpuscular volume	MCV	Electronic counting method [a)]
	Mean corpuscular hemoglobin	MCH	Calculated from Hb and RBC [a)]
	Mean corpuscular hemoglobin concentration	MCHC	Calculated from Hb and Ht [a)]
	Prothrombin time	PT	Clot method [b)]
	Activated partial thromboplastin time	APTT	Clot method [b)]
	Fibrinogen		Thromboplastin method [b)]
Blood chemistry	Glutamic oxaloacetic transaminase	GOT	UV-rate method [c) d)]
	Glutamic pyruvic transaminase	GPT	UV-rate method [c) d)]
	Lactate dehydrogenase	LDH	UV-rate method [c) d)]
	Creatine phosphokinase	CPK	UV-rate method [c) d)]
	Alkaline phosphatase	AlP	Bessey-Lowry method [c) d)]
	Glucose		Hexokinase-G6PD method [c)] or Glucose dehydorogenase mathod [d)]
	Blood urea nitrogen	BUN	Urease-GLDH method [c)] or Urease-LEDH method [d)]
	Total bilirubin	T.bilirubin	Azobilirubin method [c)] or Bilirubin oxidase method [d)]
	Creatinine		Jaffé method [c)] or craatininase-creatinase-sarcosine-oxidase-POD method [d)]
	Triglyceride	TG	UV-rate method [c)] or LPL-GK-GPO-POD method [d)]
	Total cholesterol	T.cho	CEH-COD-POD method [c) d)]
	Phospholipid	PL	PLD-ChOD-POD method [c) d)]
	Total protein	TP	Biuret method [c) d)]
	Albumin		BCG method [c) d)]
	Albumin-globulin ratio	A/G	Calculated from protein fractions
	Sodium	Na	Ion selective electrode method [c) d)]
	Potassium	K	Ion selective electrode method [c) d)]
	Chloride	Cl	Ion selective electrode method [c) d)]
	Calcium	Ca	OCPC method [c) d)]
	Inorganic phosphorus	P	Molybdic acid method [c) d)]

a) : Coulter Counter T890 (Coulter Electronics Inc.)
b) : Coagulometer ALC 100 (Instrumentation Laboratory)
c) : Automatic Analyzer Monarch (Instrumentation Laboratory) : Used before 2001.
d) : Toshiba Biochemical Analyzer Model TBA-120FR (Toshiba. Corporation.) : Used after 2001

Table2. Body weight in Crj:CD(SD)IGS Rats Fed CR-LPF

Sex	Female				Male			
Group Age(weeks)	BG data (1996)	Group 1 (2000)	Group 2 (2001)	Group 3 (2002)	BG data (1996)	Group 1 (2000)	Group 2 (2001)	Group 3 (2002)
6	141±7[a)](120)	157±8(75)	166±9(67)	161±9(95)	183±8(120)	202±11(75)	213±11(82)	212±11(95)
10	215±16(120)	231±17(75)	237±20(67)	236±21(95)	353±27(120)	384±32(75)	391±28(82)	395±32(95)
19	275±23(80)	294±24(75)	300±30(57)	301±29(75)	504±41(80)	542±54(74)	558±52(72)	559±55(75)
32	311±33(40)	333±37(45)	328±38(27)	339±39(45)	587±56(45)	618±79(45)	643±77(42)	645±78(45)

a) : Mean±S.D.
( ) : Number of amimals determined

Table3. Food consumption in Crj:CD(SD)IGS Rats Fed CR-LPF

Sex	Female				Male			
Group Age(weeks)	BG data (1996)	Group 1 (2000)	Group 2 (2001)	Group 3 (2002)	BG data (1996)	Group 1 (2000)	Group 2 (2001)	Group 3 (2002)
6	17±3[a)](120)	19±2[a)](75)	19±2(67)	20±2(95)	21±2(120)	24±2(75)	26±2(82)	26±2(95)
10	19±2(120)	21±2(75)	21±2(67)	22±2(95)	26±2(120)	30±3(75)	30±3(82)	31±3(95)
19	19±2(80)	20±2(75)	20±3(57)	21±2(75)	27±3(80)	29±3(74)	30±3(72)	31±3(75)
32	18±1(40)	20±2(45)	19±2(27)	20±2(45)	26±2(40)	28±4(45)	30±3(42)	29±3(45)

a) : Mean±S.D.
( ) : Number of amimals determined

Table 4-1. Hematology in Crj:CD(SD)IGS Female Rats Fed CR-LPF

Sex	Female							
Group	BG data		Group 1		Group 2		Group 3	
Item (Unit)	(1996)		(2000)		(2001)		(2002)	
Age : 10 weeks								
RBC (x10⁴/mm³)	771±38[a)]	(40)	—		770±36	(10)	770±41	(20)
Hemoglobin (g/dL)	16.3±0.6	(40)	—		15.9±0.8	(10)	15.4±0.8	(20)
Hematocrit (%)	44±2	(40)	—		45±3	(10)	45±3	(20)
MCV (μm³)	57.3±1.6	(40)	—		58.6±1.5	(10)	58.4±1.8	(20)
MCH (pg)	21.2±0.8	(40)	—		20.6±0.6	(10)	20.0±0.6	(20)
MCHC (%)	37.0±0.7	(40)	—		35.1±0.6	(10)	34.2±0.9	(20)
Reticulocyte (‰)	14±5	(40)	—		1.2±0.3	(10)	1.9±0.4	(20)
WBC (x10²/mm³)	74±19	(40)	—		61±13	(10)	65±17	(20)
Lymphocyte (%)	84.0±6.5	(40)	—		91.6±1.7	(10)	88.6±5.3	(20)
Stab neutrophil (%)	0.0±0.0	(40)	—		0.1±0.2	(10)	0.0±0.1	(20)
Segmented neutrophil (%)	15.1±6.5	(40)	—		7.2±1.7	(10)	10.4±5.4	(20)
Eosinophil (%)	0.9±0.7	(40)	—		0.7±0.4	(10)	0.6±0.7	(20)
Basophil (%)	0.0±0.0	(40)	—		0.0±0.0	(10)	0.0±0.0	(20)
Monocyte (%)	0.1±0.2	(40)	—		0.6±0.5	(10)	0.4±0.5	(20)
Platelet (x10⁴/mm³)	117.5±12.2	(40)	—		105.1±6.5	(10)	105.8±8.2	(20)
PT (sec)	11.9±0.5	(40)	—		—		—	
APTT (sec)	12.3±1.0	(40)	—		—		—	
Fibrinogen (mg/dL)	203±20	(40)	—		—		—	
Age : 19 weeks								
RBC (x10⁴/mm³)	793±36	(40)	797±38	(30)	791±33	(30)	792±31	(30)
Hemoglobin (g/dL)	16.1±0.6	(40)	15.4±0.7	(30)	15.1±0.4	(30)	15.0±0.6	(30)
Hematocrit (%)	44±2	(40)	45±2	(30)	44±2	(30)	45±2	(30)
MCV (μm³)	55.4±1.6	(40)	56.2±1.2	(30)	56.2±1.4	(30)	56.8±1.3	(30)
MCH (pg)	20.3±0.9	(40)	19.3±0.5	(30)	19.0±0.5	(30)	19.0±0.5	(30)
MCHC (%)	36.7±0.9	(40)	34.3±0.5	(20)	33.9±0.6	(30)	33.4±0.5	(30)
Reticulocyte (‰)	18±4	(40)	1.8±0.5	(30)	1.6±0.3	(30)	1.7±0.3	(10)
WBC (x10²/mm³)	56±17	(40)	56±16	(30)	52±14	(30)	59±24	(30)
Lymphocyte (%)	85.0±5.2	(40)	85.2±5.3	(20)	87.2±4.4	(20)	84.7±6.7	(10)
Stab neutrophil (%)	0.0±0.1	(40)	0.2±0.3	(20)	0.1±0.3	(20)	0.1±0.2	(10)
Segmented neutrophil (%)	14.0±5.2	(40)	13.5±5.3	(20)	11.3±3.8	(20)	13.8±6.4	(10)
Eosinophil (%)	0.9±0.6	(40)	0.7±0.7	(20)	0.9±1.0	(20)	1.3±1.1	(10)
Basophil (%)	0.0±0.0	(40)	0.0±0.0	(20)	0.0±0.0	(20)	0.0±0.0	(10)
Monocyte (%)	0.1±0.2	(40)	0.5±0.6	(20)	0.6±0.8	(20)	0.2±0.3	(10)
Platelet (x10⁴/mm³)	105.0±9.9	(40)	100.8±8.6	(30)	100.8±11.7	(30)	101.3±14.9	(30)
PT (sec)	12.0±0.6	(40)	—		—		12.4±0.5	(10)
APTT (sec)	13.4±1.1	(40)	—		—		15.0±1.0	(10)
Fibrinogen (mg/dL)	168±16	(40)	—		—		210±28	(10)
Age : 32 weeks								
RBC (x10⁴/mm³)	792±45	(40)	769±43	(45)	790±36	(15)	778±39	(45)
Hemoglobin (g/dL)	15.2±0.7	(40)	15.2±0.7	(45)	15.1±0.6	(15)	14.9±0.7	(45)
Hematocrit (%)	43±2	(40)	44±2	(45)	45±2	(15)	44±2	(45)
MCV (μm³)	54.8±2.0	(40)	57.1±1.6	(45)	56.9±1.7	(15)	56.9±1.8	(45)
MCH (pg)	19.2±0.7	(40)	19.7±0.7	(45)	19.1±0.6	(15)	19.1±0.7	(45)
MCHC (%)	35.1±0.7	(40)	34.6±0.7	(45)	33.6±0.6	(15)	33.5±0.7	(45)
Reticulocyte (‰)	19±4	(40)	1.6±0.5	(45)	1.4±0.2	(15)	1.3±0.4	(45)
WBC (x10²/mm³)	50±15	(40)	47±12	(45)	46±14	(15)	46±15	(45)
Lymphocyte (%)	81.3±6.9	(40)	83.9±8.2	(45)	85.7±4.8	(15)	78.8±9.6	(45)
Stab neutrophil (%)	0.0±0.1	(40)	0.2±0.5	(45)	0.0±0.0	(15)	0.6±0.6	(45)
Segmented neutrophil (%)	17.7±6.9	(40)	14.1±7.6	(45)	12.4±4.9	(15)	18.8±9.4	(45)
Eosinophil (%)	1.0±0.8	(40)	1.1±0.8	(45)	1.0±0.7	(15)	1.5±1.0	(45)
Basophil (%)	0.0±0.0	(40)	0.0±0.0	(45)	0.0±0.0	(15)	0.0±0.0	(45)
Monocyte (%)	0.1±0.2	(40)	0.7±0.5	(45)	0.8±0.6	(15)	0.3±0.4	(45)
Platelet (x10⁴/mm³)	95.3±11.4	(40)	93.7±9.5	(45)	107.7±28.7	(15)	99.0±9.8	(45)
PT (sec)	11.7±0.4	(40)	10.9±0.7	(15)	11.9±0.4	(15)	12.6±0.5	(45)
APTT (sec)	12.5±1.1	(40)	14.7±2.3	(15)	14.9±1.0	(15)	16.4±8.5	(45)
Fibrinogen (mg/dL)	170±20	(40)	169±22	(15)	183±21	(15)	187±33	(45)

a) : Mean±S.D.

( ) : Number of animals determined

Table 4-2. Hematology in Crj:CD(SD)IGS Male Rats Fed CR-LPF

Sex	Male							
Group	BG data		Group 1		Group 2		Group 3	
Item (Unit)	(1996)		(2000)		(2001)		(2002)	
Age : 10 weeks								
RBC (x10⁴/mm³)	775±33	(40)	—		782±31	(10)	775±32	(20)
Hemoglobin (g/dL)	16.5±0.6	(40)	—		15.9±0.6	(10)	15.5±0.6	(20)
Hematocrit (%)	45±2	(40)	—		47±2	(10)	46±2	(20)
MCV (μm³)	57.6±1.5	(40)	—		60.1±1.6	(10)	59.0±0.8	(20)
MCH (pg)	21.2±0.7	(40)	—		20.3±0.7	(10)	20.0±0.4	(20)
MCHC (%)	36.9±0.7	(40)	—		33.8±0.6	(10)	33.9±0.5	(20)
Reticulocyte (‰)	15±4	(40)	—		1.6±0.2	(10)	2.3±0.5	(20)
WBC (x10²/mm³)	85±22	(40)	—		92±27	(10)	87±24	(20)
Lymphocyte (%)	85.5±6.0	(40)	—		90.8±3.5	(10)	87.1±3.9	(20)
Stab neutrophil (%)	0.0±0.0	(40)	—		0.0±0.0	(10)	0.0±0.1	(20)
Segmented neutrophil (%)	13.7±6.0	(40)	—		7.6±3.4	(10)	11.6±3.6	(20)
Eosinophil (%)	0.8±0.8	(40)	—		0.6±0.6	(10)	0.9±0.6	(20)
Basophil (%)	0.0±0.0	(40)	—		0.0±0.0	(10)	0.0±0.0	(20)
Monocyte (%)	0.1±0.2	(40)	—		1.1±0.8	(10)	0.5±0.5	(20)
Platelet (x10⁴/mm³)	112.1±9.8	(40)	—		114.6±15.0	(10)	106.0±7.9	(20)
PT (sec)	12.7±0.5	(40)	—		—		—	
APTT (sec)	15.2±1.2	(40)	—		—		—	
Fibrinogen (mg/dL)	246±41	(40)	—		—		—	
Age : 19 weeks								
RBC (x10⁴/mm³)	853±39	(40)	838±38	(29)	837±38	(30)	837±61	(30)
Hemoglobin (g/dL)	16.4±0.5	(40)	15.8±0.7	(29)	15.4±0.6	(30)	15.3±1.0	(30)
Hematocrit (%)	45±2	(40)	46±2	(29)	46±2	(30)	46±3	(30)
MCV (μm³)	52.7±1.7	(40)	54.9±1.5	(29)	54.3±1.3	(30)	54.9±1.8	(30)
MCH (pg)	19.2±0.6	(40)	18.9±0.4	(29)	18.4±0.4	(30)	18.3±0.6	(30)
MCHC (%)	36.5±0.5	(40)	34.4±0.6	(29)	33.9±0.8	(30)	33.4±0.6	(30)
Reticulocyte (‰)	17±5	(40)	2.0±0.8	(19)	1.7±0.4	(30)	1.7±0.5	(10)
WBC (x10²/mm³)	86±24	(40)	83±17	(29)	95±24	(30)	88±23	(30)
Lymphocyte (%)	83.6±6.0	(40)	82.3±8.2	(19)	86.3±5.0	(20)	81.2±9.6	(10)
Stab neutrophil (%)	0.0±0.1	(40)	0.2±0.3	(19)	0.2±0.3	(20)	0.0±0.0	(10)
Segmented neutrophil (%)	15.6±6.0	(40)	16.3±8.2	(19)	12.0±5.0	(20)	17.7±9.7	(10)
Eosinophil (%)	0.7±0.7	(40)	0.6±0.7	(19)	1.0±0.5	(20)	1.0±0.7	(10)
Basophil (%)	0.0±0.0	(40)	0.0±0.0	(19)	0.0±0.0	(20)	0.0±0.0	(10)
Monocyte (%)	0.1±0.2	(40)	0.5±0.5	(19)	0.6±0.4	(20)	0.1±0.2	(10)
Platelet (x10⁴/mm³)	105.6±11.8	(40)	108.7±9.6	(29)	108.2±11.9	(30)	105.8±14.0	(30)
PT (sec)	12.0±0.6	(40)	—		—		12.9±0.5	(10)
APTT (sec)	14.3±1.6	(40)	—		—		17.6±1.3	(10)
Fibrinogen (mg/dL)	240±26	(40)	—		—		323±27	(10)
Age : 32 weeks								
RBC (x10⁴/mm³)	868±60	(40)	846±28	(45)	831±30	(30)	843±44	(45)
Hemoglobin (g/dL)	15.6±1.0	(40)	15.7±0.5	(45)	15.2±0.6	(30)	15.4±0.7	(45)
Hematocrit (%)	45±3	(40)	46±2	(45)	45±2	(30)	46±3	(45)
MCV (μm³)	52.1±1.6	(40)	54.1±1.4	(45)	54.2±1.8	(30)	54.3±1.6	(45)
MCH (pg)	18.0±0.6	(40)	18.5±0.5	(45)	18.3±0.5	(30)	18.2±0.5	(45)
MCHC (%)	34.5±0.6	(40)	34.3±0.7	(45)	33.8±0.8	(30)	33.6±0.9	(45)
Reticulocyte (‰)	21±7	(40)	1.6±0.3	(45)	1.9±0.4	(30)	1.6±0.5	(45)
WBC (x10²/mm³)	77±18	(40)	81±23	(45)	83±28	(30)	80±26	(45)
Lymphocyte (%)	76.6±8.9	(40)	81.4±6.5	(45)	77.6±7.7	(30)	76.7±8.9	(45)
Stab neutrophil (%)	0.0±0.1	(40)	0.2±0.3	(45)	0.2±0.3	(30)	0.5±0.5	(45)
Segmented neutrophil (%)	22.1±8.7	(40)	16.9±6.4	(45)	20.3±7.5	(30)	21.3±8.8	(45)
Eosinophil (%)	1.2±1.0	(40)	0.9±0.8	(45)	0.9±0.5	(30)	1.1±0.8	(45)
Basophil (%)	0.0±0.0	(40)	0.0±0.0	(45)	0.0±0.0	(30)	0.0±0.0	(45)
Monocyte (%)	0.1±0.2	(40)	0.6±0.6	(45)	1.1±1.0	(30)	0.4±0.4	(45)
Platelet (x10⁴/mm³)	96.5±12.1	(40)	111.1±15.5	(45)	115.5±14.6	(30)	109.7±19.7	(45)
PT (sec)	12.2±0.6	(40)	11.5±0.6	(15)	12.3±0.3	(30)	12.9±0.5	(45)
APTT (sec)	13.8±1.5	(40)	13.7±1.7	(15)	15.8±1.4	(30)	16.0±1.2	(45)
Fibrinogen (mg/dL)	259±25	(40)	270±27	(15)	287±31	(30)	295±44	(45)

a) : Mean±S.D.

( ) : Number of animals determined

Table 5-1. Blood chemistry in Crj:CD(SD)IGS Rats Fed CR-LPF (determined by Monarch)

Sex	Female				Male			
Group	BG data		Group 1		BG data		Group 1	
Item (Unit)	(1996)		(2000)		(1996)		(2000)	
Age : 10 weeks								
GOT(IU/L)	60±9[a)]	(40)	−		59±9	(40)	−	
GPT(IU/L)	37±5	(40)	−		44±6	(40)	−	
LDH(IU/L)	23±5	(40)	−		31±4	(40)	−	
CPK(IU/L)	94±16	(40)	−		122±22	(40)	−	
AlP(IU/L)	241±56	(40)	−		397±74	(40)	−	
T.cho(mg/dL)	63±19	(40)	−		64±14	(40)	−	
TG(mg/dL)	26±7	(40)			38±14	(40)		
PL(mg/dL)	107±23	(40)	−		93±14	(40)	−	
T.bililubin(mg/dL)	0.10±0.01	(40)	−		0.10±0.01	(40)	−	
Glucose(mg/dL)	99±9	(40)	−		112±11	(40)	−	
BUN(mg/dL)	16±2	(40)	−		14±1	(40)	−	
Creatinine(mg/dL)	0.62±0.04	(40)	−		0.61±0.05	(40)	−	
Na(mmol/L)	143±1	(40)	−		145±1	(40)	−	
K(mmol/L)	4.6±0.4	(40)	−		4.4±0.3	(40)	−	
Cl(mmol/L)	115±2	(40)	−		114±2	(40)	−	
Ca(mg/dL)	9.2±0.3	(40)	−		9.0±0.3	(40)	−	
P(mg/dL)	7.2±0.5	(40)	−		6.9±0.5	(40)	−	
TP(g/dL)	5.9±0.3	(40)	−		5.6±0.2	(40)	−	
Albumin(g/dL)	3.8±0.2	(40)	−		3.6±0.1	(40)	−	
A/G	1.04±0.07	(40)	−		0.93±0.09	(40)	−	
Age : 19 weeks								
GOT(IU/L)	72±24	(40)	63±18	(30)	64±11	(40)	53±15	(29)
GPT(IU/L)	45±12	(40)	37±13	(30)	46±6	(40)	38±7	(29)
LDH(IU/L)	28±18	(40)	31±15	(30)	42±11	(40)	52±25	(29)
CPK(IU/L)	65±10	(40)	53±9	(20)	92±15	(40)	72±11	(19)
AlP(IU/L)	94±23	(40)	82±23	(30)	181±33	(40)	181±35	(29)
T.cho(mg/dL)	76±18	(40)	89±19	(30)	75±20	(40)	77±18	(29)
TG(mg/dL)	30±8	(40)	36±9	(20)	50±14	(40)	71±25	(19)
PL(mg/dL)	133±24	(40)	150±22	(10)	101±21	(40)	110±22	(9)
T.bililubin(mg/dL)	0.11±0.02	(40)	0.11±0.02	(30)	0.11±0.01	(40)	0.12±0.02	(29)
Glucose(mg/dL)	116±12	(40)	121±9	(30)	126±14	(40)	140±14	(29)
BUN(mg/dL)	17±3	(40)	16±2	(30)	15±2	(40)	14±1	(29)
Creatinine(mg/dL)	0.65±0.06	(40)	0.67±0.05	(30)	0.63±0.05	(40)	0.63±0.06	(29)
Na(mmol/L)	143±1	(40)	143±1	(20)	144±2	(40)	145±1	(19)
K(mmol/L)	4.5±0.4	(40)	4.5±0.3	(20)	4.6±0.4	(40)	4.7±0.3	(19)
Cl(mmol/L)	114±2	(40)	112±2	(20)	113±2	(40)	110±2	(19)
Ca(mg/dL)	9.3±0.4	(40)	9.8±0.3	(20)	9.2±0.3	(40)	9.8±0.3	(19)
P(mg/dL)	6.0±15	(40)	6.0±1.2	(20)	6.1±0.6	(40)	6.9±1.0	(19)
TP(g/dL)	6.6±0.5	(40)	7.1±0.4	(30)	6.1±0.2	(40)	6.7±0.3	(29)
Albumin(g/dL)	4.0±0.2	(40)	4.4±0.2	(20)	3.6±0.1	(40)	3.9±0.2	(20)
A/G	0.96±0.08	(40)	0.94±0.13	(10)	0.76±0.05	(40)	0.78±0.08	(9)
Age : 19 weeks								
GOT(IU/L)	165±414	(40)	87±127	(45)	77±63	(40)	53±25	(45)
GPT(IU/L)	75±100	(40)	43±25	(45)	56±32	(40)	40±24	(45)
LDH(IU/L)	119±478	(40)	46±62	(45)	52±44	(40)	48±20	(45)
CPK(IU/L)	59±16	(40)	47±9	(45)	78±15	(40)	60±11	(45)
AiP(IU/L)	62±20	(40)	54±16	(45)	171±39	(40)	144±26	(45)
T.cho(mg/dL)	94±23	(40)	103±24	(45)	89±24	(40)	88±22	(45)
TG(mg/dL)	42±14	(40)	63±24	(45)	77±26	(40)	104±54	(45)
PL(mg/dL)	169±33	(40)	182±36	(45)	121±22	(40)	124±26	(45)
T.bililubin(mg/dL)	0.12±0.02	(40)	0.12±0.03	(45)	0.11±0.01	(40)	0.13±0.03	(45)
Glucose(mg/dL)	120±13	(40)	127±13	(45)	137±14	(40)	139±13	(45)
BUN(mg/dL)	16±3	(40)	16±3	(45)	16±2	(40)	13±2	(45)
Creatinine(mg/dL)	0.65±0.05	(40)	0.67±0.08	(45)	0.65±0.06	(40)	0.60±0.05	(45)
Na(mmol/L)	143±1	(40)	142±2	(45)	144±2	(40)	145±1	(45)
K(mmol/L)	4.4±0.4	(40)	4.4±0.4	(45)	4.5±0.3	(40)	4.7±0.3	(45)
Cl(mmol/L)	112±1	(40)	112±2	(45)	111±1	(40)	111±2	(45)
Ca(mg/dL)	9.6±0.3	(40)	10.3±0.4	(45)	9.3±0.2	(40)	10.0±0.4	(45)
P(mg/dL)	6.1±0.7	(40)	5.4±1.3	(45)	5.2±0.5	(40)	6.4±0.9	(45)
TP(g/dL)	7.1±0.4	(40)	7.6±0.6	(45)	6.4±0.2	(40)	6.8±0.3	(45)
Albumin(g/dL)	4.3±0.3	(40)	−		3.7±0.1	(40)	−	
A/G	1.02±0.09	(40)	1.00±0.10	(45)	0.74±0.05	(40)	0.72±0.07	(45)

a) : Mean±S.D.

( ) : Number of animals determined

Table 5-2. Blood chemistry in Crj:CD(SD)IGS Rats Fed CR-LPF (determined by TBA-120FR)

Sex	Female				Male			
Group	Group 2		Group 3		Group 2		Group 3	
Item (Unit)	(2001)		(2002)		(2001)		(2002)	
Age : 10 weeks								
GOT(IU/L)	61±6	(10)	59±8	(20)	61±6	(10)	64±7	(20)
GPT(IU/L)	22±3	(10)	25±4	(20)	29±5	(10)	29±4	(20)
LDH(IU/L)	44±6	(10)	50±17	(20)	46±9	(10)	44±9	(20)
CPK(IU/L)	90±15	(10)	91±19	(20)	118±17	(10)	120±23	(20)
AlP(IU/L)	412±107	(10)	359±109	(20)	572±86	(10)	689±122	(20)
T.cho(mg/dL)	74±15	(10)	78±10	(20)	72±15	(10)	69±11	(20)
TG(mg/dL)	20±11	(10)	17±9	(20)	44±24	(10)	49±21	(20)
PL(mg/dL)	−		−		−		−	(20)
T.bililubin(mg/dL)	0.07±0.01	(10)	0.05±0.02	(20)	0.09±0.03	(10)	0.06±0.01	(20)
Glucose(mg/dL)	103±9	(10)	117±14	(20)	128±15	(10)	128±14	(20)
BUN(mg/dL)	17±3	(10)	17±4	(20)	14±2	(10)	13±2	(20)
Creatinine(mg/dL)	0.32±0.04	(10)	0.32±0.05	(20)	0.28±0.04	(10)	0.27±0.04	(20)
Na(mmol/L)	140±2	(10)	142±1	(20)	142±1	(10)	144±1	(20)
K(mmol/L)	4.6±0.3	(10)	4.6±0.3	(20)	4.8±0.4	(10)	4.6±0.3	(20)
Cl(mmol/L)	109±1	(10)	109±2	(20)	108±1	(10)	108±2	(20)
Ca(mg/dL)	10.1±0.4	(10)	10.5±0.4	(20)	10.3±0.3	(10)	10.4±0.4	(20)
P(mg/dL)	7.0±0.8	(10)	7.5±0.5	(20)	8.1±0.7	(10)	8.0±0.8	(20)
TP(g/dL)	5.6±0.2	(10)	6.2±0.4	(20)	5.6±0.2	(10)	5.9±0.3	(20)
Albumin(g/dL)	−		2.9±0.2	(10)	−		2.6±0.1	(10)
A/G	0.99±0.07	(10)	0.99±0.08	(10)	0.87±0.06	(10)	0.87±0.05	(10)
Age : 19 weeks								
GOT(IU/L)	69±22	(20)	59±9	(30)	63±6	(20)	65±9	(30)
GPT(IU/L)	32±15	(20)	29±7	(30)	29±5	(20)	32±7	(30)
LDH(IU/L)	50±18	(20)	46±12	(30)	49±12	(20)	51±14	(30)
CPK(IU/L)	60±12	(20)	59±8	(10)	78±12	(20)	68±10	(10)
AlP(IU/L)	165±48	(20)	161±54	(30)	301±52	(20)	300±57	(30)
T.cho(mg/dL)	80±17	(20)	88±16	(30)	73±15	(20)	76±14	(30)
TG(mg/dL)	13±6	(20)	22±17	(20)	58±21	(20)	54±33	(20)
PL(mg/dL)	−		153±27	(10)	−		112±23	(10)
T.bililubin(mg/dL)	0.08±0.02	(20)	0.07±0.02	(30)	0.09±0.02	(20)	0.07±0.02	(30)
Glucose(mg/dL)	127±14	(20)	123±17	(30)	141±13	(20)	145±19	(30)
BUN(mg/dL)	15±2	(20)	16±3	(30)	13±1	(20)	13±2	(30)
Creatinine(mg/dL)	0.35±0.04	(20)	0.36±0.04	(30)	0.30±0.04	(20)	0.30±0.03	(30)
Na(mmol/L)	142±2	(20)	141±2	(20)	143±1	(20)	143±2	(20)
K(mmol/L)	4.6±0.3	(20)	4.4±0.3	(20)	4.8±0.3	(20)	4.5±0.4	(20)
Cl(mmol/L)	111±2	(20)	110±2	(20)	108±1	(20)	108±1	(20)
Ca(mg/dL)	10.3±0.3	(20)	10.4±0.4	(20)	10.2±0.3	(20)	9.9±0.4	(20)
P(mg/dL)	5.3±1.2	(20)	5.8±1.1	(20)	6.5±0.7	(20)	6.1±0.7	(20)
TP(g/dL)	6.5±0.4	(20)	6.9±0.4	(30)	6.1±0.3	(20)	6.3±0.2	(30)
Albumin(g/dL)	3.0±0.2	(20)	3.1±0.3	(20)	2.7±0.1	(20)	2.8±0.1	(20)
A/G	−		0.86±0.08	(10)	−		0.74±0.04	(10)
Age : 19 weeks								
GOT(IU/L)	106±101	(15)	142±223	(45)	74±52	(30)	65±18	(45)
GPT(IU/L)	51±47	(15)	70±107	(45)	36±22	(30)	35±16	(45)
LDH(IU/L)	74±71	(15)	114±167	(45)	69±61	(30)	55±16	(45)
CPK(IU/L)	52±7	(15)	66±61	(30)	66±11	(30)	63±12	(30)
AlP(IU/L)	116±39	(15)	111±44	(45)	266±68	(30)	246±65	(45)
T.cho(mg/dL)	99±27	(15)	104±25	(45)	92±23	(30)	89±21	(45)
TG(mg/dL)	23±14	(15)	43±35	(45)	80±38	(30)	80±40	(45)
PL(mg/dL)	171±42	(15)	191±34	(15)	131±27	(30)	121±23	(15)
T.bililubin(mg/dL)	0.07±0.03	(15)	0.07±0.03	(45)	0.05±0.02	(30)	0.05±0.02	(45)
Glucose(mg/dL)	130±12	(15)	125±15	(45)	114±15	(30)	136±14	(45)
BUN(mg/dL)	15±2	(15)	16±2	(45)	13±2	(30)	13±2	(45)
Creatinine(mg/dL)	0.37±0.05	(15)	0.36±0.04	(45)	0.30±0.04	(30)	0.31±0.05	(45)
Na(mmol/L)	142±1	(15)	142±1	(45)	143±1	(30)	144±2	(45)
K(mmol/L)	4.6±0.3	(15)	4.4±0.3	(45)	4.7±0.3	(30)	4.5±0.3	(45)
Cl(mmol/L)	111±2	(15)	110±2	(45)	109±1	(30)	109±2	(45)
Ca(mg/dL)	10.3±0.4	(15)	10.4±0.4	(45)	10.1±0.3	(30)	10.1±0.3	(45)
P(mg/dL)	4.9±1.2	(15)	4.6±1.2	(45)	5.9±1.0	(30)	5.9±0.9	(45)
TP(g/dL)	7.1±0.5	(15)	7.1±0.6	(45)	6.6±0.2	(30)	6.5±0.2	(45)
Albumin(g/dL)	−		−		−		−	
A/G	−		0.96±0.10	(45)	−		0.70±0.06	(45)

a) : Mean±S.D.

( ) : Number of animals determined

Table 6-1. Relative Organ Weights in Crj:CD(SD)IGS Female Rats Fed CR-LPF

Sex	Female							
Group	BG data		Group 1		Group 2		Group 3	
Item (Unit)	(1996)		(2000)		(2001)		(2002)	
Age : 10 weeks								
Final Body Weight (g)	197±12[a)]	(40)	−		216±8	(10)	225±22	(20)
Brain (g%)	0.92±0.06	(40)	−		0.85±0.05	(10)	0.84±0.08	(20)
Thymus (mg%)	196±27	(40)	−		201±38	(10)	207±43	(20)
Heart (g%)	0.36±0.03	(40)	−		0.35±0.03	(10)	0.35±0.03	(20)
Lung (g%)	0.48±0.03	(40)	−		0.48±0.02	(10)	0.45±0.04	(20)
Liver (g%)	2.80±0.14	(40)	−		2.76±0.14	(10)	2.92±0.25	(20)
Spleen (g%)	0.21±0.03	(40)	−		0.22±0.03	(10)	0.20±0.03	(20)
Kidney (R+L, g%)	0.75±0.05	(40)	−		0.77±0.06	(10)	0.76±0.07	(20)
Adrenal (R+L, mg%)	32±4	(40)	−		30±4	(10)	27±4	(20)
Ovary (R+L, mg%)	39.7±5.8	(40)	−		37.81±9.49	(10)	35.88±4.35	(20)
Uterus (g%)	223±55	(40)	−		181±25	(10)	190±39	(20)
Age : 19 weeks								
Final Body Weight (g)	254±21	(40)	276±17	(30)	286±29	(30)	289±28	(30)
Brain (g%)	0.76±0.06	(40)	0.70±0.04	(30)	0.70±0.07	(30)	0.69±0.07	(30)
Thymus (mg%)	93±21	(40)	92±25	(30)	92±16	(30)	88±20	(30)
Heart (g%)	0.33±0.03	(40)	0.32±0.02	(30)	0.31±0.03	(30)	0.30±0.02	(30)
Lung (g%)	0.42±0.03	(40)	0.40±0.03	(30)	0.40±0.04	(30)	0.38±0.03	(30)
Liver (g%)	2.46±0.11	(40)	2.47±0.14	(30)	2.45±0.16	(30)	2.50±0.13	(30)
Spleen (g%)	0.18±0.03	(40)	0.18±0.02	(30)	0.18±0.03	(30)	0.17±0.02	(30)
Kidney (R+L, g%)	0.68±0.05	(40)	0.67±0.06	(30)	0.66±0.04	(30)	0.64±0.05	(30)
Adrenal (R+L, mg%)	26±3	(40)	23±4	(30)	23±3	(30)	22±4	(30)
Ovary (R+L, mg%)	29.6±5.3	(40)	25.43±4.31	(30)	25.63±3.64	(30)	26.52±4.46	(30)
Uterus (g%)	223±45	(40)	184±38	(10)	185±31	(10)	183±54	(10)
Age : 32 weeks								
Final Body Weight (g)	291±33	(40)	312±33	(45)	308±37	(27)	317±37	(45)
Brain (g%)	0.68±0.07	(40)	0.64±0.07	(45)	0.65±0.07	(27)	0.63±0.08	(45)
Thymus (mg%)	49±11	(40)	51±12	(45)	47±15	(27)	47±12	(45)
Heart (g%)	0.32±0.02	(40)	0.30±0.03	(45)	0.30±0.02	(27)	0.30±0.02	(45)
Lung (g%)	0.37±0.04	(40)	0.36±0.03	(45)	0.36±0.03	(27)	0.36±0.03	(45)
Liver (g%)	2.36±0.17	(40)	2.34±0.17	(45)	2.31±0.20	(27)	2.39±0.19	(45)
Spleen (g%)	0.15±0.02	(40)	0.15±0.02	(45)	0.15±0.02	(27)	0.15±0.02	(45)
Kidney (R+L, g%)	0.63±0.06	(40)	0.63±0.06	(45)	0.62±0.05	(27)	0.62±0.05	(45)
Adrenal (R+L, mg%)	22±4	(40)	20±3	(45)	19±3	(27)	20±3	(45)
Ovary (R+L, mg%)	23.9±5.9	(40)	20.03±4.74	(45)	20.46±4.49	(27)	18.89±4.79	(45)
Uterus (g%)	241±56	(40)	230±45	(45)	210±48	(27)	227±33	(15)

a) : Mean±S.D.
( ) : Number of animals determined

Table 6-2. Relative Organ Weights in Crj:CD(SD)IGS Male Rats Fed CR-LPF

Sex	Male							
Group	BG data		Group 1		Group 2		Group 3	
Item (Unit)	(1996)		(2000)		(2001)		(2002)	
Age : 10 weeks								
Final Body Weight (g)	316±22[a)]	(40)	−		359±20	(10)	360±31	(20)
Brain (g%)	0.62±0.05	(40)	−		0.56±0.03	(10)	0.56±0.04	(20)
Thymus (mg%)	122±27	(40)	−		137±26	(10)	159±27	(20)
Heart (g%)	0.33±0.02	(40)	−		0.31±0.02	(10)	0.33±0.02	(20)
Lung (g%)	0.37±0.02	(40)	−		0.36±0.02	(10)	0.36±0.02	(20)
Liver (g%)	2.69±0.14	(40)	−		2.80±0.27	(10)	2.82±0.18	(20)
Spleen (g%)	0.19±0.03	(40)	−		0.19±0.01	(10)	0.19±0.02	(20)
Kidney (R+L, g%)	0.74±0.05	(40)	−		0.73±0.04	(10)	0.73±0.04	(20)
Adrenal (R+L, mg%)	17±2	(40)	−		15±2	(10)	15±2	(20)
Testis (R+L, g%)	0.97±0.09	(40)	−		0.81±0.07	(10)	0.85±0.07	(20)
Prostate (g%)	0.25±0.05	(40)	−		0.24±0.05	(10)	0.23±0.04	(20)
Age : 19 weeks								
Final Body Weight (g)	470±40	(40)	514±48	(29)	525±43	(30)	531±48	(30)
Brain (g%)	0.44±0.04	(40)	0.42±0.03	(29)	0.41±0.03	(30)	0.41±0.04	(30)
Thymus (mg%)	54±13	(40)	55±13	(29)	54±13	(30)	54±10	(30)
Heart (g%)	0.28±0.02	(40)	0.27±0.02	(29)	0.27±0.02	(30)	0.27±0.02	(30)
Lung (g%)	0.30±0.03	(40)	0.30±0.03	(29)	0.29±0.02	(30)	0.29±0.02	(30)
Liver (g%)	2.44±0.14	(40)	2.45±0.18	(29)	2.45±0.13	(30)	2.51±0.19	(30)
Spleen (g%)	0.15±0.02	(40)	0.15±0.02	(29)	0.15±0.02	(30)	0.15±0.02	(30)
Kidney (R+L, g%)	0.61±0.05	(40)	0.6±0.05	(29)	0.6±0.05	(30)	0.6±0.05	(30)
Adrenal (R+L, mg%)	11±2	(40)	11±2	(29)	11±2	(30)	11±2	(30)
Testis (R+L, g%)	0.69±0.07	(40)	0.65±0.07	(29)	0.63±0.05	(30)	0.61±0.11	(30)
Prostate (g%)	0.26±0.05	(40)	0.26±0.04	(9)	0.23±0.03	(30)	0.24±0.03	(20)
Age : 32 weeks								
Final Body Weight (g)	558±56	(40)	587±77	(45)	612±75	(42)	610±75	(45)
Brain (g%)	0.39±0.04	(40)	0.37±0.04	(45)	0.36±0.04	(42)	0.37±0.05	(45)
Thymus (mg%)	27±7	(40)	26±7	(45)	27±8	(42)	27±9	(45)
Heart (g%)	0.26±0.02	(40)	0.25±0.02	(45)	0.24±0.02	(42)	0.24±0.02	(45)
Lung (g%)	0.27±0.02	(40)	0.26±0.03	(45)	0.26±0.03	(42)	0.26±0.02	(45)
Liver (g%)	2.36±0.16	(40)	2.33±0.17	(45)	2.35±0.16	(42)	2.40±0.19	(45)
Spleen (g%)	0.14±0.02	(40)	0.14±0.02	(45)	0.13±0.02	(42)	0.14±0.02	(45)
Kidney (R+L, g%)	0.54±0.04	(40)	0.53±0.04	(45)	0.54±0.04	(42)	0.55±0.05	(45)
Adrenal (R+L, mg%)	9±1	(40)	9±2	(45)	9±1	(42)	9±1	(45)
Testis (R+L, g%)	0.62±0.05	(40)	0.58±0.10	(45)	0.57±0.08	(42)	0.58±0.07	(45)
Prostate (g%)	0.23±0.04	(40)	0.22±0.04	(45)	0.22±0.05	(42)	0.21±0.04	(45)

a) : Mean±S.D.
( ) : Number of animals determined

Effects of Food Restriction on Result of Hematology Examination and Urinalysis in CD (SD) IGS Rats- A Four-Week Restricted Feeding Examination with Two-Week Recovery Period in Six-Week-Old Rats -

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ABSTRACT. Fifty-two male Crj: CD (SD) IGS rats were examined for changes in and recovery of clinical data and body weight after 4-week food restriction and a 2-week recovery period. From 6 weeks of age, 10 rats for each group (one control group and three for restriction group) were used in this study. Standard diet was given *ad libitum* to the control group and 85%, 70% and 55% of the food consumption of the control group was permitted in the restriction groups. Two recovery groups (one for the control and the other for the 55% restriction group) were set and 6 rats for each group were used to examine recovery. Diet was given *ad libitum* to both of these groups. The main finding was that body weight gains were suppressed, with suppression correlated with the rate of decrease in feeding, that with differences in restoration of weight by feed condition. Reduction of water consumption was correlated with rate of decrease in feeding. On urinalysis, reduction of excretion of each ingredient in urine with time and rapid reduction of electrolytes in urine were found, and these change exhibited recovery with re-feeding. On hematology examination, changes in erythrocyte system parameters considered due to blood concentration were found, and these parameters exhibited opposite changes from the restriction groups by re-feeding. The change after re-feeding appeared to originate the rapid recovery of the body water with increase of the water consumption. Moreover, although changes in the leukocyte system considered affects of stress were also found, no large change in total number of leukocytes or actual number of lymphocytes was found, unlike previous findings. These results of suggest that confirmation of reproducibility by accumulation of the same types of examination data is probably required to determine whether these findings are due to food restriction or other factors. — Key words: food restriction, hematology, urinalysis.

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INTRODUCTION

In general toxicological studies, decrease of body weight accompanying decrease in food consumption may be found after drug administration. Moreover, it is reported that changes in clinical parameters occur with decrease in food consumption. In a preliminary examination, changes in body weight, food consumption, hematological parameters, and findings on urinalysis were observed in the non-treated rats under fasted condition. It was found that changes in electrolytes in urine and hematological parameters occur in the initial stage of the fasting. We therefore carried out a 4-week feed restriction examination with 2-week recovery period in 6-week old rats to confirm changes in and recovery of clinical data and body weight.

MATERIALS AND METHODS

Animals and housing

Fifty-two male Crj: CD (SD) IGS rats 4 weeks of age were purchased from Charles River Japan Inc. (Atsugi Farm). The animals were quarantined and acclimated for 2 weeks. During this period, those exhibiting healthy and favorable growth were selected for the study. The animals were randomly allocated to 6 groups in accordance with body weight. The animal room was maintained at a temperature of 21-25°C and humidity of 46-65% with 12-hour light/dark cycle (light 7:00-19:00). The animals were individually housed in plastic cages (W270 × D442 × H185 mm).

Experimental design

Restriction of feeding was started of 6 weeks of age and confirmed for 4 weeks. Two-week recovery groups were prepared for the control group and the 55% restriction feed group. The control groups (the main study group and recovery group) were fed a standard diet *ad libitum*. Animals of the restricted feeding groups were given approximately 85%, 70% and 55% of the daily diet calculated from food consumption by the control group. The recovery groups were fed *ad libitum*.

Study design

The first day of restriction of feeding was considered restriction day 0. The final day of restriction (initial day of the recovery period) was considered recovery day 0.

Clinical signs:

Clinical signs were monitored once a day from restriction day 0 to restriction day 3,and twice a week thereafter.

Body weight:

Each animal was weighed once a day from restriction day 0 to restriction day 3, and twice a week thereafter.

Food consumption:

For the control group, food consumption was measured once a day from restriction day 0 to restriction day 3. Daily food consumption after restriction day 3 was calculated from food consumption over 3- or 4-day periods.

Water consumption:

Water consumption was measured once a day from restriction day 0 to restriction day 3, and once a week thereafter.

Urinalysis:

At the 1st, 2nd, 3rd and 4th weeks of restricted feeding (for the 85% and 70% restriction groups, the control and 55% restriction groups of the recovery group) and the 1st week of recovery (for the control and 55% restriction recovery groups), 4hr- and 18hr-urine samples were collected for six animals per group using metabolic cages. The urine extracted within 4 hours (fasting) of the beginning of urine sampling was considered the fresh urine sample. Protein, pH, ketone bodies, glucose, occult blood, bilirubin, urobilinogen, nitrite and specific gravity (Multistix test paper method) were determined using an automatic urine analyzer (Clinitek 100, Beyer and Sankyo Ltd.). Urine subsequently extracted within 18 hours (feeding) was considered the accumulated urine sample. Volume of urine was measured using a calibrated cylinder. Urine was grossly examined for color. Sodium and potassium were determined using a Na/K ISE analyzer (Model 614, Beyer-Medical Ltd.).

Hematology:

At restriction day 29 and recovery day 14, blood was taken from the iliac vein under ethyl ether anesthesia after an overnight fast. The following hematological tests were performed after treatment with EDTA-2K anticoagulant using a Technicon H*1E (Bayer-Medical Co., Ltd.): red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell count (WBC). White blood cell classification was also measured as absolute counts and percentages of total WBC; neutrophils (NEUT), lymphocytes (LYMP), monocytes (MONO), eosinophils (EOS), basophils (BASO) and large unstained cells (LUC). Reticulocytes (RET) were microscopically estimated on smears stained by the Brecher method.

At restriction day 29, blood was taken from the abdominal aorta under ethyl ether anesthesia after an overnight fast. The following hematological tests were performed on the plasma after centrifugal separation with treatment with 3.8% sodium citrate anticoagulant using a Coagulometer KC 4A (Heinrich Amelung GmbH): prothrombin (PT) and activated partial thromboplastin time (APTT).

Electrolytes in blood:

At restriction day 29 and recovery day 14, blood was taken from the abdominal aorta under ethyl ether anesthesia after an overnight fast. Serum concentrations of sodium and potassium were measured with Na/K ISE analyzer (Model 614, Beyer-Medical Ltd.)

Necropsy and organ weights:

All the animals were sacrificed under ether anesthesia at the end of the study. The following organs were removed and weighed : pituitary, adrenal glands (right and left), kidneys (right and left), thymus, liver, spleen, testes (right and left), prostate, seminal vesicle and epididymides (right and left). Paired organ was measured separately in this study.

Statistical analysis:

Body weights, food consumption, water consumption, organ weights, urine volume, electrolytes in urine and hematological data include electrolytes were statistically analyzed by one-way ANOVA or Student's t-test.

The cumulative chi-square test was used for qualitative parameters in urinalysis.

RESULTS

Clinical signs:

There were no deaths and no abnormal clinical signs during the observation period.

Body weights:

Body weight change during the examination period is shown in Fig. 1 and Tables 1-1a, -1b, -2a and -2b. Body weight gains were significantly suppressed in the 55% restriction group (from restriction day 1), the 70% restriction group (from restriction day 2) and the 85% restriction group (from restriction day 7) compared with the control group. Body weights on restriction day 28 (the end of the restriction period) were 88% (85% restriction group), 80% (70% restriction group) and 68% (55% restriction group) of the control group.

Over the 2-week recovery period, body weight in the 55% restriction feed group recovered to 85% of the control group value.

Food consumption:

Change food consumption during the examination period is shown in Table 2a and 2b. During the recovery periods the 55% restriction group exhibited a transient tendency toward overeating until recovery day 7, but thereafter food consumption was not more than that in the control group.

Water consumption:

Water consumption during the examination period is shown in Table 3a and 3b. Throughout the restriction period, water consumption was reduced in proportion to the rate of restriction of feeding, and the 55% and 70% restriction groups exhibited significant reductions compared with the control group, although the 55% restriction group exhibited rapid recovery to 83% of the control group value recovery day 3 and to 78% of the control group value at recovery day 10.

Urinalysis:

Results for fresh urine samples are shown in Tables 4a-1, -2, -3, -4, 4b-1 and -2, and the results for accumulated urine samples in Table 5a and 5b. On the qualitative urine examination, the fresh urine samples exhibited significant decreased specific gravity (from the 1st week of the restriction feed period), WBC (from the 2nd week of the restriction period), protein (at the 2nd and 4th weeks of the restriction period) and ketone bodies (at

the 4th week of the restriction period), with rates of decrease correlated with the rate of restriction of feeding. Although ketone bodies showed the significant decreased at the 1st week of the recovery period, normalization was observed at the 2nd week. For the other changes, normalization was observed in the 1st week of the recovery period.

For the accumulated urine samples, no significant change was observed in urine volume during the examination period. Urine color tone was normal but slightly light from the 2nd week of the restriction period. With regard to electrolytes in urine, reduction of potassium level was correlated with rate of restriction of feeding and the change in potassium level was significantly different from the 1st week of the restricted feeding period. Sodium level was significantly reduced from the 1st week of the restriction period in the 55% restriction group. Potassium and sodium levels in urine at the 4th week of the restriction period were respectively 108% and 90% in the 85% restriction group, 94% and 82% in the 70% restriction group and 73% and 65% in the 55% restriction group compared with the control group. For these changes, normalization was observed in the 1st week of the recovery period.

Hematology:

The results of hematology examination are shown in Table 6a and 6b. Increases in number of RBC, HGB and HCT were correlated with the rate of restriction of feeding, and were significant in the 70% and 55% restriction groups. Significant increase in MCHC was also found in the 55% restriction group. Moreover, decrease in MCV was correlated with rate of restriction of feeding. Reduction of RET was also correlated with the rate of restriction of feeding and was significant in the 70% and 55% restriction groups.

At the end of the recovery period, significant decreases were observed in RBC and HGB, with reduction of HCT and MCHC and significant increases in MCV, MCH and RET.

No remarkable change was observed in WBC, although significant decreases in NEUT, MONO and LUC and significant increase in LYMP were found in the 55% restriction group. Monocytes were significantly decreased in the 85% restriction group. Moreover, in absolute counts, of significant decreases in NEUT and MONO were found in the 55% restriction group.

These changes in WBC differential recovered by the end of the recovery period.

Electrolytes in blood:

Results for electrolytes in blood are shown in Table 7. Significant decrease in sodium in serum and significant increase of potassium in serum were found in the 55% restriction group. These changes had normalized by the end of the recovery period.

Necropsy and organ weights:

A white area in liver was observed in one animal in the control group. A red area in the prostate was observed in one animal in the 70% restriction group. Red spots in the seminal vesicle (in one animal), small liver (in one animal), small prostate (in two animals) and small seminal vesicle (in three animals) were observed in the 55% restriction group. In the recovery group, dilatation of the pelvic cavity was observed in one animal each in the control and 55% restriction groups.

Absolute weights of organs are shown in Table 8a. The absolute weights of the pituitary, adrenal (right and left), kidney (right and left), liver, spleen, testis (right and left), prostate, seminal vesicle and epididymis (right and left) of the restriction groups were significantly lower than those of the control group.

Relative organ weights are shown in Table 8b. The relative weights of liver in the restriction groups were significantly lower than that of the control group. The relative weights of the adrenal (right and left), kidney (right and left), thymus, testis (right and left) and epididymis (right and left) of the restriction group were significantly higher than those of the control group.

At the end of the recovery period, the absolute weights of the kidney (right and left), seminal vesicle and liver were significantly lower and the relative weight of the testis (right and left) significantly higher than those of the control. Changes in the other organs mentioned above had recovered.

DISCUSSION

The main finding of our study was that body weight gain was suppressed by restricted feeding, with the amount of suppression correlated with the rate of restriction of feeding.

Reduction of water consumption was correlated with rate of restriction of feeding. This change was thought to be related to restriction of feeding and the suppression of body weight gain. In the recovery period, water consumption exhibited changes similar to food consumption but different from that in the control group.

On qualitative urine examination, reduction of specific gravity was the main finding. Reduction of protein and WBC was found later, and reduction of ketone bodies was the last finding in the last week. It appeared that reduction of specific gravity and protein was due to reduction of food consumption. Decrease in ketone bodies, which are lipid metabolites, was thought to be related to decrease in internal lipid levels caused by long-term restriction of feeding. No change was found in urine volume and urine color tone was within the normal range under restriction of feeding, but a tendency light urine color tone was observed in the restriction groups. This change was considered related to decrease of excretion of ingredients of urine. Moreover, low urine volume compared with the control group was found in the recovery period, and appeared to result from increase in the amount of water required for maintenance of internal osmotic pressure with recovery of body weight. Electrolytes in urine, especially potassium exhibited remarkable reduction in level correlated with the rate of decrease in from the 1st week of the restricted feeding period. On the other hand, sodium in urine was 10% reduced in the 70% restriction group and 22% reduced in the 55% restriction group compared with the control group. It was thought that these changes resulted from regulation of excretion in urine in order to maintain internal homeostasis. In electrolytes in serum, significant difference from the control group was observed in the restriction groups, but was considered unimportant since it this was caused

by very slight change in serum electrolyte level in restriction feed group.

On hematology examination, increases in RBC, HGB, HCT, and MCHC, considered to be effects of blood concentration, were found[1, 2]. The changes in these parameters at the end of the recovery period were thought to be largely due to rapid recovery of the amount of internal water by re-feeding. This reaction was thought to be due to decrease in erythrocytes in blood and induced production of erythrocytes, with remarkable in increase of RET. Furthermore, we speculated that the increases in MCV and MCHC were reactive changes that compensated for the number of RET. Decreases in the percentages of NEUT, MONO, and LUC and increase in LYMP were observed. Absolute counts were decreased for NEUT and MONO but not for lymphocytes. Generally, it is known that secretion of adrenocortical hormones is induced by stress, resulting in thymic atrophy and loss of lymphocytes[3, 4]. We therefore speculated that the changes in these parameters were related to stress. However, the changes with stress observed in this examination differed from the changes that occurred with short-term examination or the restriction feeding examination reported in the past[4, 5].

The results mentioned above suggest confirmation of reproducibility by accumulation of the same types of examination data is probably required to determine whether these findings are caused by restriction of feeding or other factors.

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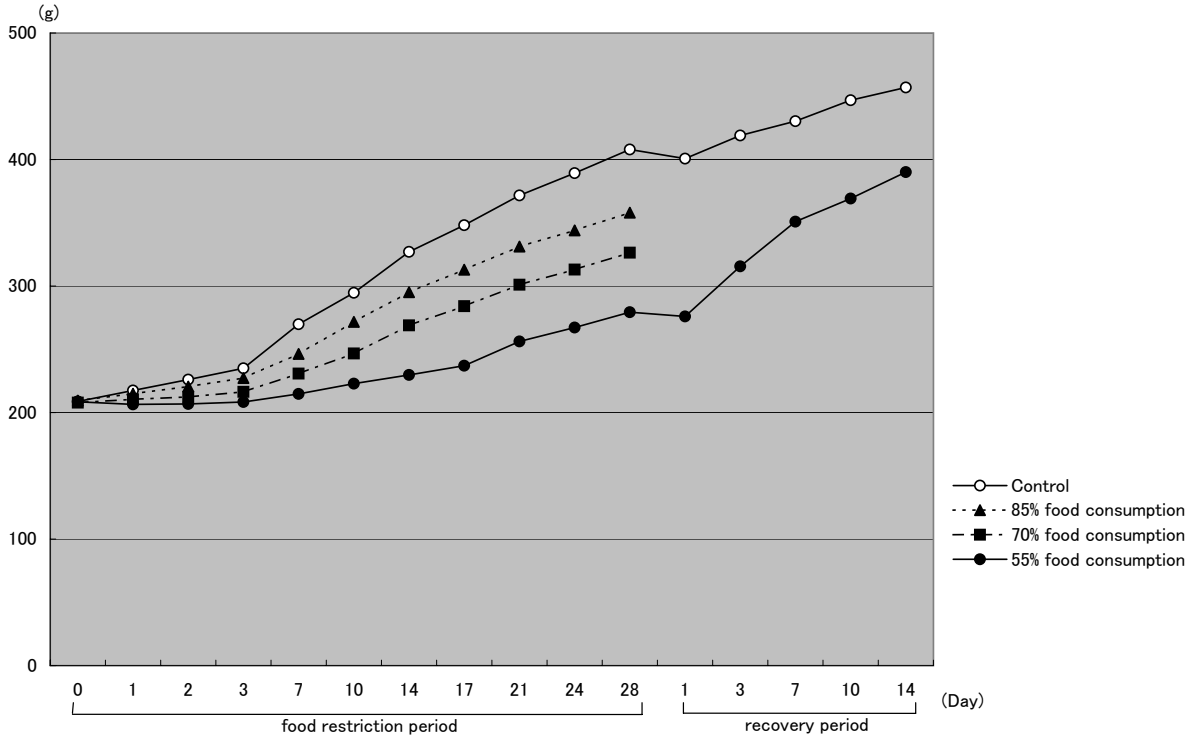

Fig 1. Body weight data at the end of 4-week food restriction period and 2-week recovery period

Table 1-1a. Body weight data at the end of 4-week food restriction period

(g)

Group	n	food restriction day 0	1	2	3
Control	16	208.7 ± 10.2	217.5 ± 10.7	226.1 ± 10.8	235.0 ± 11.5
85% food consumption	10	209.5 ± 10.8	215.0 ± 9.9	220.6 ± 8.6	227.3 ± 8.1
70% food consumption	10	207.9 ± 8.2	210.5 ± 7.7	212.4 ± 7.1**	216.4 ± 7.6**
55% food consumption	16	208.6 ± 9.8	206.5 ± 9.4**	206.8 ± 9.1**	208.4 ± 8.4**

Group	n	food restriction day 7	10	14	17	21	24	28
Control	16	269.8 ± 13.6	294.6 ± 14.4	327.1 ± 16.2	348.1 ± 18.7	371.6 ± 19.9	389.3 ± 19.5	407.9 ± 23.0
85% food consumption	10	246.4 ± 6.5**	271.7 ± 8.4**	295.2 ± 11.4**	313.0 ± 12.9**	331.3 ± 13.6**	344.0 ± 15.3**	358.0 ± 15.5**
70% food consumption	10	230.8 ± 5.9**	246.8 ± 6.8**	268.9 ± 7.8**	284.1 ± 8.8**	301.1 ± 9.2**	313.0 ± 9.0**	326.4 ± 10.8**
55% food consumption	16	214.8 ± 8.6**	222.9 ± 8.6**	229.8 ± 9.6**	237.0 ± 10.9**	256.2 ± 10.4**	267.2 ± 9.5**	279.3 ± 9.9**

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 1-1b. Body weight data at the end of 2-week recovery period

(g)

Group	n	recovery day 1	3	7	10	14
Control	6	400.7 ± 22.6	419.0 ± 23.4	430.3 ± 26.2	446.9 ± 27.5	457.0 ± 24.8
55% food consumption	6	276.0 ± 13.4**	315.6 ± 15.2**	350.9 ± 21.5**	369.2 ± 22.7**	390.1 ± 24.1**

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 1-2a. Body weight gain data at the end of 4-week food restriction period

(g)

Group	n	food restriction day 1	2	3
Control	16	8.7 ± 2.1	17.3 ± 2.0	26.3 ± 2.7
85% food consumption	10	5.4 ± 3.5	11.1 ± 4.1	17.8 ± 4.7
70% food consumption	10	2.6 ± 1.9	4.5 ± 2.0	8.5 ± 2.9
55% food consumption	16	-2.0 ± 2.2	-1.7 ± 2.8	-0.2 ± 3.2

Group	n	food restriction day 7	10	14	17	21	24	28
Control	16	61.1 ± 5.1	85.9 ± 5.8	118.3 ± 8.8	139.4 ± 12.4	162.9 ± 14.3	180.5 ± 14.7	199.2 ± 17.8
85% food consumption	10	36.9 ± 6.4	62.2 ± 8.3	85.7 ± 10.0	103.5 ± 12.1	121.7 ± 13.5	134.5 ± 14.5	148.5 ± 14.9
70% food consumption	10	22.9 ± 6.5	38.9 ± 5.9	61.0 ± 8.7	76.2 ± 8.7	93.2 ± 10.2	105.1 ± 8.6	118.5 ± 11.1
55% food consumption	16	6.2 ± 6.8	14.3 ± 7.0	21.2 ± 8.7	28.5 ± 13.4	47.7 ± 12.6	58.6 ± 12.4	70.8 ± 12.4

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)

Table 1-2b. Body weight gain data at the end of 2-week recovery period

(g)

Group	n	recovery day 3	7	10	14
Control	6	18.3 ± 5.3	29.6 ± 6.7	46.1 ± 8.4	56.3 ± 10.3
55% food consumption	6	39.6 ± 3.0	74.9 ± 9.3	93.2 ± 10.9	114.1 ± 11.5

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)

Table 2a. Food consumption data at the end of 4-week food restriction period

(g/day)

		food restriction day		
Group	n	1	2	3
Control	16	21.0 ± 2.1	23.3 ± 1.6	23.7 ± 2.3

		food restriction day						
Group	n	3 ～ 7	7 ～ 10	10 ～ 14	14 ～ 17	17 ～ 21	21 ～ 24	24 ～ 28
Control	16	25.0 ± 1.6	26.1 ± 1.7	27.2 ± 2.0	27.3 ± 2.0	27.7 ± 1.7	27.5 ± 1.7	27.4 ± 1.8

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)

Table 2b. Food consumption data at the end of 2-week recovery period

(g/day)

		recovery day			
Group	n	0,1 ～ 3	3 ～ 7	7 ～ 10	10 ～ 14
Control	6	27.7 ± 1.9	27.1 ± 2.5	27.7 ± 2.3	27.0 ± 1.7
55% food consumption	6	28.6 ± 2.1	27.8 ± 3.0	26.3 ± 3.2	26.6 ± 2.0

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)

Table 3a. Water consumption data at the end of 4-week food restriction period

(g)

		food restriction day					
Group	n	1	2	3	10	17	24
Control	16	34.7 ± 3.3	32.2 ± 3.8	34.6 ± 3.8	38.7 ± 6.3	42.7 ± 6.3	41.4 ± 6.7
85% food consumption	10	31.8 ± 4.6	30.2 ± 4.1	32.0 ± 4.4	34.5 ± 4.5	36.8 ± 5.2*	36.1 ± 6.4
70% food consumption	10	29.3 ± 4.2**	27.5 ± 4.0**	29.3 ± 4.7**	30.2 ± 4.7**	33.1 ± 6.7**	32.3 ± 8.2**
55% food consumption	16	25.0 ± 2.9**	25.0 ± 3.3**	25.3 ± 3.0**	24.1 ± 3.3**	25.2 ± 4.2**	27.6 ± 4.0**

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 3b. Water consumption data at the end of 2-week recovery period

(g)

		recovery day	
Group	n	3 日	10 日
Control	6	41.5 ± 8.9	43.1 ± 5.2
55% food consumption	6	34.6 ± 2.3	33.7 ± 12.6

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ～ 12:00)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 4a-1. Urinalysis data measured at 1-week of food restriction

Group	n	GLU						PLO					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	3	3	0	0	0
85% food consumption	6	6	0	0	0	0	0	4	2	0	0	0	0
70% food consumption	6	6	0	0	0	0	0	4	2	0	0	0	0
55% food consumption	6	6	0	0	0	0	0	4	1	1	0	0	0

Group	n	KET						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	2	4	0	0	0	0	6	0	0	0	6	0	0	0	0
85% food consumption	6	5	1	0	0	0	0	6	0	0	0	6	0	0	0	0
70% food consumption	6	3	3	0	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	6	3	3	0	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG*						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	0	0	2	0	4	0	0	0	0	0	2	4	0
85% food consumption	6	0	1	3	1	1	0	0	0	0	0	1	4	1	0
70% food consumption	6	0	3	0	3	0	0	0	0	0	0	1	5	0	0
55% food consumption	6	2	3	1	0	0	0	0	0	0	0	1	4	1	0

Group	n	OB					NIT		WBC				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	6	0	0	0	0	6	0	0	5	1	0	0
85% food consumption	6	6	0	0	0	0	6	0	0	6	0	0	0
70% food consumption	6	5	0	0	1	0	6	0	3	3	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	3	3	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 4a-2. Urinalysis data measured at 2-week of food restriction

Group	n	GLU						PLO*					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	0	4	2	0	0
85% food consumption	6	6	0	0	0	0	0	0	3	3	0	0	0
70% food consumption	6	6	0	0	0	0	0	2	4	0	0	0	0
55% food consumption	6	6	0	0	0	0	0	2	2	2	0	0	0

Group	n	KET						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	0	6	0	0	0	0	6	0	0	0	5	1	0	0	0
85% food consumption	6	2	4	0	0	0	0	6	0	0	0	6	0	0	0	0
70% food consumption	6	3	3	0	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	6	3	3	0	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG*						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	0	0	1	3	2	0	0	0	0	0	1	5	0
85% food consumption	6	1	2	2	1	0	0	0	0	0	0	1	3	2	0
70% food consumption	6	2	0	4	0	0	0	0	0	0	0	0	5	1	0
55% food consumption	6	5	1	0	0	0	0	0	0	0	0	0	1	3	2

Group	n	OB					NIT		WBC*				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	5	0	1	0	0	6	0	0	5	0	1	0
85% food consumption	6	5	0	1	0	0	6	0	4	2	0	0	0
70% food consumption	6	6	0	0	0	0	6	0	6	0	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	5	1	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 4a-3. Urinalysis data measured at 3-week of food restriction

Group	n	GLU						PLO					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	2	3	1	0	0
85% food consumption	6	6	0	0	0	0	0	2	2	2	0	0	0
70% food consumption	6	6	0	0	0	0	0	0	5	1	0	0	0
55% food consumption	6	6	0	0	0	0	0	0	4	2	0	0	0

Group	n	KET						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	0	4	2	0	0	0	6	0	0	0	5	1	0	0	0
85% food consumption	6	0	6	0	0	0	0	6	0	0	0	6	0	0	0	0
70% food consumption	6	1	4	1	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	6	2	4	0	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG*						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	1	0	1	2	2	0	0	0	0	0	2	4	0
85% food consumption	6	0	3	1	1	1	0	0	0	0	1	0	3	2	0
70% food consumption	6	1	1	1	3	0	0	0	0	0	0	0	4	2	0
55% food consumption	6	2	3	0	1	0	0	0	0	0	0	0	1	5	0

Group	n	OB					NIT		WBC*				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	5	1	0	0	0	6	0	2	4	0	0	0
85% food consumption	6	6	0	0	0	0	6	0	5	1	0	0	0
70% food consumption	6	6	0	0	0	0	6	0	6	0	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	6	0	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 4a-4. Urinalysis data measured at 4-week of food restriction

Group	n	GLU						PLO*					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	1	4	1	0	0
85% food consumption	6	6	0	0	0	0	0	2	3	1	0	0	0
70% food consumption	6	6	0	0	0	0	0	3	2	1	0	0	0
55% food consumption	6	6	0	0	0	0	0	2	4	0	0	0	0

Group	n	KET*						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	0	3	3	0	0	0	6	0	0	0	6	0	0	0	0
85% food consumption	6	0	6	0	0	0	0	6	0	0	0	6	0	0	0	0
70% food consumption	6	2	4	0	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	6	4	2	0	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG*						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	1	1	0	2	2	0	0	0	0	0	2	4	0
85% food consumption	6	1	1	3	1	0	0	0	0	0	1	0	2	3	0
70% food consumption	6	2	1	2	1	0	0	0	0	0	0	0	3	3	0
55% food consumption	6	3	3	0	0	0	0	0	0	0	0	0	1	3	2

Group	n	OB					NIT		WBC*				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	5	1	0	0	0	6	0	2	4	0	0	0
85% food consumption	6	6	0	0	0	0	6	0	5	1	0	0	0
70% food consumption	6	6	0	0	0	0	6	0	6	0	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	5	1	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 4b-1. Urinalysis data measured at 1-week of recovery

Group	n	GLU						PLO					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	2	2	2	0	0
55% food consumption	6	6	0	0	0	0	0	0	3	3	0	0	0

Group	n	KET*						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	0	3	3	0	0	0	6	0	0	0	5	1	0	0	0
55% food consumption	6	0	6	0	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	2	0	2	1	1	0	0	0	0	0	0	6	0
55% food consumption	6	0	0	0	1	4	1	0	0	0	0	0	0	5	1

Group	n	OB					NIT		WBC				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	6	0	0	0	0	6	0	3	3	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	5	1	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 4b-2. Urinalysis data measured at 2-week of recovery

Group	n	GLU						PLO					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	4	1	1	0	0
55% food consumption	6	6	0	0	0	0	0	0	2	4	0	0	0

Group	n	KET						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	0	4	2	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	6	1	3	2	0	0	0	6	0	0	0	6	0	0	0	0

Group	n	SG						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	0	1	4	1	0	0	0	0	0	0	1	4	1
55% food consumption	6	0	0	1	3	2	0	0	0	0	0	0	0	4	2

Group	n	OB					NIT		WBC				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	6	0	0	0	0	6	0	2	4	0	0	0
55% food consumption	6	6	0	0	0	0	6	0	3	3	0	0	0

Parameter : n, Significantly different from control, * : $p < 0.05$

Table 5a. Urinalysis data at the end of 4-week food restriction period

Data examined			Color (n)		Urine vol	U-Na		U-K	
period	Group	n	Y	S	(mL/18h)	(mmol/L)	(mmol/18h)	(mmol/L)	(mmol/18h)
	Control	6	6	0	8.8 ± 1.7	171 ± 38	1.4610 ± 0.1893	316 ± 56	2.7109 ± 0.2806
food restriction	85% food consumption	6	6	0	10.6 ± 2.8	143 ± 25	1.4557 ± 0.2315	218 ± 55	2.1790 ± 0.1304**
1-week	70% food consumption	6	6	0	9.7 ± 3.2	147 ± 37	1.3302 ± 0.1149	192 ± 54	1.7205 ± 0.1255**
	55% food consumption	6	4	2	10.7 ± 6.0	131 ± 58	1.1435 ± 0.2557*	158 ± 73	1.3467 ± 0.2590**
	Control	6	4	2	11.1 ± 3.0	149 ± 45	1.5579 ± 0.2831	282 ± 79	2.9541 ± 0.2755
food restriction	85% food consumption	6	3	3	13.1 ± 2.4	136 ± 20	1.7492 ± 0.1006	208 ± 42	2.6447 ± 0.0924
2-week	70% food consumption	6	4	2	12.9 ± 5.1	131 ± 34	1.5644 ± 0.2971	190 ± 51	2.2358 ± 0.2394**
	55% food consumption	6	4	2	11.4 ± 6.6	121 ± 61	1.0704 ± 0.0691**	171 ± 74	1.5764 ± 0.2259**
	Control	6	2	4	13.6 ± 5.0	146 ± 39	1.8251 ± 0.2856	249 ± 83	3.0620 ± 0.3244
food restriction	85% food consumption	6	4	2	15.2 ± 4.9	120 ± 36	1.6848 ± 0.2164	175 ± 68	2.3836 ± 0.3154*
3-week	70% food consumption	6	3	3	15.4 ± 8.5	116 ± 48	1.4822 ± 0.2652	184 ± 95	2.2137 ± 0.1335**
	55% food consumption	6	4	2	11.7 ± 5.4	109 ± 39	1.1021 ± 0.1592**	183 ± 66	1.8522 ± 0.2296**
	Control	6	4	2	14.2 ± 4.9	129 ± 41	1.6783 ± 0.2873	228 ± 82	2.9329 ± 0.2476
food restriction	85% food consumption	6	4	2	16.2 ± 7.0	126 ± 43	1.8115 ± 0.1829	191 ± 82	2.6412 ± 0.2052
4-week	70% food consumption	6	4	2	15.3 ± 8.3	124 ± 53	1.5712 ± 0.1322	188 ± 77	2.4174 ± 0.2632*
	55% food consumption	6	3	3	14.7 ± 6.8	100 ± 44	1.2176 ± 0.2443**	161 ± 85	1.8955 ± 0.2028**

Color, Y : Yellow, S : Straw
Parameter : mean ± S.D., Data : examined by overnight collection urine sample (approximately 18 hr)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 5b. Urinalysis data at the end of 2-week recovery period

Data examined			Color (n)		Urine vol	U-Na		U-K	
period	Group	n	Y	S	(mL/18h)	(mmol/L)	(mmol/18h)	(mmol/L)	(mmol/18h)
recovery	Control	6	5	1	15.0 ± 7.5	125 ± 34	1.6774 ± 0.2327	238 ± 76	3.1173 ± 0.4590
1-week	55% food consumption	6	6	0	10.3 ± 3.0	163 ± 39	1.5981 ± 0.2899	305 ± 72	2.9824 ± 0.4443
recovery	Control	6	1	5	15.8 ± 4.9	101 ± 45	1.4349 ± 0.4340	212 ± 86	3.0131 ± 0.5086
2-week	55% food consumption	6	4	2	11.3 ± 2.3	152 ± 39	1.6622 ± 0.3508	272 ± 71	2.9475 ± 0.4084

Color, Y : Yellow, S : Straw
Parameter : mean ± S.D., Data : examined by overnight collection urine sample (approximately 18 hr)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 6a. Hematology data at the end of 4-week food restriction period and 2-week recovery period

Data examined period	Group	n	CBC						
			WBC ($\times 10^3/\mu$ L)	RBC ($\times 10^6/\mu$ L)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
food restriction period	Control	10	11.09 ± 2.66	8.03 ± 0.39	15.5 ± 0.5	46.2 ± 1.5	57.6 ± 2.1	19.3 ± 0.7	33.6 ± 0.5
	85% food consumption	10	10.65 ± 1.93	8.20 ± 0.27	15.8 ± 0.5	46.6 ± 1.1	57.0 ± 2.1	19.3 ± 0.9	33.9 ± 0.5
	70% food consumption	10	10.83 ± 3.16	8.43 ± 0.32*	16.1 ± 0.6*	47.7 ± 1.6	56.6 ± 1.8	19.1 ± 0.6	33.8 ± 0.5
	55% food consumption	10	10.61 ± 3.15	8.64 ± 0.28**	16.7 ± 0.5**	48.5 ± 1.4**	56.2 ± 1.2	19.3 ± 0.7	34.4 ± 0.7**
recovery period	Control	6	12.16 ± 2.98	8.49 ± 0.25	15.8 ± 0.3	46.1 ± 1.4	54.4 ± 2.2	18.6 ± 0.5	34.2 ± 0.6
	55% food consumption	6	12.00 ± 1.96	7.77 ± 0.16**	15.1 ± 0.4**	44.3 ± 1.6	57.1 ± 1.2*	19.4 ± 0.4*	33.9 ± 0.7

Data examined period	Group	n	CBC		Coagulation test	
			PLT ($\times 10^3/\mu$ L)	Reticulocyte (%)	PT (sec)	APTT (sec)
food restriction period	Control	10	1031 ± 107	26.6 ± 3.2	12.0 ± 0.5	17.3 ± 1.7
	85% food consumption	10	1121 ± 99	25.5 ± 5.9	12.3 ± 0.8	17.0 ± 2.5
	70% food consumption	10	1059 ± 91	20.2 ± 3.3*	11.7 ± 0.6	16.1 ± 1.2
	55% food consumption	10	1095 ± 56	19.7 ± 5.2**	12.1 ± 0.5	17.3 ± 1.1
recovery period	Control	6	1031 ± 128	24.5 ± 4.1	12.5 ± 0.8	18.9 ± 1.8
	55% food consumption	6	1182 ± 129	43.4 ± 6.5**	12.7 ± 0.9	17.9 ± 0.9

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$
n=9 (because one sample coagulated) : 55% food consumption group PLT data at the end of food restriction period and control group PT,APTT data at the end of recovery period

Table 6b. Differential cell data at the end of 4-week food restriction period and 2-week recovery period

Data examined period	Group	n	DIFF (%)					
			NEUT	LYMP	MONO	EOS	BASO	LUC
food restriction period	Control	10	12.0 ± 3.6	83.1 ± 3.9	1.9 ± 0.6	0.9 ± 0.4	0.3 ± 0.1	1.8 ± 0.4
	85% food consumption	10	10.4 ± 2.5	85.6 ± 2.8	1.3 ± 0.4*	1.0 ± 0.4	0.3 ± 0.1	1.5 ± 0.2
	70% food consumption	10	10.2 ± 3.0	85.3 ± 3.2	1.5 ± 0.5	1.3 ± 0.7	0.3 ± 0.1	1.5 ± 0.3
	55% food consumption	10	7.8 ± 1.8**	88.3 ± 2.4**	1.1 ± 0.5**	1.2 ± 0.4	0.3 ± 0.1	1.3 ± 0.3**
recovery period	Control	6	12.4 ± 2.9	82.2 ± 3.4	2.1 ± 0.5	1.1 ± 0.3	0.3 ± 0.1	1.9 ± 0.5
	55% food consumption	6	11.8 ± 3.2	83.5 ± 2.9	1.7 ± 0.4	1.0 ± 0.3	0.3 ± 0.0	1.7 ± 0.5

Data examined period	Group	n	DIFF ($\times 10^3/\mu$ L)					
			NEUT	LYMP	MONO	EOS	BASO	LUC
food restriction period	Control	10	1.30 ± 0.39	9.24 ± 2.44	0.21 ± 0.07	0.10 ± 0.06	0.04 ± 0.01	0.19 ± 0.05
	85% food consumption	10	1.11 ± 0.40	9.11 ± 1.67	0.13 ± 0.03	0.11 ± 0.05	0.03 ± 0.01	0.16 ± 0.03
	70% food consumption	10	1.09 ± 0.35	9.25 ± 2.82	0.17 ± 0.10	0.13 ± 0.07	0.03 ± 0.02	0.16 ± 0.07
	55% food consumption	10	0.83 ± 0.31*	9.37 ± 2.79	0.12 ± 0.06*	0.12 ± 0.05	0.03 ± 0.01	0.14 ± 0.05
recovery period	Control	6	1.47 ± 0.36	10.03 ± 2.66	0.26 ± 0.10	0.14 ± 0.05	0.04 ± 0.02	0.22 ± 0.08
	55% food consumption	6	1.41 ± 0.45	10.02 ± 1.66	0.21 ± 0.08	0.12 ± 0.03	0.04 ± 0.01	0.21 ± 0.07

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 7. Serum electrolyte data at the end of 4-week food restriction period and 2-week recovery period

Data examined period	Group	n	Na (mmol/L)	K (mmol/L)
food restriction period	Control	10	145 ± 1	4.62 ± 0.17
	85% food consumption	10	145 ± 2	4.89 ± 0.26
	70% food consumption	10	145 ± 1	4.89 ± 0.17
	55% food consumption	10	144 ± 1*	4.94 ± 0.38*
recovery period	Control	6	144 ± 1	4.59 ± 0.19
	55% food consumption	6	144 ± 1	4.82 ± 0.27

Parameter : mean ± S.D.
Significantly different from control, * : $p<0.05$, ** : $p<0.01$

Table 8a. Organ Weight (Absolute weight) data at the end of 4-week food restriction period and 2-week recovery period

(mg)

Data examined period	Group	n	Pituitary	Adrenal-R	Adrenal-L	Kidney-R	Kidney-L	Thymus
food restriction period	Control	10	11.0 ± 2.1	27.3 ± 4.3	29.5 ± 3.4	1343 ± 116	1344 ± 107	594 ± 78
	85% food consumption	10	9.8 ± 2.3	25.4 ± 4.5	27.1 ± 5.2	1199 ± 64**	1212 ± 74**	512 ± 90
	70% food consumption	10	8.6 ± 1.8*	26.5 ± 4.5	27.6 ± 2.5	1109 ± 49**	1094 ± 52**	519 ± 113
	55% food consumption	10	7.6 ± 1.8**	21.2 ± 2.9**	22.2 ± 2.3**	1003 ± 54**	996 ± 53**	494 ± 78
recovery period	Control	6	12.8 ± 1.3	28.6 ± 5.3	30.9 ± 6.8	1404 ± 141	1395 ± 92	477 ± 84
	55% food consumption	6	11.6 ± 1.6	25.3 ± 2.2	25.8 ± 1.6	1208 ± 144*	1252 ± 102*	448 ± 96

Data examined period	Group	n	Liver (g)	Spleen
food restriction period	Control	10	11.787 ± 1.499	765 ± 99
	85% food consumption	10	9.208 ± 0.526**	644 ± 80**
	70% food consumption	10	7.811 ± 0.346**	592 ± 56**
	55% food consumption	10	6.676 ± 0.231**	546 ± 49**
recovery period	Control	6	11.910 ± 1.281	773 ± 81
	55% food consumption	6	9.863 ± 0.881**	745 ± 93

Data examined period	Group	n	Testis-R	Testis-L	Prostate	Seminal Vesicle	Epididymis-R	Epididymis-L
food restriction period	Control	10	1642 ± 111	1632 ± 115	498 ± 124	1249 ± 153	478 ± 24	471 ± 31
	85% food consumption	10	1602 ± 110	1589 ± 136	403 ± 95	1053 ± 263	446 ± 32	445 ± 27
	70% food consumption	10	1471 ± 127**	1447 ± 107**	382 ± 68*	946 ± 162**	428 ± 35**	410 ± 41**
	55% food consumption	10	1514 ± 102*	1490 ± 79*	316 ± 90**	697 ± 155**	420 ± 24**	400 ± 23**
recovery period	Control	6	1659 ± 169	1662 ± 150	576 ± 60	1555 ± 217	571 ± 53	572 ± 44
	55% food consumption	6	1579 ± 63	1587 ± 37	497 ± 102	1295 ± 178*	535 ± 43	525 ± 30

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$
n=9 (because one sample was broken) : 85% food consumption group Pituitary data at the end of food restriction period

Table 8b. Organ Weight (Relative weight) data at the end of 4-week food restriction period and 2-week recovery period

(mg/100gBW)

Data examined period	Group	n	Pituitary	Adrenal-R	Adrenal-L	Kidney-R	Kidney-L	Thymus
food restriction period	Control	10	2.69 ± 0.61	6.66 ± 1.28	7.16 ± 0.84	325.6 ± 15.7	326.0 ± 19.1	144.1 ± 17.3
	85% food consumption	10	2.74 ± 0.60	7.09 ± 1.19	7.56 ± 1.36	335.1 ± 11.7	338.5 ± 15.5	143.3 ± 26.8
	70% food consumption	10	2.64 ± 0.49	8.13 ± 1.32*	8.46 ± 0.64*	339.8 ± 11.2	335.3 ± 15.7	158.5 ± 32.0
	55% food consumption	10	2.70 ± 0.65	7.55 ± 1.08	7.88 ± 0.82	356.5 ± 17.6**	354.0 ± 13.8**	175.6 ± 26.3*
recovery period	Control	6	2.80 ± 0.25	6.22 ± 0.84	6.74 ± 1.26	307.2 ± 26.0	305.4 ± 13.7	104.5 ± 18.8
	55% food consumption	6	2.99 ± 0.41	6.50 ± 0.65	6.63 ± 0.67	308.9 ± 19.0	320.7 ± 13.9	114.7 ± 24.4

Data examined period	Group	n	Liver (g/100gBW)	Spleen
food restriction period	Control	10	2.8566 ± 0.3033	185.6 ± 20.4
	85% food consumption	10	2.5716 ± 0.0736**	179.8 ± 19.0
	70% food consumption	10	2.3937 ± 0.0925**	181.2 ± 15.6
	55% food consumption	10	2.3747 ± 0.1058**	194.1 ± 17.9
recovery period	Control	6	2.6016 ± 0.1766	169.7 ± 21.0
	55% food consumption	6	2.5257 ± 0.1061	191.6 ± 26.3

Data examined period	Group	n	Testis-R	Testis-L	Prostate	Seminal Vesicle	Epididymis-R	Epididymis-L
food restriction period	Control	10	399.3 ± 33.5	396.5 ± 29.4	120.5 ± 28.6	303.6 ± 39.1	116.2 ± 8.9	114.5 ± 9.3
	85% food consumption	10	448.8 ± 42.7*	445.1 ± 47.0*	112.5 ± 27.5	295.7 ± 81.5	125.0 ± 11.8	124.7 ± 11.6
	70% food consumption	10	450.9 ± 36.4**	443.5 ± 32.8*	117.2 ± 22.0	290.7 ± 53.5	131.4 ± 11.3**	125.7 ± 13.4
	55% food consumption	10	538.4 ± 33.0**	529.9 ± 27.9**	112.4 ± 31.8	248.2 ± 57.5	149.1 ± 7.5**	142.3 ± 8.2**
recovery period	Control	6	363.4 ± 36.6	364.0 ± 30.9	126.3 ± 14.5	340.4 ± 45.2	125.4 ± 14.3	125.6 ± 12.2
	55% food consumption	6	406.0 ± 27.6*	408.4 ± 29.6*	128.6 ± 30.1	332.5 ± 48.6	137.4 ± 11.2	134.8 ± 7.1

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$
n=9 (because one sample was broken) : 85% food consumption group Pituitary data at the end of food restriction period

Effects of Food Restriction on Findings of Hematology Examination and Urinalysis in CD (SD) IGS rats - A Four-Week Restricted Feeding Examination in Rats over 20 Weeks of Age -

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ABSTRACT. Twenty male Crj: CD (SD) IGS rats were examined for changes in clinical data and body weight after 4-week food restriction. Two groups, one a control group and the other a restricted feeding group, were set in this study and 10 rats (23 weeks of age) were used for each group. Standard diet was fed *ad libitum* to the control group and approximately 55% of the diet calculated from food consumption by the control group was fed to the restricted feeding group for 4 weeks. Body weight was decreased in the restricted feeding group. Not only significant change in WBC on urinalysis but also the changes in RBC, HGB, MCHC, RET and WBC on hematology examination were observed in the restricted feeding group. Therefore, it is required to whether these findings are due to food restriction or other factors. — Key words: food restriction, hematology, urinalysis

CD(SD)IGS-2002/2003 : 85-90

INTRODUCTION

Body weight reduction caused by decrease in food consumption may be found in toxicity studies. Moreover, it is reported that changes in clinical parameters occur with decrease in food consumption[1-4]. However, it is difficult to judge whether such changes are secondary ones due to reduction of food consumption and body weight or direct ones caused by drug administration. We therefore carried out a restricted feeding examination for 4 weeks in rats over 20 weeks of age.

MATERIALS AND METHODS

Animals and housing

Twenty male Crj: CD (SD) IGS rats were purchased from Charles River Japan Inc. (Atsugi Farm). The animal was raised until 20 weeks of age. After exceeding 20 week of age, animals that were healthy and exhibited favorable growth were selected for this study. The animals were randomly allocated to 2 groups based on body weight. The animal room was maintained at a temperature of 21-25°C and humidity of 46-65% with a 12-hour light/dark cycle (light 7:00-19:00). The animals were housed individually in plastic cages (W270 × D442 × H185 mm) with beta chips for bedding during the test period.

Experimental design

Restriction of feeding was started at 23 weeks of age and confirmed for 4 weeks. The control group was fed a standard diet *ad libitum*. Animals of the restricted feeding group were fed approximately 55% of the daily diet calculated for the control group.

Examinations and methods

The initial day of restriction of feeding was considered restriction day 0.

Clinical signs:

Clinical signs were observed once a week throughout the examination period.

Body weight:

Body weight was measured once a week throughout the examination period.

Food consumption:

For the control group, daily food consumption was calculated from the residual food that had accumulated for 7 days.

Urinalysis:

At the 4th week of the restricted feeding period, urine samples were collected for 6 animals per group using a metabolic cage. The 4-hour urine sample was considered the fresh urine sample. Protein, pH, ketone bodies, glucose, occult blood, bilirubin, urobilinogen, nitrite and specific gravity (Multistix test paper method) were determined using an automated urine analyzer (Clinitek 100, Beyer and Sankyo Ltd.). An 18-hour urine sample was collected from the same animals and urine volume and color were measured grossly and sodium and potassium were determined using a Na/K ISE analyzer (Model 614, Beyer-Medical Ltd.).

Hematology:

At restriction day 29, blood was taken from the iliac vein under ether anesthesia after an overnight fast. The following hematological tests were performed after treatment with EDTA-2K anticoagulant using the Technicon H*1E (Bayer-Medical Co., Ltd.): red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell count (WBC). White blood cell classification was also measured as absolute counts and

percentages of WBC: neutrophils (NEUT), lymphocytes (LYMP), monocytes (MONO), eosinophils (EOS), basophils (BASO) and large unstained cells (LUC). Reticulocytes (RET) were microscopically estimated on smears stained by the Brecher method.

At restriction day 29, blood was taken from the abdominal aorta under ether anesthesia after an overnight fast. The following hematological tests were performed on the plasma after centrifugal separation with treatment with 3.8% sodium citrate anticoagulant using a Coagulometer KC 4A (Heinrich Amelung GmbH): prothrombin (PT) and activated partial thromboplastin time (APTT).

Electrolytes in blood:

At restriction day 29, blood was taken from the abdominal aorta under ether anesthesia after an overnight fast. Serum concentration of sodium and potassium were measured with a Na/K ISE analyzer (Model 614, Beyer-Medical Ltd.).

Necropsy and organ weights:

All the animals were sacrificed under ether anesthesia at the end of the study. The following organs were removed and weighed: pituitary, adrenal glands (right and left), kidney (right and left), thymus, liver, spleen, testes (right and left), prostate, seminal vesicle and epididymides (right and left). Paired organs were measured separately in this study.

Statistical analysis:

Body weights, organ weights, urine volume, electrolytes in urine and hematological data were statistically analyzed by Student's t-test.

The cumulative chi-square test was used for qualitative parameters in urinalysis.

RESULTS

Clinical signs:

There were no deaths and no abnormal clinical signs during the observation period.

Body weight:

Body weight change during the examination period is shown in Fig. 1and Table 1-1 and 1-2. Body weight was significant decreased in the restriction group compared with the control group from the first week of the restricted feeding period. Body weight in the restricted feeding group on restriction day 28 was 80% of that in the control group.

Food consumption:

Food consumption change during the examination period is shown in Table 2. Food consumption by the control group and that by the restriction feed group were almost the same throughout the examination period.

Urinalysis:

Result for the 4-hour urine samples are shown in Table 3-1, and for 18-hour urine samples in Table 3-2. On qualitative examination of 4-hour urine sample, a significant low WBC value was found in the restricted feeding group.

For the 18-hour urine sample, no significant change was found in urine volume. Slightly light urine tone was observed in the restricted feeding group. Examination of electrolytes in urine revealed reduction of potassium and sodium levels. Potassium level was significantly reduced in the restricted feeding group. Reduction of sodium level was observed in the restricted feeding group, but was not significant because of the large dispersion of values in the control group. The potassium and sodium levels in urine of the restricted feeding group were 72% and 69% of those in the control group, respectively.

Hematology:

Results of hematology examination are shown in Table 4-1 and 4-2.

Significant increases in numbers of RBC, HGB and MCHC and significant decrease in number of RET were observed in the restricted feeding group. Increase in HCT and decrease in MCV were also observed, but were not significant.

Significant decrease in total number of WBC was observed in the restricted feeding group. Significant decrease in BASO and decreases in NEUT, MONO and LUC as well as increases in LYMP and EOS were observed in the restricted feeding group. Moreover, on absolute counts, significant decreases in NEUT, MONO, BASO and LUC and decrease in LYMP were observed in the restricted feeding group.

Electrolytes in blood:

Results for electrolytes in blood are shown in Table 5. Significant increase in sodium level in serum was found in the restricted feeding group.

Necropsy and organ weights:

Small prostate (in four animals) and small seminal vesicle (in one animal) were observed in the restricted feeding group.

Absolute organ weights are shown in Table 6-1. Pituitary, kidney (right and left), thymus, liver, spleen, prostate, seminal vesicle and epididymides (right and left) of the restricted feeding group were significantly lower in absolute weight than in the control group.

Relative organ weights are shown in Table 6-2. Thymus, liver, prostate and seminal vesicle of the restricted feeding group were significantly lower in relative weight than in the control group. Adrenals (right and left), testes (right and left) and epididymides (right and left) of the restriction feed group were significantly higher than those of the control group.

DISCUSSION

In order to observe the effects on various clinical parameters of restriction of feeding, a restricted feeding examination was carried out for 4 week using rats older than 20 weeks of age.

Results confirmed body weight reduction due to restriction of feeding, to 55% of the control group value. The rate of body weight change on the end of the restricted feeding period (day 28) was 107% in the control group and 85% in the 55%

restriction group compared with the beginning of the restricted feeding period.

On urinalysis, reductions of specific gravity, protein and ketone bodies were found, with significant reduction of leukocytes.

Reduction of urine volume was found in this study. Generally, in fasted rats, reduction of water consumption and urine volume occur[5]. Urine color tone was light in the restricted feeding group, but within normal range, and appeared to depend on the reduction of excretionof ingredients in urine, which participates in determination of urine color tone. In urine, there was a 28% reduction of sodium and 31% reduction of potassium, compared with the control group.

On hematology examination, increases in RBC, HGB and MCHC that appeared to be due to hemoconcentration were found[2]. Moreover, remarkable reductions in the number of RET considered to be changes due to apparent increase in RBC by hemoconcentration were found. No remarkable changes were found in percentages on WBC classification, but reductions in numbers of NEUT, MONO, BASO and LUC were found.

Decrease or the increase in absolute or relative organ weight was observed for several measured organs.

These results confirmed that changes in toxicological parameters occurred as a result of restriction of feeding. Feeding time and stress resulting from the metabolism cage at the time of urine sample collection could also have affected toxicological parameters. These matters should be considered when toxicity studies are performed. Finally, it is known that restriction of feeding has various effects on reproductive function and rate of metabolism. Therefore it is required to whether these findings are due to food restriction or other factors.

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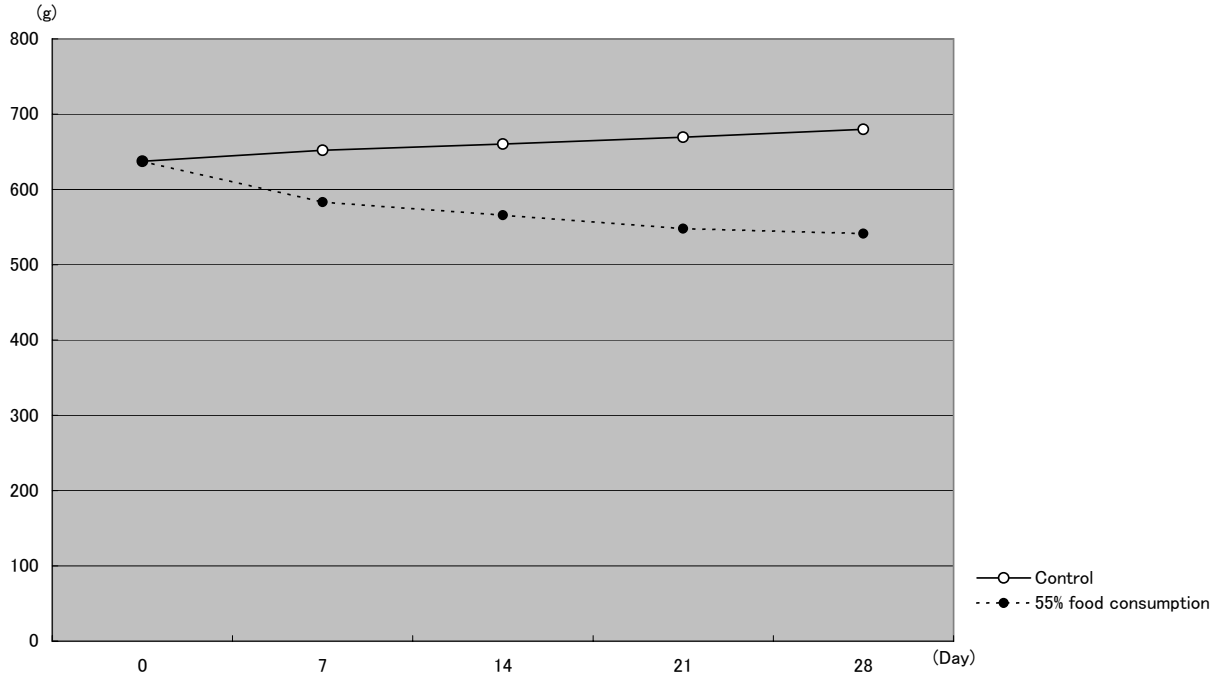

Fig. 1 Body weight data at the end of 4-week food restriction period in young-adult rats

Table 1-1. Body weight data at the end of 4-week food restriction period in young-adult rats

(g)

		food restriction day				
Group	n	0	7	14	21	28
Control	10	637.4 ± 15.4	652.1 ± 16.5	660.4 ± 18.4	669.3 ± 18.3	679.9 ± 18.6
55% food consumption	10	637.2 ± 16.8	583.2 ± 22.0**	565.8 ± 24.7**	548.0 ± 21.5**	541.3 ± 18.4**

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ∽ 12:00)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 1-2. Body weight gain data at the end of 4-week food restriction period in young-adult rats

(g)

Group	n	7	14	21	28
Control	10	14.7 ± 7.5	23.0 ± 12.8	31.9 ± 12.1	42.5 ± 12.5
55% food consumption	10	-54.0 ± 8.6	-71.4 ± 13.6	-89.2 ± 8.6	-95.8 ± 10.0

Parameter : mean ± S.D., Data : examined at A.M.(10:00 ∽ 12:00)

Table 2. Food consumption data at the end of 4-week food restriction period in young-adult rats

(g/day)

		food restriction day			
Group	n	0 ∽ 7	7 ∽ 14	14 ∽ 21	21 ∽ 28
Control	10	29.1 ± 1.5	29.7 ± 1.9	29.7 ± 1.6	28.9 ± 2.0
55% food consumption	10	13.5	16.5	16.5	16.5

Parameter : mean ± S.D.
Data of control group : examined at A.M.(10:00 ∽ 12:00)
Data of 55% food consumption group : estimate by the number of pellet

Table 3-1. Urinalysis data measured at 4-week of food restriction in young-adult rats

Group	n	GLU						PLO					
		-	+/-	+	++	+++	++++	-	+/-	+	++	+++	++++
Control	6	6	0	0	0	0	0	0	1	3	2	0	0
55% food consumption	5	5	0	0	0	0	0	0	3	2	0	0	0

Group	n	KET						BIL				URO				
		-	+/-	+	++	+++	++++	-	+	++	+++	0.1	1.0	2.0	4.0	8.0
Control	6	1	4	1	0	0	0	6	0	0	0	6	0	0	0	0
55% food consumption	5	2	2	1	0	0	0	5	0	0	0	5	0	0	0	0

Group	n	SG						PH							
		≦ 1.005	1.010	1.015	1.020	1.025	≧ 1.030	5.0	6.0	6.5	7.0	7.5	8.0	8.5	≧ 9.0
Control	6	0	1	1	2	0	2	0	0	0	0	0	1	4	1
55% food consumption	5	0	3	0	2	0	0	0	0	0	0	1	2	2	0

Group	n	OB					NIT		WBC*				
		-	+/-	+	++	+++	-	+	-	+/-	+	++	+++
Control	6	5	0	1	0	0	6	0	0	3	2	1	0
55% food consumption	5	4	0	1	0	0	5	0	3	2	0	0	0

Parameter : n
Significantly different from control, * : $p < 0.05$

Table 3-2. Urinalysis data of 4-week food restriction period in young-adult rats

Data examined period	Group	n	Color (n) Y	S	A	Urine vol (mL/18h)	U-Na (mmol/L)	U-Na (mmol/18h)	U-K (mmol/L)	U-K (mmol/18h)
food restriction 4-week	Control	6	4	2	0	18.4 ± 9.6	95 ± 26	1.6417 ± 0.7135	210 ± 68	3.3512 ± 0.6252
	55% food consumption	6	3	2	1	12.3 ± 4.8	104 ± 38	1.1758 ± 0.3493	207 ± 64	2.3169 ± 0.4726**

Parameter : mean ± S.D., Data : examined by overnight collection urine sample (approximately 18 hr)
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 4-1. Hematology data at the end of 4-week food restriction period in young-adult rats

Group	n	CBC WBC (× 10^3/ μ L)	RBC (× 10^6/ μ L)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	10	11.65 ± 2.16	9.06 ± 0.26	15.7 ± 0.6	46.1 ± 1.4	50.9 ± 1.3	17.3 ± 0.6	33.9 ± 0.6
55% food consumption	10	9.75 ± 1.70*	9.45 ± 0.22**	16.3 ± 0.7*	47.0 ± 1.7	49.7 ± 1.8	17.2 ± 0.7	34.6 ± 0.6*

Group	n	CBC PLT (× 10^3/ μ L)	Reticulocyte (%)
Control	10	1006 ± 85	14.9 ± 3.7
55% food consumption	10	1078 ± 122	8.3 ± 1.8**

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 4-2. Differential cell data at the end of 4-week food restriction period in young-adult rats

Group	n	DIFF (%) NEUT	LYMP	MONO	EOS	BASO	LUC
Control	10	15.0 ± 3.9	78.5 ± 3.5	2.6 ± 0.4	1.5 ± 0.6	0.3 ± 0.1	2.2 ± 0.4
55% food consumption	10	13.0 ± 3.7	81.0 ± 4.4	2.1 ± 0.9	1.9 ± 0.5	0.2 ± 0.1*	1.7 ± 0.7

Group	n	DIFF (× 10^3/ μ L) NEUT	LYMP	MONO	EOS	BASO	LUC
Control	10	1.79 ± 0.67	9.11 ± 1.58	0.30 ± 0.08	0.16 ± 0.05	0.03 ± 0.01	0.25 ± 0.06
55% food consumption	10	1.22 ± 0.25*	7.94 ± 1.69	0.21 ± 0.11*	0.19 ± 0.05	0.02 ± 0.01*	0.17 ± 0.08*

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 5. Serum electrolyte data at the end of 4-week food restriction period in young-adult rats

Group	n	Na (mmol/L)	K (mmol/L)
Control	10	144 ± 1	4.91 ± 0.23
55% food consumption	10	145 ± 1*	4.89 ± 0.14

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 6-1. Organ Weight (Absolute weight) data at the end of 4-week food restriction period in young-adult rats

(mg)

Group	n	Pituitary	Adrenal-R	Adrenal-L	Kidney-R	Kidney-L	Thymus
Control	10	14.4 ± 1.5	28.7 ± 2.7	29.9 ± 2.1	1798 ± 147	1809 ± 99	327 ± 97
55% food consumption	10	12.2 ± 1.0**	28.9 ± 3.5	31.7 ± 4.6	1495 ± 128**	1498 ± 143**	196 ± 52**

Group	n	Liver (g)	Spleen
Control	10	15.491 ± 0.818	908 ± 135
55% food consumption	10	10.751 ± 0.989**	713 ± 94**

Group	n	Testis-R	Testis-L	Prostate	Seminal Vesicle	Epididymis-R	Epididymis-L
Control	10	1851 ± 130	1846 ± 147	883 ± 172	1957 ± 169	789 ± 67	764 ± 51
55% food consumption	10	1791 ± 124	1794 ± 139	479 ± 146**	1185 ± 273**	689 ± 69**	697 ± 87

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

Table 6-2. Organ Weight (Relative weight) data at the end of 4-week food restriction period and 2-week recovery period

(mg/100gBW)

Group	n	Pituitary	Adrenal-R	Adrenal-L	Kidney-R	Kidney-L	Thymus
Control	10	2.11 ± 0.22	4.22 ± 0.44	4.40 ± 0.33	264.5 ± 20.0	266.2 ± 14.6	48.1 ± 14.5
55% food consumption	10	2.26 ± 0.20	5.34 ± 0.69**	5.84 ± 0.81**	276.5 ± 24.9	276.8 ± 25.3	36.2 ± 9.8*

Group	n	Liver (g/100gBW)	Spleen
Control	10	2.2787 ± 0.1104	133.8 ± 21.3
55% food consumption	10	1.9854 ± 0.1584**	131.8 ± 17.6

Group	n	Testis-R	Testis-L	Prostate	Seminal Vesicle	Epididymis-R	Epididymis-L
Control	10	272.5 ± 21.5	271.8 ± 24.9	129.7 ± 24.5	288.7 ± 21.2	116.1 ± 10.2	112.5 ± 9.1
55% food consumption	10	331.5 ± 29.1**	332.2 ± 33.4**	88.5 ± 26.6**	218.9 ± 50.3**	127.4 ± 13.3*	128.8 ± 15.5*

Parameter : mean ± S.D.
Significantly different from control, * : $p < 0.05$, ** : $p < 0.01$

The Effect of Aging on the Results of Rat Micronucleus Assay

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ABSTRACT. We conducted the micronucleus assay in 3-, 5-, 7-, 9-, 11-, and 13-week-old male rats to determine whether the results varied with age. We administered cyclophosphamide orally at 0 (vehicle), 5, 10, or 20 mg/kg, twice, 24 h apart, to five rats per dosage group and collected bone marrow and peripheral blood 24 h after the second treatment. We observed an age-related decrease in micronucleus-inducing activity in both polychromatic erythrocytes (bone marrow) and reticulocytes (peripheral blood), which we attributed to an age-related decrease in hematopoiesis. In spite of the age-related decrease in sensitivity to the model chemical studied here, all age groups tested showed positive responses. We concluded that the rat is suitable for the micronucleus assay regardless of age. —Key words: micronucleus assay, rat, aging, cyclophosphamide, CSGMT

CD(SD)IGS-2002/2003 : 91-94

INTRODUCTION

The mouse is conventionally used for the micronucleus assay while the rat is usually used for general toxicologic and toxicokinetic studies. If micronucleus induction can be evaluated in the same rats that are used for general toxicology studies, a lot of information can be gathered concomitantly. We explored the possibility of incorporating micronucleus assays into 28 day repeat dose general toxicology studies, where the effect of aging could be important, using the same animals [6]. The Collaborative Study Group for the Micronucleus Test (CSGMT), a working group of the Mammalian Mutagenicity Study Group (MMS), which is a subgroup of the Japanese Environmental Mutagen Society (JEMS), previously evaluated the effects of aging on the micronucleus assay in mice [3, 5, 10, 12]. In the present study, we investigated the aging effects in rats using cyclophosphamide (CP) as the model chemical.

MATERIALS AND METHODS

Chemicals

Cyclophosphamide (CAS no. 50-18-0, lot no. 9014), obtained from Shionogi & Co., Ltd. (Osaka, Japan), was dissolved in distilled water immediately before use.

Animals

Male Crj:CD(SD)IGS rats 3, 5, 7, 9, 11, and 13 weeks old were purchased from Charles River Japan Inc. They were given commercial pellets and tap water ad libitum throughout the acclimatization and experimental periods and were subjected to a 12 h light/dark cycle.

Micronucleus assay protocols

To perform the micronucleus assay using bone marrow and peripheral blood simultaneously in the same animal, a double-dosing regimen [2, 4] was used. CP dissolved in distilled water at 0 (vehicle), 5, 10, or 20 mg/kg was administered orally twice, 24 h apart, to five rats per dosage group. Both peripheral blood and bone marrow were collected 24 h after the second treatment. Acridine orange staining [8] was used for analysis. Micronucleated polychromatic erythrocyte (MNPCE), micronucleated normochromatic erythrocyte (MNNCE) and micronucleated reticulocyte (MNRET) frequencies were recorded based on the observation of 2000 polychromatic erythrocytes (PCEs), 2000 normochromatic erythrocytes (NCEs), or 2000 reticulocytes (RETs). Cytotoxicity was evaluated based on the observation of 300 bone marrow erythrocytes or 2000 peripheral blood reticulocytes.

Statistical analysis

To compare the responses of each dosage group with those of the concurrent control group, we used Kastenbaum and Bowman's tables [11] for the frequency of MNPCEs, MNRETs, and MNNCEs and the *t*-test for the frequency of PCEs and RETs.

RESULTS

Table I shows the results of both assays. In the bone marrow assay, the MNPCE frequency increased significantly in the treated animals in all dosage and age groups. The magnitude of this increase decreased with increasing age in the 10 and 20 mg/kg/day groups. There were no significant increases in MNNCE frequency in any group treated at 5 mg/kg per day. The PCE frequency was highest in the 3-week-old age group and decreased significantly at all doses in the 9-, 11-, and 13-week-old groups. The decreases were age-related.

In the peripheral blood micronucleus assay, the MNRET frequency increased significantly in all dosage groups by the CP at 5, 7, 11, and 13 weeks of age. The magnitude of the increases decreased in an age-related manner within the 5, 10, and 20 mg/kg/day groups at up to 7-9 weeks age, but not in the vehicle control groups. There was no age- or dose-dependent increase in the frequencies of MNNCE. The RET frequency decreased significantly in treated animals at all dosages in the 3-, 7-, and 9-week-old groups and at 20 mg/kg/day in all age groups, relative to the 3-week-old group. An age-related decrease was seen in all dosage groups.

Note: This paper is duly permitted to reprint from original paper (Mutagenesis, 18(3), 273-275, 2003).

DISCUSSION

There was a tendency towards an age-related decrease in micronucleus assay sensitivity to CP in the rat bone marrow assay. The sensitivity of the assay may depend on the metabolic activity of cytochrome P450 2C enzyme (CYP2C), which metabolizes CP to a genotoxic intermediate [1], but this needs further evaluation. Yajima *et al*. [16-18] and Handa and Yajima [7] speculated that the increased MNPCE and MNRET frequencies are the result of errors that occur in the processes of denucleation, differentiation and repair of genetic damage. They also suggested a close relationship between erythropoiesis and micronucleus induction. In the present study, there was a close relationship between PCE and MNPCE frequencies at the same CP dose (Figure1). These results suggested that the young animals, which had a high level of erythropoietic activity, tended to show high micronucleus induction. We confirmed an age-related reduction in sensitivity to CP, although all dosages induced MNPCE significantly. At 5 mg/kg/day, CP did not significantly increase MNNCE frequency in any age group. These results might suggest that the micronucleus-inducing activity of compounds could be evaluated in bone marrow cells regardless of age of rat if MNPCEs, but not MNNCEs, were scored.

The results of the peripheral blood assay were similar to those of the bone marrow assay. The micronucleus-inducing activity of compounds could be evaluated regardless of age if MNRETs, but not MNNCEs, were scored. A good correlation between RET and MNRET frequencies was seen within the same CP dosages, but the peripheral blood data varied more widely than the bone marrow data (Figure 2), probably because some circulating micronucleated erythrocytes were destroyed in the spleen [9, 13-15], but that needs further evaluation.

The effect of aging on the micronucleus assay was not so clear in the mouse [5] as in this study of the rat. The lowering of erythropoiesis with age in the mouse was less [5] than in rat, which was assumed to be the primary cause of the small effect of aging in the mouse micronucleus assay.

In summary, these results suggest that the effect of aging in the micronucleus assay depend mainly on age-related changes in erythropoietic function and the chemical induction of micronuclei in bone marrow cells can be evaluated by scoring MNPCEs in the bone marrow or MNRETs in the peripheral blood in the rat regardless of age, with marginal age-dependent differences in sensitivity.

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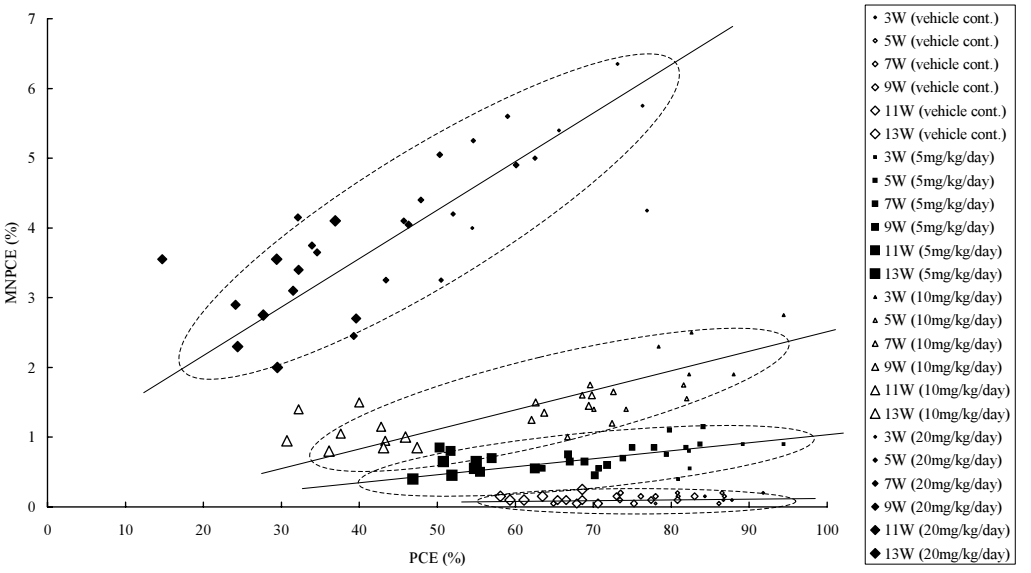

Figure 1. Relationship of MNPCE and PCE frequencies in bone marrow of male SD rats following oral administration of CP.

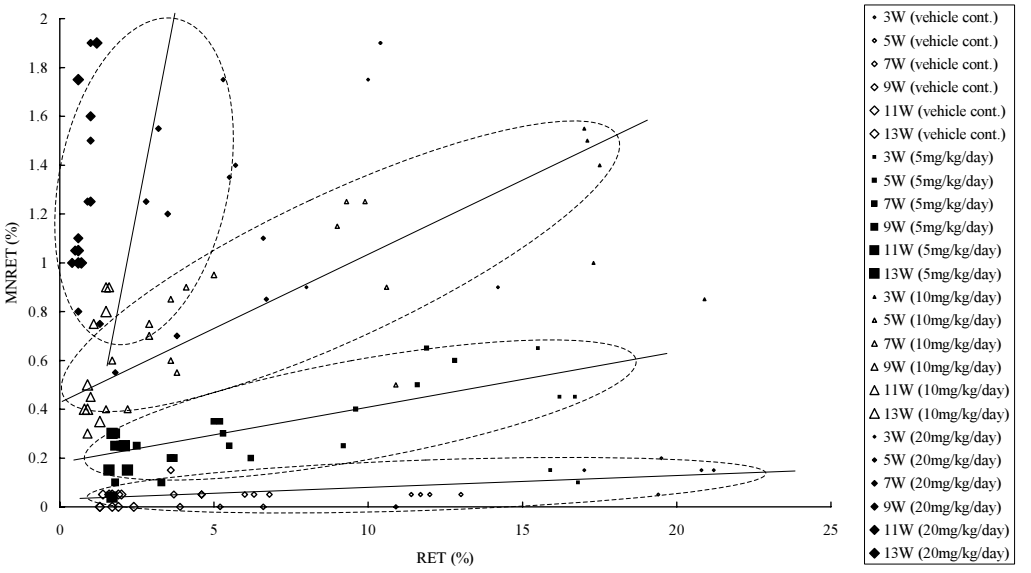

Figure 2. Relationship of MNRET and RET frequencies in peripheral blood of male SD rats following oral administration of CP.

Table I. Micronucleus induction in male SD rats treated with cyclophosphamide (*n*=5).

Age(weeks old)	Dose(mg/kg)	Bone marrow			Peripheral blood		
		MNPCE (%)	MNNCE (%)	PCE (%)	MNRET (%)	MNNCE (%)	RET (%)
3	0	0.12±0.06	0.01±0.02	85.72±5.10	0.14±0.06	0.03±0.05	19.58±1.64
	5	0.71±0.23[a]	0.02±0.03	85.84±5.77	0.36±0.23	0.01±0.02	16.62±0.54[a]
	10	2.27±0.37[a]	0.08±0.06[b]	85.14±6.20	1.26±0.32[a]	0.04±0.04	17.96±1.65
	20	5.15±1.00[a]	0.20±0.13[a]	69.28±9.41[b]	1.47±0.52[a]	0.04±0.07	10.60±2.24[a]
5	0	0.15±0.06	0.02±0.03	84.22±3.09	0.04±0.02	0.00±0.00	11.80±0.78
	5	0.95±0.17[a]	0.02±0.03	81.78±2.16	0.48±0.16[a]	0.01±0.02	11.02±1.55
	10	1.55±0.15[a]	0.07±0.06	76.10±5.41[b]	1.01±0.32[a]	0.02±0.03	9.94±0.81[a]
	20	4.66±0.94[a]	0.19±0.07[a]	55.72±4.98[a]	1.29±0.34[a]	0.01±0.02	5.96±0.65[a]
7	0	0.13±0.06	0.02±0.03	73.18±5.01	0.03±0.03	0.00±0.00	6.18±0.63
	5	0.70±0.15[a]	0.01±0.02	72.16±5.47	0.29±0.27[b]	0.00±0.00	5.44±0.46[b]
	10	1.44±0.32[a]	0.04±0.04	69.98±2.53	0.77±0.18[a]	0.01±0.02	4.02±0.58[a]
	20	4.34±0.72[a]	0.14±0.07[a]	49.48±6.47[a]	1.05±0.41[a]	0.02±0.03	3.02±0.78[a]
9	0	0.11±0.04	0.02±0.03	77.88±4.06	0.06±0.06	0.01±0.02	4.08±0.49
	5	0.62±0.11[a]	0.02±0.03	68.94±2.13[a]	0.17±0.07	0.02±0.03	2.98±0.81[b]
	10	1.43±0.14[a]	0.04±0.04	65.52±3.77[a]	0.57±0.16[a]	0.00±0.00	2.24±0.65[a]
	20	3.61±0.68[a]	0.17±0.08[a]	37.24±5.72[a]	1.24±0.48[a]	0.01±0.02	0.96±0.25[a]
11	0	0.08±0.03	0.01±0.02	67.80±2.00	0.02±0.03	0.01±0.02	1.72±0.46
	5	0.68±0.15[a]	0.01±0.02	55.40±4.81[a]	0.24±0.06[b]	0.00±0.00	1.86±0.19
	10	1.21±0.23[a]	0.06±0.04	36.18±4.53[a]	0.66±0.27[a]	0.01±0.02	1.22±0.31[b]
	20	3.13±0.35[a]	0.08±0.03[b]	28.42±9.43[a]	1.20±0.24[a]	0.00±0.00	0.70±0.28[a]
13	0	0.15±0.06	0.02±0.05	62.12±4.15	0.03±0.03	0.00±0.00	1.72±0.24
	5	0.54±0.11[a]	0.04±0.04	51.88±3.33[a]	0.25±0.11[a]	0.01±0.02	1.86±0.27
	10	0.89±0.08[a]	0.05±0.05	40.64±7.05[a]	0.49±0.18[a]	0.00±0.00	1.08±0.30[a]
	20	2.94±0.87[a]	0.20±0.04[a]	29.58±4.58[a]	1.34±0.45[a]	0.01±0.02	0.74±0.26[a]

Values shown are means±SD. MNNCE, micronucleated normochromatic erythrocytes; MNPCE, micronucleated PCE; MNRET, micronucleated RET; PCE, polychromatic erythrocytes; RET, reticulocytes.

[a]P<0.01 compared with the results of vehicle control group in each age.

[b]P<0.05 compared with the results of vehicle control group in each age.

Spontaneous Ocular Lesions in Aged Crj:CD(SD)IGS Rats

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ABSTRACT. Spontaneous ocular lesions in the 57- and 109-week-old Crj:CD(SD)IGS (IGS) rats (20 males and 20 females at 57 weeks of age, 17 males and 29 females at 109 weeks of age) were investigated histopathologically. At 57 weeks of age, corneal degeneration characterized by focal deposition of basophilic materials in the corneal epithelial basement membrane was observed in both sexes. Preretinal arteriolar loop, retinal dysplasia, and peripheral and diffuse retinal degeneration were detected in the 57-week-old female rats. At 109 weeks of age, corneal degeneration, cataract, and peripheral and diffuse retinal degeneration were observed in male and female rats. The incidences of corneal degeneration in the 109-week-old rats (11.8% in males and 41.4% in females) were significantly lower than those in the 57-week-old rats (50.0% in males and 75.0% in females). In contrast, the incidences of retinal degeneration in the 109-week-old rats (52.9% in males and 75.9% in females) were significantly higher than those in the 57-week-old rats (0% in males and 30.0% in females). It seems that these results contribute to characterize spontaneous ocular lesions in the aged IGS rats. —Key words: aging, IGS rat, incidence, ocular lesions.

CD(SD)IGS-2002/2003 : 95-99

INTRODUCTION

Crj:CD(SD)IGS (IGS) rats have been induced by the international genetic standard system and begun to be widely used in various toxicity studies[8]. The international genetic standard system, which has been developed by Charles River Laboratories, is a new breeding procedure of laboratory rats. The system makes it possible to produce uniform laboratory rats owing to the genetic ramification control[8]. Therefore, the IGS rats are expected to meet internationalization of research and development of new drugs. To evaluate toxic effects on the eye, it is important to accumulate comparable historical data on the animals used. Previously, we reported on historical data of spontaneous ophthalmic lesions in young-adult Sprague-Dawley, Wistar, and F344 strains of the rats between 8 and 62 weeks of age[10], in IGS rats between 5 and 31 weeks of age[11], and in aged, 110-week-old, Fischer344 rats[12]. However, historical control data on spontaneous ocular lesions of the aged IGS rats are not yet fully accumulated. Therefore, we investigated histopathologically spontaneous ocular lesions in the aged, 57- and 109-week-old, IGS rats.

MATERIALS AND METHODS

Seventy male and 70 female Crj:CD(SD)IGS (IGS) rats at 4 weeks of age were purchased from Charles River Japan, Inc. (Kanagawa). They were individually housed in wire mesh cages and maintained in a barrier-sustained room controlled at 21 - 25 ℃ and 30 - 70% relative humidity, ventilated 10 times per hr, and illuminated by white fluorescent ceiling lights with a 12-hr light-dark cycle. The light intensity within each cage in the racks ranged from 38 to 250 lux depending on a position of the cages. The animals had free access to a low protein commercial diet (18% protein content) for rats (CR-LRF with γ -ray irradiation, Oriental Yeast Co. Ltd., Tokyo) and tap water. They cared for and were treated humanely in accordance with *the Guidelines for Animal Experimentation*, published by the Japanese Association for Laboratory Animal Science (1987). The rats survived at the end of studies (20 males and 20 females at 57 weeks of age and 17 males and 29 females at 109 weeks of age) were euthanatized under deep anesthesia with ether and subjected to a complete necropsy. The eyeballs were fixed in Davidson's fixative, embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin (HE). The incidences of ocular lesions in the 109-week-old rats were compared with those in the 57-week-old rats and the statistical significance was assessed by the Fisher's extract test for differences in the incidences.

RESULTS

Spontaneous ocular lesions and their incidences in the aged IGS rats were shown in Table 1.

At 57 weeks of age, corneal degeneration was observed in male and female rats, and preretinal arteriolar loop, retinal dysplasia, peripheral retinal degeneration and diffuse retinal degeneration were observed in female rats. Unilateral corneal degeneration was detected in 8 males (40.0%) and 8 females (40.0%) and bilateral corneal degeneration was detected in 2 males (10.0%) and 7 females (35.0%). The numbers of rats with the corneal degeneration were 10 in males (50.0%) and 15 in females (75.0%). In the corneal lesions, basophilic materials or basophilic fine granules deposited focally in the corneal epithelial basement membrane (Fig. 1) and the corneal epithelial cells adjacent the deposits were slightly edematous and swelling. Preretinal arteriolar loop was detected in unilateral eyeballs of 4 females (20.0%) and was characterized by heterotopic arterioles including red blood cells in the preretinal region of the vitreous (Fig. 2). Retinal dysplasia developed bilaterally in one female (5.0%) and was characterized by focal disorganization of the retinal layers, showing pseudorosettes (Fig. 3). Peripheral degeneration of the retina was detected unilaterally in 5 females (25.0%). In the retinal lesions, retinal cells of the outer layers of the retina, such as pigment epithelium, photoreceptor, outer nuclear, and outer plexiform layers, were atrophic, degenerative or lost at the peripheral portion of the retina (Fig. 4). Diffuse degeneration of unilateral retina developed in one female (5.0%), in which atrophy, degeneration or missing of the outer retinal

Note : This paper is duly permitted to reprint from original paper (Animal Eye Research, 20, 15-19, 2001)

Table 1. Numbers of Aged IGS Rats with Histopathological Ocular Lesions

Tissues and lesions	57 weeks of age		109 weeks of age	
	Male	Female	Male	Female
No. of rats examined	20	20	17	29
Eyelid	-	-	-	-
Conjunctiva	-	-	-	-
Cornea				
Degeneration, U	8(40.0)	8(40.0)	1(5.6)*	6(20.7)
Degeneration, B	2(10.0)	7(35.0)	1(5.9)	6(20.7)
Degeneration, Total	10(50.0)	15(75.0)	2(11.8)*	12(41.4)*
Anterior chamber	-	-	-	-
Iris	-	-	-	-
Ciliary body	-	-	-	-
Lens				
Cataract, U	0(0)	0(0)	1(5.9)	7(24.1)*
Vitreous				
Preretinal arteriolar loop, U	0(0)	4(20.0)	0(0)	0(0)*
Retina				
Dysplasia, B	0(0)	1(5.0)	0(0)	0(0)
Peripheral degeneration, U	0(0)	5(25.0)	6(35.3)**	11(37.9)
Peripheral degeneration, B	0(0)	0(0)	2(11.8)	3(10.3)
Diffuse degeneration, U	0(0)	1(5.0)	3(17.6)	5(17.2)
Diffuse degeneration, B	0(0)	0(0)	0(0)	8(27.6)**
Degeneration, Total	0(0)	6(30.0)	9(52.9)**	22(75.9)**
Optic disc	-	-	-	-
Choroid	-	-	-	-

Values in parentheses represent percentages.
U: unilateral. B: bilateral. -: no lesions.
*: $p < 0.05$. **: $p < 0.01$.

layers was observed in the all area (Fig. 5). The numbers of rats with the retinal degeneration were 6 in females (30.0%).

At 109 weeks of age, corneal degeneration, cataract, peripherel retinal degeneration, and diffuse retinal degeneration were observed in male and female rats. Unilateral corneal degeneration was detected in one male (5.9%) and 6 females (20.7%) and bilateral corneal degeneration was detected in one male (5.9%) and 6 females (20.7%). The numbers of rats with the corneal degeneration were 2 in males (11.8%) and 12 in females (41.4%). The corneal lesions observed in the 109-week-old rats were histologically similar to those in the 57-week-old rats. Cataract was detected unilaterally in one male (5.9%) and 7 females (24.1%). The lesions were characterized by edematous, fragmented or liquefactive degeneration of the lens fibers in the peripheral or posterior cortical portion (Fig. 6) or in the whole cortical area of the lens (Fig. 7). The eyeballs of the rats with cataract (one male and 6 females) were affected with severe diffuse retinal degeneration (Fig. 8). Unilateral peripheral degeneration of the retina was detected in 6 males (35.3%) and 11 females (37.9%) and bilateral peripheral degeneration of the retina was detected in 2 males (11.8%) and 3 females (10.3%). These retinal lesions in the 109-week-old rats revealed similar findings to those in the 57-weeks-old rats. Unilateral diffuse degeneration of the retina occurred in 3 males (17.6%) and 5 females (17.2%) and bilateral diffuse degeneration of the retina developed in 8 female (27.6%). These lesions in the 109-week-old rats were essentially similar to those in the 57-week-old rats. However, the degrees of the retinal degeneration in the 109-week-old rats were severer than those in the 57-week-old rats. The numbers of rats with the retinal degeneration were 9 in males (52.9%) and 22 in females (75.9%).

The incidence of unilateral corneal degeneration in the 109-week-old males was significantly ($p < 0.05$) lower than that in the 57-week-old males. The incidences of corneal degeneration in the 109-week-old males and females were significantly ($p < 0.05$) lower than those in the 57-week-old males and females, respectively. The incidence of unilateral cataract in the 109-week-old females was significantly ($p < 0.05$) higher than that in the 57-week-old females. The incidence of unilateral preretinal arteriolar loop in the 109-week-old females was significantly ($p < 0.05$) lower than that in the 57-week-old females. The incidence of unilateral peripheral degeneration of the retina in the 109-week-old males was significantly ($p < 0.01$) higher than that in the 57-week-old males. The incidence of bilateral diffuse degeneration of the retina in the 109-week-old females was significantly ($p < 0.01$) higher than that in the 57-week-old females. The incidences of retinal degeneration in the 109-week-old males and females were significantly ($p < 0.01$) higher than those in the 57-week-old males and females, respectively.

DISCUSSION

Various spontaneous lesions were histopathologically observed in the IGS rats at 57 and 109 weeks of age. Previously we reported on spontaneous histopathological ocular lesions in the 9-, 18- and 31-week-old IGS rats[11]. In the study, focal granulomatous lesions in the conjunctiva, corneal degeneration, persistent hyaloid artery, preretinal arteriolar loop, retinal dysplasia, retinal hypoplasia and retinal degeneration were histologically detected[11]. It is well known that persistent hyaloid artery, retinal dysplasia and retinal hypoplasia have been described as congenital ocular lesions in the rats[14]. In the present study, retinal dysplasia in a 57-week-old female rat may be one of the congenital lesions, because the lesions occurred sporadic and the incidence was not related to the aging. It has been suggested that preretinal arteriolar loops in the dogs were caused by a failure of incorporation of membrana vasculosa retinae, indicating one of the congenital ocular defects[9]. On the other hand, preretinal arteriolar loops in the rats have been reported to originate directly from the central artery[13]. However, the precise pathogenesis of preretinal arteriolar loops in the rats is still obscure.

Corneal degeneration was detected in unilateral and bilateral eyeballs in the present study. The corneal lesions characterized by basophilic deposits in the basement membrane and slight degeneration of the epithelial cells resembled the lesions described as spontaneous corneal degeneration, corneal calcification and corneal dystrophy of the rats[2,3,7]. The deposits in the corneal epithelial basement membrane have been revealed to be minerals containing calcium and phosphorous[2,7]. Ophthalmoscopically the corneal lesions were observed as

central corneal opacity, i.e. multiple deposits of white fine granules on the central corneal surface[2,5,11]. Our previous study disclosed that the incidence of corneal degeneration in the IGS rats increased with age between 9 and 31 weeks of age[11]. In the present study, however, the incidence of corneal degeneration in the IGS rats at 109 weeks of age was lower than that in the IGS rats at 57 weeks of age. The correlation between corneal degeneration and aging in the IGS rats remains to be elucidated in further studies. It has been suggested that hypercalcemia, corneal desiccation or noxious substances such as ammonia would be considerable causes of the corneal degeneration in the rats[2,7].

In the present study, cataracts were observed in the 109-week-old IGS rats but not in the 57-week-old IGS rats. In addition, our previous study revealed that no cataracts were detected histologically in any IGS rats at 9, 18 and 31 weeks of age[11]. The present study showed that all cases of the cataracts developed unilaterally. It has been described that posterior capsular cataracts were detected usually in the unilateral eyeballs of the Sprague-Dawley rats[4]. However, the pathogenesis of spontaneous lens opacities in the aged rats is not known[14].

The incidences of both peripheral and diffuse retinal degeneration in the IGS rats increased with age, coinciding with the previous results describing the incidences of retinal atrophy in the IGS, Sprague-Dawley, Wistar, and F344 rats[10,11]. In addition, an ophthalmoscopic study revealed that no retinal atrophy was observed in any Sprague-Dawley rats between 6 and 8 weeks of age[5]. Peripheral retinal degeneration in aged Fischer 344 rats has been revealed to occur without any degeneration of other areas of the retina[6]. Hence, it seemed to be primarily a lesion of aging rather than a light-associated lesion[6]. On the bases of these results, it is likely that retinal atrophy in the rats may develop in association with aging. On the other hand, cataract were nearly always accompanied by diffuse retinal degeneration in the aged IGS rats. It is probable that the retinal degeneration in these cases may develop secondary to cataract. However, it is necessary to refer the consideration that retinal atrophy in the rats would be caused by confounding factors such as lighting, diet, temperature, age of initial exposure, and different pigmentation characteristics of the strains[14].

Several ocular lesions have been reported to occur spontaneously in the rats in association with aging and lighting in the animal environment[1,4,14]. The environmental condition, such as light intensity, housing, and feeding, of the present study was the same as our previous study using the IGS rats[11]. Therefore, it seems that these results contribute to characterize spontaneous ocular lesions in the aged IGS rats.

ACKNOWLEDGMENTS

We are grateful to Y. Ogata, Y. Amano, M. Kaneko, H. Suzaki, and M. Yamazaki for their technical assistance.

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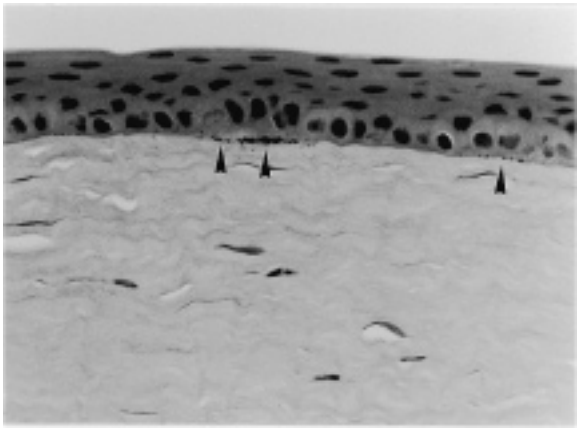

Figure 1. Corneal degeneration in a 57-week-old female IGS rat. Note basophilic fine granular deposits (arrowheads) in the basement membrane of the corneal epithelium. HE stain, x 400.

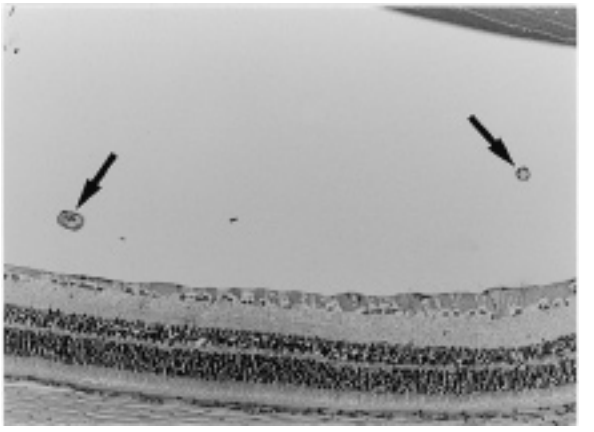

Figure 2. Preretinal arteriolar loop in a 57-week-old female IGS rat. Note two heterotopic arterioles (arrows) in the preretinal region of the vitreous. HE stain, x 100.

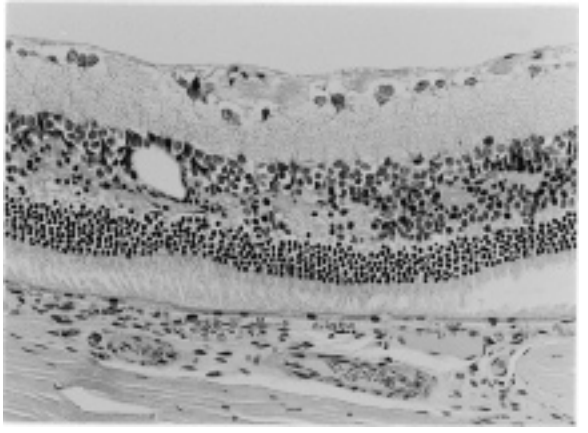

Figure 3. Focal retinal dysplasia in a 57-week-old female IGS rat. Note a foci of psuedorosette formation in the retina. HE stain, x 180.

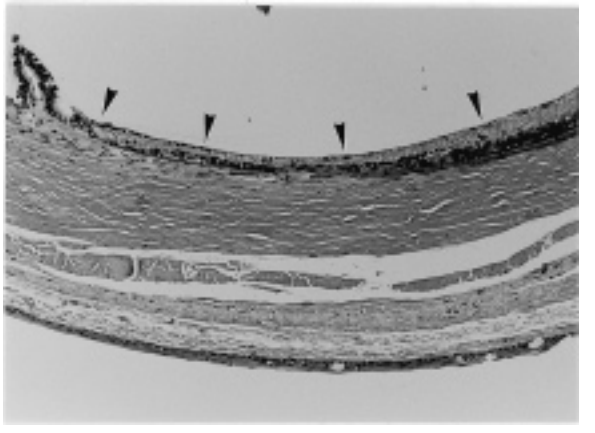

Figure 4. Peripheral retinal degeneration in a 57-week-old female IGS rat. Note degeneration and dysorganization of the outer retinal layers in the peripheral portion (arrowheads). HE stain, x 100.

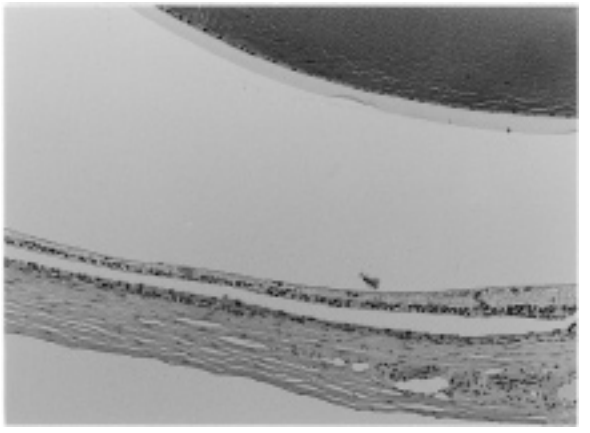

Figure 5. Diffuse retinal degeneration in a 57-week-old female IGS rat. Note severe degeneration and thinning of the retina. HE stain, x 100.

Figure 6. Posterior subcapsular cataract in a 109-week-old female IGS rat. Note fragmentation and liquefactive degeneration of the lens fibers in the posterior subcapsular portion (arrowheads) of the lens. HE stain, x 100.

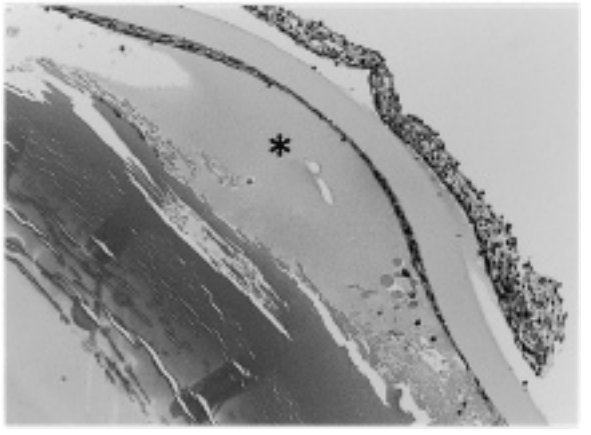

Figure 7. Cortical cataract in a 109-week-old female IGS rat. Note severe liquefactive degeneration (asterisk) of the lens fibers in the whole cortical portion of the lens. HE stain, x 100.

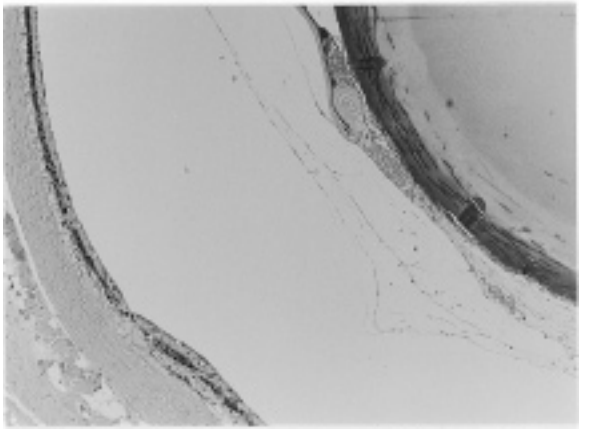

Figure 8. Severe diffuse retinal degeneration associated with cortical cataract in a 109-week-old female IGS rat. X 40.

Ectopic Retina in the Optic Nerve of Crj:CD(SD)IGS Rats

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Summary. Ectopic retina was observed histopathologically within the beginning of the optic nerve in 19 of 80 male (23.8%) and 21 of 80 female (26.3%) Crj:CD(SD)IGS rats. No abnormal findings were detected ophthalmoscopically in the optic disk of these affected eyes. The ectopic retinal tissues consisted of irregularly-aggregated photoreceptor cells or of tissue segments of the photoreceptor segment, outer nuclear, outer plexiform, and inner nuclear layers. There were no age-related changes or no sex difference in the occurrence of the lesions. These results suggest that the ectopic retina in the optic nerve in Crj:CD(SD)IGS rats may develop in a congenital manner such as overgrowth of the inner layer of the optic cup. —Key words: ectopic retina, optic nerve, optic disk, Crj CD(SD)IGS rat.

CD(SD)IGS-2002/2003 : 100-102

Table 1 Incidence of ophthalmoscopic lesions in Crj:CD(SD)IGS rats

	Weeks of age							
	5		9		18		31	
	Male	Female	Male	Female	Male	Female	Male	Female
No. of rats examined	80	80	60	60	40	40	20	20
Vitreous								
Hyaloid artery remnant, U	19	30	14	23	6	2	2	0
Hyaloid artery remnant, B	29	12	13	5	4	0	0	0
Hemorrhage, U	0	0	0	1	0	0	0	0
Fundus								
Retinal atrophy, U	0	0	1	1	2	2	1	1

U : unilateral. B : bilateral.

Table 2 Incidence of histopathological ocular lesions in Crj:CD(SD)IGS rats

	Weeks of age							
	6		10		19		32	
	Male	Female	Male	Female	Male	Female	Male	Female
No. of rats examined	20	20	20	20	20	20	20	20
Vitreous								
Hyaloid artery remnant, U	7	3	7	10	4	1	1	0
Hyaloid artery remnant, B	3	1	1	0	0	0	0	0
Hemorrhage, U	0	0	2	0	0	0	0	0
Retina								
Dysplasia, U	7	7	2	5	2	2	3	3
Dysplasia, B	0	1	0	0	0	1	0	0
Atrophy, U	0	0	0	0	1	1	1	1
Optic nerve								
Ectopic retina, U	8	1	7	3	2	1	1	9
Ectopic retina , B	0	0	0	2	1	2	0	3

U : unilateral. B : bilateral.

Note: This paper is duly permitted to reprint from original paper (Anim. Eye Res. 21 143-147, 2002).

Table 3 Incidences of the ocular lesions found in the eyes with ectopic retina in Crj:CD(SD)IGS rats

	Weeks of age								Total eyes
	6		10		19		32		with lesions (%)
	Male	Female	Male	Female	Male	Female	Male	Female	
No. of eyes with ectopic retina	8	1	7	7	4	5	1	15	48
Ophthalmoscopic/ histopathological lesions found in the eyes with ectopic retina									
Hyaloid artery remnant [a)]	7	0	5	3	1	4	0	6	26(54.2)
Retinal atrophy [a)]	0	0	0	0	0	0	0	1	1(2.1)
Retinal dysplasia [b)]	2	1	0	1	0	0	0	2	6(12.5)

a) : Ophthalmoscopic lesion.
b) : Histopathological lesion

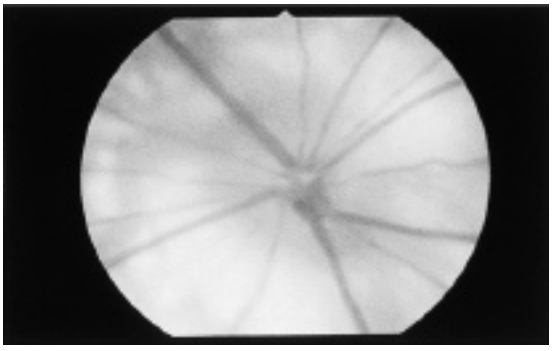

Fig. 1A fundus of the right eye of a 9-week-old male Crj:CD(SD)IGS rat. No abnormal finding is seen in the optic disk, whereas ectopic retina was histopathologically found in the beginning of the optic nerve.

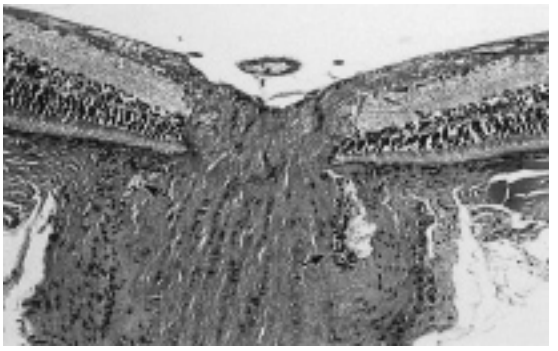

Fig. 2 Ectopic retina in the beginning of the optic nerve in the right eye of a 19-week-old female Crj:CD(SD)IGS rat. Ectopic retinal tissues are observed along the periphery on the optic nerve (arrows). The ectopic retinal tissues resemble photoreceptor cells. HE stain, ×130

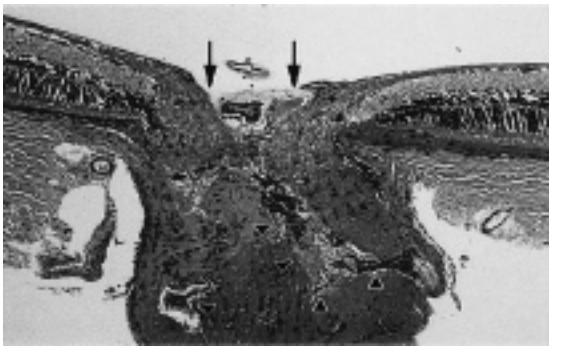

Fig. 3 Ectopic retina in the beginning of the optic nerve in the right eye of a 10-week-old female Crj:CD(SD)IGS rat. The optic disk is slightly excavated (arrows). Ectopic retinal tissues are involved within the obtic nerve (arrowheads). Note poorly developed and dislocated lamina cribrosa (*). HE stain, ×110

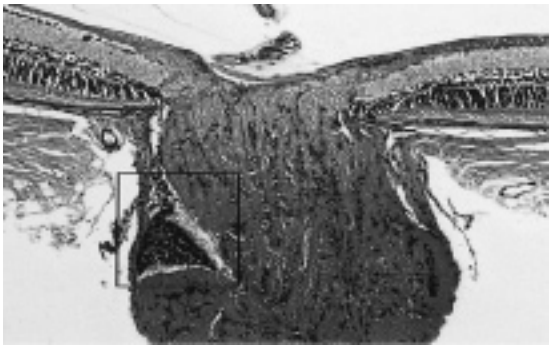

Fig. 4 Serial section of Fig.3. The ectopic retinal tissues closely resemble outer retinal layers (□). HE stain, ×110

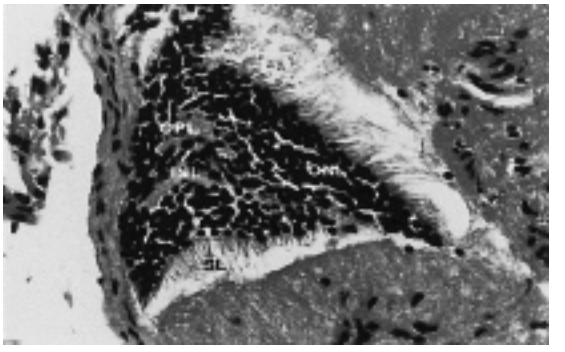

Fig. 5 High magnification of Fig.4. The ectopic retinal tissues consist of segments of the photoreceptor segment layer (SL), outer nuclear layer (ONL), outer plexiform layer (OPL) and inner nuclear layer (INL). HE stain, ×440

Age-Related Spontaneous Ocular Lesions in Crj:CD(SD) IGS Rats

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ABSTRACT. The eyes were periodically examined on 150 male and 150 female Crj:CD(SD)IGS rats for up to 1 year with a slit lump and an indirect ophthalmoscope in order to survey age-related spontaneous ocular lesions. The pupil was dilated instillation of 0.5% tropicamide to facilitate the examination. Most of the ocular lesions observed in this study were of the type commonly seen in the Sprague-Dawley strain rat. The incidences of corneal opacity and lenticular opacity were generally increased with increasing age. Anterior subcapsular lenticular opacity found in Crj:CD(SD)IGS rats was a new spontaneous ocular lesion which has not been reported in Japan.

CD(SD)IGS-2002/2003 : 103-104

Table 1. Incidence of Spontaneous ophthalmic lesions in Crj:CD(SD)IGS rats at pretest

	Female	Male
No. of rats	175	175
Anatomic site		
Normal	55 (31)	58 (33)
Cornea		
Opacity	85 (48)	87 (50)
Keratitis	1 (1)	0 (0)
Anterior chamber		
Hyphema	1 (1)	1 (1)
Iris		
Anterior synechia	0 (0)	2 (1)
Hemorrhage	4 (2)	3 (2)
PPM	0 (0)	2 (1)
Lens		
Anterior suture prominence	7 (4)	11 (6)
Opacity	17 (10)	12 (7)
Anterior capsular	0 (0)	2 (1)
Equatorial	0 (0)	0 (0)
Posterior	0 (0)	0 (0)
Nuclear	17 (10)	11 (6)
Vitreous		
Hyaloid artery remnant	31 (18)	41 (23)
Hemorrhage	2 (1)	1 (1)
PHPV	1 (1)	1 (1)
Fundus		
Chorioretinopathy	3 (2)	0 (0)
Retinal fold	1 (1)	0 (0)

Data are expressed as number and (%) of affected rats.
PPM: Persistent pupillary membrane
PHPV: Persistent hyperplastic primary vitreous

Note: This paper is duly permitted to reprint from original paper (Anim. Eye Res. 20, 21-25, 2001).

Table 2. Incidence of Spontaneous ophthalmic lesions in Crj:CD(SD)IGS female rats with age

Weeks of age	5	16	28	41	54
No. of rats	150	149	149	100	100
Anatomic site					
Normal	50 (33)	66 (44)	17 (11)	1 (1)	0 (0)
Cornea					
Opacity	73 (49)	66 (44)	122 (82)	89 (89)	90 (90)
Edema	0 (0)	0 (0)	0 (0)	0 (0)	1(1)
Neovascularization	0 (0)	0 (0)	0 (0)	0 (0)	1(1)
Iris					
Hemorrhage	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)
Anterior synechia	0 (0)	0 (0)	2 (1)	1 (1)	1 (1)
Lens					
Anterior suture prominence	7 (5)	0 (0)	0 (0)	0 (0)	0 (0)
Opacity	15 (10)	27 (18)	54 (36)	83 (83)	98 (98)
Anterior	0 (0)	2 (1)	8 (5)	9 (9)	19 (19)
Capsular	0	0	0	0	2
Subcapsular/central	0	0	8	7	5
Cortical	0	2	0	1	14
Nuclearcortical junction	0	0	0	1	0
Nuclear	15 (10)	26 (17)	48 (32)	82 (82)	97 (97)
Fundus					
Chorioretinopathy	0 (0)	5 (3)	5 (3)	3 (3)	5 (5)
Retinal fold	1 (1)	1 (1)	1 (1)	0 (0)	0 (0)
Retinal hyperreflectivity	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)

Data are expressed as number and (%) of affected rats.

Table 3. Incidence of Spontaneous ophthalmic lesions in Crj:CD(SD)IGS male rats with age

Weeks of age	5	16	28	41	54
No. of rats	150	150	150	99	96
Anatomic site					
Normal	52 (35)	31 (21)	14 (9)	2 (2)	1 (1)
Cornea					
Opacity	74 (49)	114 (72)	130 (87)	90 (91)	89 (93)
Iris					
Hemorrhage	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
PPM	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Lens					
Anterior suture prominence	9 (6)	0 (0)	0 (0)	0 (0)	1 (1)
Opacity	10 (7)	24 (16)	51 (34)	61 (62)	84 (88)
Anterior	0 (0)	0 (0)	3 (2)	11 (11)	14 (15)
Capsular	0	0	0	1	1
Subcapsular/central	0	0	3	8	9
Cortical	0	0	0	2	5
Equatorial	0 (0)	3 (2)	3 (2)	0 (0)	2 (2)
Cortical	0	1	1	0	0
Nuclearcortical junction	0	2	2	0	2
Posterior	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)
Cortical	0	1	0	0	1
Nuclear	10 (7)	20 (13)	46 (31)	55 (56)	80 (83)
Fundus					
Chorioretinopathy	0 (0)	1 (1)	2 (1)	1 (1)	1 (1)
Retinal opacity	0 (0)	0 (0)	0 (0)	1 (1)	2 (2)

Data are expressed as number and (%) of affected rats.
PPM: Persistent pupillary membrane

Comparative Study of Toxicity of 4-Nitrophenol and 2,4-Dinitrophenol in Newborn and Young Rats

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ABSTRACT. The toxicities of 4-nitrophenol and 2,4-dinitrophenol in newborn and young rats was examined and the susceptibility of newborn rats was analyzed in terms of presumed unequivocally toxic and no observed adverse effect levels (NOAELs). In the 18-day repeated dose newborn rat study, 4-nitrophenol was orally given from day 4 to day 21 after birth but did not induce any toxicity up to 160 mg/kg in the main study, although it induced death in one of six males at 160 mg/kg, and three of six males and one of six females at 230 mg/kg in a prior dose-finding study. In the 28-day repeated dose oral toxicity study starting at 6 weeks of age, 4-nitrophenol caused the death of most males and females at 1,000 mg/kg but was not toxic at 400 mg/kg except for male rat specific renal toxicity. As unequivocally toxic levels were considered to be 230 mg/kg/day in newborn rats and 600 to 800 mg/kg/day in young rats, and NOAELs were 110 mg/kg/day in newborn rats and 400 mg/kg/day in young rats, the susceptibility of the newborn to 4-nitrophenol appears to be 2.5 to 4 times higher than that of young animals. In the newborn rat study of 2,4-dinitrophenol, animals died at 30 mg/kg in the dose-finding study and significant lowering of body and organ weights was observed at 20 mg/kg in the main study. In the 28-day young rat study, clear toxic signs followed by death occurred at 80 mg/kg but there was no definitive toxicity at 20 mg/kg. As unequivocally toxic levels and NOAELs were considered to be 30 and 10 mg/kg/day in newborn rats and 80 and 20 mg/kg/day in young rats, respectively, the toxicity of 2,4-dinitrophenol in newborns again seems to be 2 to 3 times stronger than in young rats. Abnormalities of external development and reflex ontogeny in the newborn were not observed with either chemical. Based on these results, it can be concluded that the toxic response in newborn rats is at most 4 times higher than that in young rats, at least in the cases of 4-nitrophenol and 2,4-dinitrophenol. — Key words: toxic response in the newborn rat, 4-nitrophenol, 2,4-dinitrophenol

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INTRODUCTION

Endocrine disrupting chemicals have been demonstrated to induce adverse effects on reproductive organs with *in utero* [5, 10, 11, 12] or perinatal exposure [10, 21]. Furthermore, it was shown that postnatal exposure may also exert detrimental influence in both male and female animals. Nonylphenol reduced organ weights of testes, epididymides, seminal vesicles and prostates in male rats at day 31 when the animals were intraperitoneally injected at more than 0.08 mg/kg/day during days 1 to 15 after birth [17]. As a worst case, cryptorchidism was observed in 30 to 60 % of male infants at a dose of 8 mg/kg/day, leading to infertility. *p-tert*-Octylphenol also induced earlier vaginal opening, persistent estrus after sexual maturation and atrophy of ovaries in female rats on subcutaneous administration at 100 mg/kg every other day during days 1 to 15 after birth [14]. Diethylstilbestrol depressed spermatogenesis with severe testicular atrophy in hamsters at 90 days of age (adults) when a single injection of 100 μg/animal was given on the day of birth, but the testes had no abnormalities at 42 days of age (pubertal) [15].

Based on these available information, it is very important to examine the toxicity profiles and levels of chemicals in newborn to lactating animals and compare them with test results using young animals. As infants developing so quickly might be very strongly affected, safety of chemical exposure is particularly problematic. Unfortunately, there is no standard experimental protocol for infants and the above studies on estrogenic chemicals were conducted only for reproductive organs and functions. Therefore, a new protocol for infant repeated dose toxicity study, described in the "Materials and Methods", was established.

In an existing chemical testing program of Japan in 1999, 45 chemicals were selected for an ordinary 28-day repeated dose oral toxicity study because there was no sufficient oral toxicity information of these chemicals for hazard assessment. Among the chemicals, 14 phenolic derivatives were selected to compare toxicological profiles and toxicity levels in newborn and young animals using the same lot number of chemicals, same rat strain and a newly established protocol, considering the potential for estrogenic action in the early development period. In the present study, two nitrophenols, 4-nitrophenol (4NP) and 2,4-dinitrophenol (DNP) were chosen for evaluation as the first trial in our series. Both chemicals were listed in the OECD High Production Volume Chemical Table in 1999, meaning that they are produced at levels greater than 1,000 tonnes per year in at least one OECD member country. 4NP has found use in the production of dyes, pigments, medicines, photographic chemicals and pesticides, but there was insufficient information on oral toxicity except for an unpublished study described in Toxicological Profiles for nitrophenols: 2-nitrophenol and 4-nitrophenol [1]. DNP has been used in the production of black sulfur dye, herbicides, pesticides and wood preservatives. Furthermore, it had been used as diet pills once [2] and has been widely employed as an uncoupling agent for oxidative

Note: This paper is duly permitted to reprint from original paper (J. Toxicol. Sci., 26(5), 299-311, 2001).

phosphorylation especially for *in vitro* biochemical purposes. Regarding toxicity, various effects in animals and humans likely due to oxidative phosphorylation uncoupling effects, such as death, hyperthermia, and body weight loss are reported [2].

Our 28-day repeated dose oral toxicity studies of these chemicals in young rats have been already published [19, 20]. Under the same experimental condition, we have now examined the toxicity of these chemicals in newborn rats. Based on analysis of both studies including their dose-finding processes, appropriate unequivocally toxic levels, defined in this study as a dose inducing severe toxic signs including death or critical histological damages, and no observed adverse effect levels (NOAELs) for newborn and young rats were established, and differences in toxicity levels between them were estimated with reference to infant protection from chemical exposure.

MATERIALS AND METHODS

Materials

Sodium 4-nitrophenoxide dihydrate (CAS No. 42083-62-5, purity: 98.5%) for studies on 4-nitrophenol (4NP: CAS No. 100-02-7) was obtained from Mitsui Chemicals Inc. (Tokyo, Japan) and suspended in 0.5 w/v% carboxymethylcellulose-Na solution. 2,4-Dinitrophenol (CAS No. 51-28-5, purity: 85.2 %, 13.9 % water, 0.6 % 2,6-dinitrophenol and 0.3 % unknown compounds as impurities) was also obtained from Mitsui Chemicals Inc. (Tokyo, Japan) and suspended in 1 w/v% methylcellulose solution.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Atsugi, Japan) and maintained in an environmentally controlled room at 23±3℃ with a relative humidity of 50±20 %, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. In the 18-day study of newborn rats, 20 pregnant rats (gestation day 14～15) were purchased and normally delivered. Among all newborn separated from each dam at ages of days 3, 4 males and 4 females were randomly selected and assigned to 4 dose groups including controls. Twelve foster mothers suckled the 4 males and 4 females assigned to each group up to weaning on day 21 after birth (termination of dosing). After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks. In the 28-day study of young rats, 4～5 week-old male and female rats were obtained and used at ages of 5 ～ 6 weeks old after acclimation. All animals were allowed free access to sterilized basal diet (4NP: CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan, DNP: LABO MR stock, Nihon Nosan Kogyo Inc., Yokohama, Japan) and tap water.

Study Design (Time schedule described in Scheme 1)

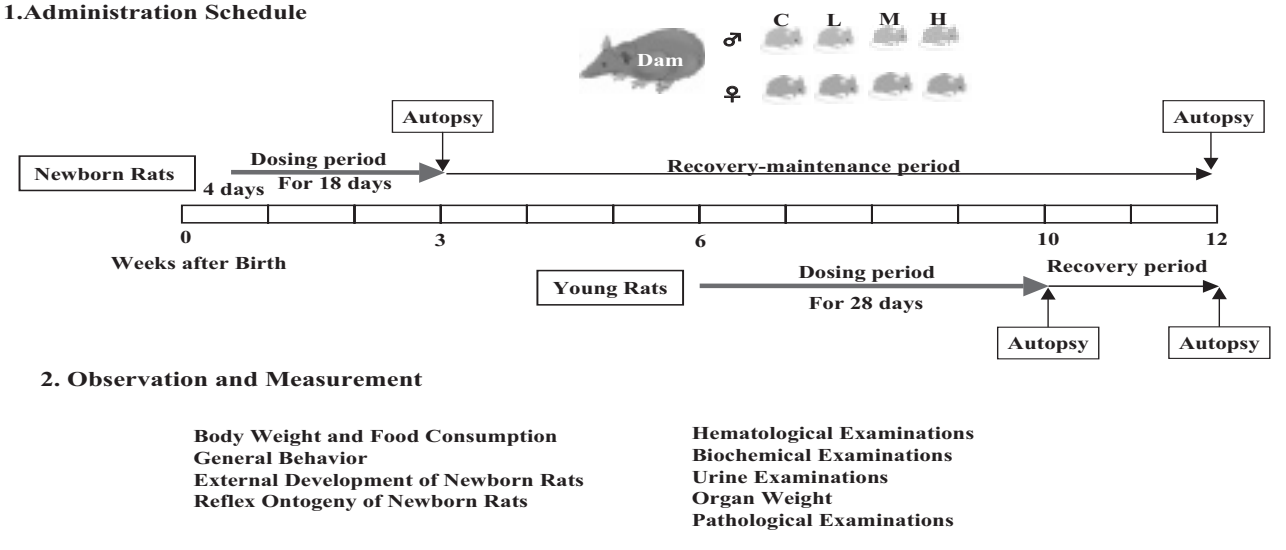
1. 18-Day Repeated Dose Study in Newborn Rats

1) Dose-finding study

Newborn rats (4NP: 6/sex/dose, DNP: 5/sex/dose) were administered 4NP at 0, 110, 160, 230 or 320 mg/kg/day, or DNP at 0, 3, 10 or 30 mg/kg/day by gastric intubation from days 4 to 21 after birth. Rats were examined for general behavior and body weights during the dosing period, and sacrificed at 22 days old after overnight starvation for examination of hematology, blood biochemistry, macro findings and organ weights.

2) Main study

Newborn rats were administered 4NP at 0, 80, 110 or 160 mg/kg/day, or DNP at 0, 3, 10 or 20 mg/kg/day by gastric intubation daily from days 4 to 21 after birth, based on results of the dose-finding study, and sacrificed after overnight starvation following the last treatment. Recovery-maintenance groups at the same dosages were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks old, the same age as at the end of the recovery period of the present 28-day study of



young rats. The number of animals at each sex/dose was 6 for both scheduled-sacrifice and recovery-maintenance.

General behavior was daily observed for newborn rats (separated from each foster mother) and foster mothers. Body weight and food consumption were measured more than once a week (food consumption, after weaning). At treatment day 17 or 18, pupillary reflex, corneal reflex, surface righting, mid-air righting and auricular reflexes were examined as parameters of reflex ontogeny. Furthermore, fur appearance, incisor eruption and eye opening were noted in lactating period as evidence of physical development, and preputial separation (4NP) or testes descent (DNP) and vaginal opening during the early recovery-maintenance period for assessment of sexual maturation. Color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment and volume of the urine were examined only at the end of recovery-maintenance period. For hematology and blood biochemistry, blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for scheduled-sacrifice and recovery-maintenance groups. One part of the blood was treated with EDTA-2K or 3.8% sodium citrate and examined for hematological parameters such as the red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet counts, reticulocyte ratio, differential leukocyte count, and blood clotting parameters such as prothrombin time and activated thromboplastin time. Plasma or serum obtained from another portion of the blood was analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase, γ-glutamyl transpeptidase, lactate dehydrogenase, alkaline phosphatase, calcium, inorganic phosphorus, sodium, potassium, chlorine). After macro findings for all organs of animals sacrificed under ether anesthesia, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes, epididymides, prostates (DNP), ovaries and uterus were removed and weighed. The trachea, pancreas, lymphatic glands, stomach, intestine, urinary bladder, spinal cord, sciatic nerve, seminal vesicles and prostates as well as the above organs were fixed with 10% buffered formalin-phosphate (Bouin's fixation for testes and epididymis) and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopic examination.

2. 28-Day Repeated Dose Study in Young Rats

1) Dose-finding study (14 days study)

Five to six-week old rats (4NP: 5/sex/dose, DNP: 4/sex/dose) were administered 4NP at 3, 12.5, 50 or 200 mg/kg/day, or DNP at 0, 0.6, 2, 6, 20 or 60 mg/kg/day by gastric intubation for 14 days. Rats were examined for general behavior, body weight and food consumption during dosing and sacrificed at day 15 after overnight starvation for examination of hematology, blood biochemistry, macro findings and organ weights (and urinalysis for DNP).

2) Main study

Five to six-week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment. Referring to the results of preliminary studies including the above dose-finding study, 5 doses including the control were established (4NP: 0, 60, 160, 400, 1,000 mg/kg/day, DNP: 0, 3, 10, 30, 80 mg/kg/day). Recovery groups (4NP: 0, 160, 400, 1,000 mg/kg/day, DNP: 0, 30, 80 mg/kg/day) were maintained for 2 weeks without chemical treatment and fully examined at 11 to 12 weeks of age. The number of animals for each sex/dose was 6 for both scheduled-sacrifice and recovery. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy finding, organ weights and histopathological finding in compliance with the Test Guideline of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Continuous data were analyzed by Bartlett's test [3] for homogeneity of distribution ($p < 0.01$ or 0.05). When homogeneity was recognized, Dunnett's test [7] or Scheffe's test [25] ($p < 0.01$ or 0.05) was conducted for group comparison (control vs treatment). If not homogenous, the data were analyzed using the Kruskal-Wallis ranking analysis [16] ($p < 0.05$) or the mean rank test of Dunnett type [13] ($p < 0.01$ or 0.05). Quantitative data for general appearance, functional tests and histopathology were analyzed by the Mann-Whitney's U test [18] or the Fisher's exact test [9] ($p < 0.005$ or 0.025).

RESULTS

4-Nitrophenol

1. 18-day study in newborn rats (including the dose-finding study)

In the dose-finding study, 5/6 males and 6/6 females at 320 mg/kg, 3/6 males and 1/6 females at 230 mg/kg, and 1/6 males at 160 mg/kg died during the early dosing period, and most of

Table 1: Clinical signs and mortality in the 18-day dose-finding study of 4-nitrophenol in newborn rats

	Dose (mg/kg)			
	110	160	230	320
Males				
No. of animals	6	6	6	6
-Convulsions	-	-	2	4
Death	-	1	3	5
Females				
No. of animals	6	6	6	6
-Convulsions	-	-	1	3
Death	-	-	1	6

-: No animals with the clinical sign

Convulsions were observed before death during early dosing period.

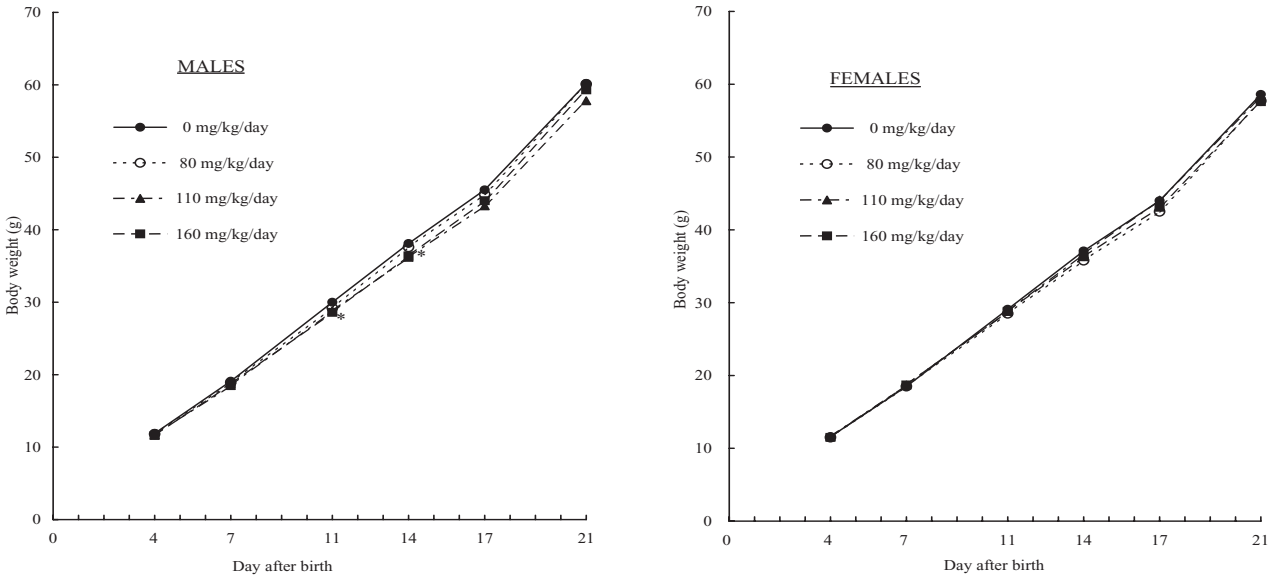


Fig. 1: Body weight change during the dosing period in the 18-day study of 4-nitrophenol in newborn rats
*: Significantly different from the controls ($p < 0.05$)

Table 2. Organ weights after 18-day repeat dosing of 4-nitrophenol in newborn rats (main study)

	Dose (mg/kg)			
	0	80	110	160
Males				
No. of animals	6	6	6	6
Body weight [a] (g)	57.2±1.3	55.5±2.9	56.3±1.8	54.9±4.9
Brain (g)	1.59±0.04[b]	1.55±0.04	1.56±0.06	1.55±0.09
	(2.78±0.11[c])	(2.80±0.15)	(2.78±0.13)	(2.84±0.20)
Pituitary (mg)	2.3±0.2	2.5±0.1	2.4±0.4	2.4±0.1
	(4.1±0.4)	(4.5±0.2)	(4.2±0.7)	(4.4±0.4)
Liver (g)	1.74±0.07	1.71±0.16	1.71±0.09	1.77±0.20
	(3.04±0.08)	(3.07±0.14)	(3.04±0.09)	(3.22±0.12*)
Kidney (g)	0.66±0.03	0.64±0.07	0.64±0.03	0.62±0.06
	(1.16±0.06)	(1.14±0.09)	(1.14±0.07)	(1.13±0.09)
Testis (g)	0.29±0.02	0.28±0.02	0.27±0.02	0.26±0.02
	(0.50±0.04)	(0.51±0.03)	(0.48±0.03)	(0.48±0.04)
Epididymis (mg)	40.2±4.4	40.7±3.6	37.5±3.3	39.5±2.1
	(70.5±8.1)	(73.0±5.5)	(66.5±4.9)	(72.2±2.7)
Females				
No. of animals	6	6	6	6
Body weight [a] (g)	54.7±2.9	54.2±2.1	54.9±1.3	54.1±1.9
Brain (g)	1.50±0.03	1.54±0.05	1.50±0.04	1.48±0.08
	(2.74±0.14)	(2.85±0.17)	(2.73±0.09)	(2.73±0.18)
Pituitary (mg)	2.3±0.3	2.5±0.2	2.5±0.2	2.5±0.1
	(4.3±0.6)	(4.5±0.3)	(4.6±0.3)	(4.7±0.3)
Liver (g)	1.64±0.12	1.62±0.06	1.63±0.08	1.68±0.07
	(2.99±0.09)	(3.00±0.08)	(2.97±0.14)	(3.10±0.10)
Kidney (g)	0.63±0.06	0.65±0.05	0.62±0.03	0.60±0.03
	(1.16±0.08)	(1.20±0.05)	(1.13±0.07)	(1.12±0.06)
Ovary (mg)	11.8±1.8	10.7±0.7	10.7±2.2	12.0±2.4
	(21.6±3.6)	(19.8±1.6)	(19.5±3.9)	(22.1±4.0)
Uterus (mg)	47.0±11.0	42.7±7.1	44.0±6.2	48.5±6.9
	(85.8±19.8)	(78.7±12.9)	(80.3±11.7)	(89.7±11.9)

[a]: Body weight after overnight starvation following the last dosing, [b]: Absolute weight, [c]: Relative weight (g or mg/100 g body weight)
*: Significantly different from the control group ($p<0.05$)

them exhibited convulsions before death (Table 1). However, no toxic signs or deaths were observed in the main study, even at the highest dose of 160 mg/kg (data not shown). Body weights demonstrated a few transient changes in the dosing period (Fig. 1) and tendency for increase in the recovery-maintenance period (data not shown) of males in the main study, not considered due to the chemical treatment. No definitive changes in abdominal fur appearance, incisor eruption, eye opening and preputial separation or vaginal opening as well as reflex ontogeny parameters were detected in any of the dose groups (data not shown). There were also no significant changes in absolute and relative organ weights of the scheduled-sacrifice and recovery-maintenance groups, except for slight increase in relative liver weights in 160 mg/kg males of the scheduled-sacrifice group (Major results are shown in Table 2). No other chemical related changes were observed for hematology, blood biochemistry, histopathology or urinalysis.

The unequivocally toxic level in newborn rats was concluded to be 230 mg/kg/day because some animals died at this dose in the dose-finding study, but only one of 36 animals in the dose-finding and main studies died at 160 mg/kg. The NOAEL was concluded to be 110 mg/kg/day, at which there was no evidence of toxicity in the main study.

2. 28-day study in young rats (including the dose-finding study)

4NP did not exert any significant toxicity up to 200 mg/kg in the dose-finding study, the doses being set from an LD_{50} value of approximate 500 mg/kg [19]. At 1,000 mg/kg in the main study, more than half animals died after oligopnea and adoption of a prone/lateral position on the first dosing day, some of them also showing tonic convulsions (Table 3). As 10 of 12 males and females finally died, the surviving animals at this dose were sacrificed at the end of the administration period. The only significant pathological change was a higher incidence of eosinophilic bodies in proximal tubular cells of the kidneys in 400 mg/kg and 1,000 mg/kg males, as shown in Table 4. No other chemical related changes in body weight, food consumption, organ weight, hematology, blood biochemistry and urinalysis were observed in any of the groups including the recovery groups.

Table 3. Clinical signs and mortality in the 28-day study of 4-nitrophenol in young rats

	Dose (mg/kg)			
	60	160	400	1,000
Males				
No. of animals	12	12	12	12
-Decrease in locomotor activity	-	-	-	12
-Oligopnea	-	-	-	12
-Prone/Lateral position	-	-	-	10
-Tonic convulsions	-	-	-	3
Deaths	-	-	-	10
Females				
No. of animals	12	12	12	12
-Decrease in locomotor activity	-	-	-	12
-Oligopnea	-	-	-	12
-Prone/Lateral position	-	-	-	10
-Tonic convulsions	-	-	-	4
Deaths	-	-	-	10

-: No animals with the clinical sign

Almost all of these clinical signs were observed just after the first dosing and disappeared within 2 hours. 7 male and 6 female rats died on the first dosing day and 3 males and 4 females died sporadically during the dosing period.

Table 4. Histological findings in the kidneys of male rats after the dosing period in the 28-day study of 4-nitrophenol in young rats

		Dose (mg/kg)				
	Grade	0	60	160	400	1,000
No. of animals		6	6	6	6	2[a)]
Kidneys						
-eosinophilic bodies	-	3	4	5	0	0
in proximal tubular cells	+	3	1	1	3	0
	++	0	1	0	3	2
Total observed number		3	2	1	6*	2

-: No remarkable changes, +: Slight, ++: Mild

[a)]: Only two of 12 male rats survived until the end of the dosing period.

*: Significantly different from the control group ($p<0.05$)

As the male rat specific change in kidney is considered to be due to α_{2U}-globulin complex formation and not relevant to humans, the NOAEL for repeated dose toxicity in young rats was concluded to be 400 mg/kg/day for both sexes. It is also speculated that the appropriate unequivocally toxic level might be in the range from 600 to 800 mg/kg/day because most animals died at 1,000 mg/kg.

2,4-Dinitrophenol

1. 18-day study in newborn rats (including the dose-finding study)

No clinical signs or deaths were encountered in the main study, but decrease in locomotor activity and panting were observed in both sexes, and 4/5 males and 1/5 females died at 30 mg/kg in the dose-finding study (Table 5). The body weights at 20 mg/kg were significantly below control values from dosing day 7 in males and dosing day 10 in females in the scheduled-sacrifice group (Fig. 2). There was also statistically significant lowering of body weights in the 20 mg/kg males for the first quarter of the recovery-maintenance period, but not in females (data not shown). No definitive changes in abdominal fur appearance, incisor eruption, eye opening and testes descent or vaginal opening as well as reflex ontogeny parameters were detected in any dose groups (data not shown). There were significant changes in several absolute and relative organ weights in scheduled-sacrifice animals of both sexes at 20 mg/kg (Table 6), but these were no longer evident after the recovery-maintenance period. The only significant change at 10 mg/kg was decreased absolute weights of testes. No chemical related histopathological changes were noted in either scheduled-sacrifice and recovery-maintenance groups. Significant increase in RBC was observed in females receiving 20 mg/kg after the treatment but not after the recovery-maintenance period in the

Table 5. Clinical signs and mortality in the repeated dose studies of 2,4-dinitrophenol in newborn rats

	18-day study (dose-finding study)			18-day study		
Dose (mg/kg)	3	10	30	3	10	20
Males						
No. of animals	5	5	5	12	12	12
-Decrease in locomotor activity	-	-	3	-	-	-
-Panting	-	-	2	-	-	-
-Prone position	-	-	-	-	-	-
-Convulsions	-	-	1	-	-	-
Deaths	-	-	4	-	-	-
Females						
No. of animals	5	5	5	12	12	12
-Decrease in locomotor activity	-	-	2	-	-	-
-Panting	-	-	1	-	-	-
-Prone position	-	-	1	-	-	-
-Convulsions	-	-	-	-	-	-
Deaths	-	-	1	-	-	-

-: No animals with the clinical sign

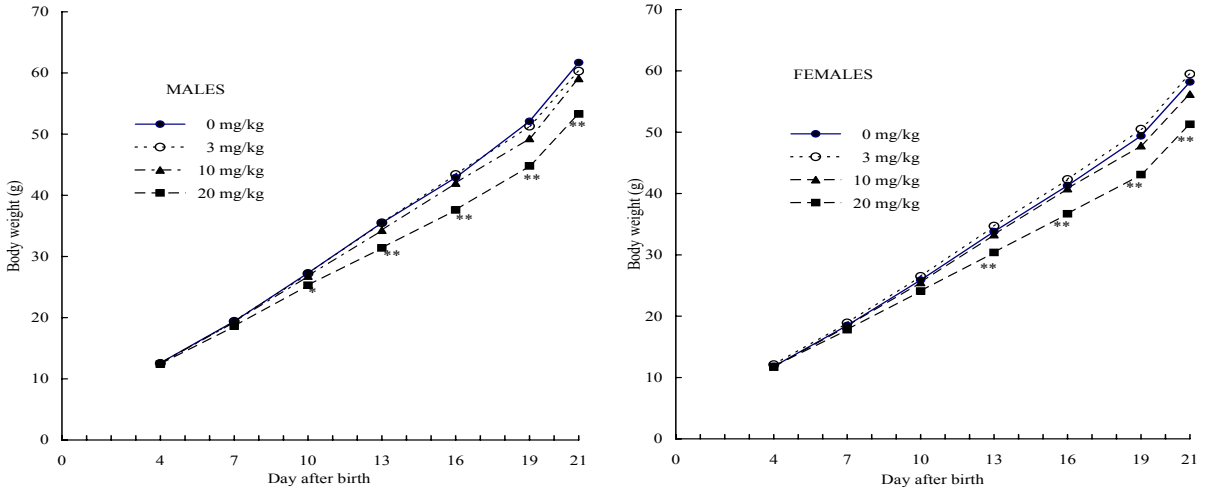


Fig 2. Body weight change during the dosing period in the 18-day study of 2,4-dinitrophenol in newborn rats
*, **: Significantly different from the controls ($p < 0.05$, $p < 0.01$)

main study and in RBC, Hb and Ht in both sexes at 30 mg/kg in the dose-finding study (data not shown). Although increases in serum GOT in males and total bilirubin in females were detected at 10 and 20 mg/kg after treatment, those were not considered to be chemical-induced because they were very slight and there was no dose-relationship (data not shown).

The unequivocally toxic level in newborn rats was concluded to be 30 mg/kg/day, as high lethality at this dose was shown in the dose-finding study but only lowered body and organ weights were observed at 20 mg/kg. The NOAEL is considered to be 10 mg/kg/day, at which only the lowering of absolute testis weights was observed.

2. 28-day study in young rats (including the dose-finding study)

Clear toxic signs, such as decrease in locomotor activity, prone position, ptosis, panting, crawling position and salivation, were observed repeatedly during the dosing period at 80 mg/kg in both sexes, and 2 males and 6 females died, as shown in Table 7. However, decrease in locomotor activity and salivation in the 30 mg/kg group were mostly observed only after the first dosing. The relative liver weights were increased in both sexes of the 80 mg/kg scheduled-sacrifice group and this persisted through the recovery period (data not shown). Relative organ weights for brain, kidneys and testes were increased only in 80 mg/kg males. On histopathological examination (Table 8),

Table 6: Organ weights after the dosing period in the 18-day study of 2,4-dinitrophenol in newborn rats

	Dose (mg/kg)			
	0	3	10	20
Males				
No. of animals	6	6	6	6
Body weight [a] (g)	56.6±3.6	54.2±3.2	53.0±3.7	48.6±5.5**
Brain (g)	1.50±0.03[b] (2.66±0.18[c])	1.52±0.06 (2.82±0.14)	1.54±0.06 (2.93±0.25)	1.47±0.07 (3.06±0.33)
Pituitary (mg)	3.1±0.2 (5.5±0.2)	3.1±0.1 (5.6±0.2)	3.0±0.3 (5.6±0.3)	2.6±0.3** (5.5±0.5)
Liver (g)	1.85±0.16 (3.25±0.13)	1.71±0.10 (3.16±0.07)	1.73±0.17 (3.26±0.14)	1.61±0.21 (3.30±0.11)
Kidney (g)	0.66±0.06 (1.16±0.08)	0.66±0.05 (1.23±0.08)	0.66±0.05 (1.24±0.08)	0.61±0.08 (1.26±0.07)
Testis (g)	0.31±0.03 (0.05±0.04)	0.29±0.01 (0.53±0.03)	0.27±0.01* (0.51±0.04)	0.26±0.04* (0.54±0.05)
Prostate (mg)	82.6±12.2 (146.1±20.2)	79.9±13.7 (148.3±28.7)	81.4±7.7 (154.3±19.1)	76.4±13.6 (158.3±29.5)
Epididymis (mg)	50.8±5.1 (89.7±6.0)	55.5±6.4 (103.1±15.9)	47.0±2.9 (89.3±11.2)	48.2±4.6 (99.6±9.0)
Females				
No. of animals	6	6	6	6
Body weight [a] (g)	52.7±2.8	53.5±3.8	50.1±3.9	45.5±3.4**
Brain (g)	1.47±0.05 (2.80±0.20)	1.47±0.05 (2.75±0.17)	1.50±0.04 (3.00±0.22)	1.43±0.04 (3.14±0.23*)
Pituitary (mg)	3.1±0.1 (5.9±0.4)	3.1±0.3 (5.9±0.4)	3.1±0.3 (6.2±0.5)	2.9±0.2 (6.4±0.4)
Liver (g)	1.69±0.12 (3.21±0.21)	1.74±0.15 (3.24±0.13)	1.61±0.19 (3.20±0.16)	1.53±0.11 (3.36±0.16)
Kidney (g)	0.63±0.05 (1.19±0.05)	0.68±0.05 (1.26±0.06)	0.63±0.06 (1.26±0.05)	0.60±0.02 (1.32±0.08**)
Ovary (mg)	14.1±2.7 (27.0±5.8)	14.4±1.8 (26.9±3.3)	14.4±2.0 (28.7±3.4)	12.2±2.9 (26.9±6.3)
Uterus (mg)	48.1±9.0 (91.2±16.0)	47.8±9.0 (89.8±26.6)	36.0±4.7 (71.9±7.2)	44.6±4.9 (98.9±16.2)

[a]: Body weight after overnight starvation following the last dosing, [b]: Absolute weight, [c]: Relative weight (g or mg/100 g body weight)
*, **: Significantly different from the control group ($p<0.05$, $p<0.01$)

Table 7: Clinical signs and mortality in repeated dose studies of 2,4-dinitrophenol in young rats

	14-day study (dose-finding study)					28-day study			
Dose (mg/kg)	0.6	2	6	20	60	3	10	30	80
Males									
No. of animals	4	4	4	4	4	6	6	12	12
-Decrease in locomotor activity	-	-	-	-	-	-	-	6[b]	12[c]
-Prone position	-	-	-	-	2[a]	-	-	-	12[c]
-Ptosis	-	-	-	-	-	-	-	-	12[c]
-Panting	-	-	-	-	-	-	-	-	12[c]
-Crawling position	-	-	-	-	-	-	-	-	12[c]
-Salivation	-	-	-	-	-	-	-	2[b]	12[c]
-Tonic convulsions	-	-	-	-	-	-	-	-	2
-Rigidity	-	-	-	-	-	-	-	-	2
Death	-	-	-	-	-	-	-	-	2
Females									
No. of animals	4	4	4	4	4	6	6	12	12
-Decrease in locomotor activity	-	-	-	-	-	-	-	5[b]	12[c]
-Prone position	-	-	-	-	1[a]	-	-	-	12[c]
-Ptosis	-	-	-	-	-	-	-	-	12[c]
-Panting	-	-	-	-	-	-	-	-	12[c]
-Crawling position	-	-	-	-	-	-	-	-	12[c]
-Salivation	-	-	-	-	-	-	-	1[b]	12[c]
-Tonic convulsions	-	-	-	-	-	-	-	-	4
-Rigidity	-	-	-	-	-	-	-	-	5
Death	-	-	-	-	-	-	-	-	6

-: No animals with the clinical sign
[a]: Observed during the early dosing period, [b]: Mostly observed only on the first dosing day, [c]: Observed repeatedly during the dosing period

Table 8. Histological findings in the 28-day study of 2,4-dinitrophenol in young rats

		Scheduled-sacrificed group					Recovery group		
Dose (mg/kg)	Grade	0	3	10	30	80	0	30	80
Males									
No. of animals		6	6	6	6	4[a)]	6	6	6
Kidneys									
- Mineralization,	-	6	6	6	6	1	6	6	4
corticomedullary junction	+	0	0	0	0	1	0	0	2
	++	0	0	0	0	2	0	0	0
Total observed number		0	0	0	0	3*	0	0	2
Females									
No. of animals		6	6	6	6	3[b)]	6	6	3[b)]
Kidneys									
- Mineralization,	-	6	6	6	6	1	6	6	1
corticomedullary junction	+	0	0	0	0	2	0	0	2
	++	0	0	0	0	0	0	0	0
Total observed number		0	0	0	0	2	0	0	2

-: No remarkable changes, +: Slight, ++: Mild

[a)]: 2/6 male rats in scheduled-sacrificed group died during dosing the period, [b)]: Each 3/6 female rats in scheduled-sacrificed group and recovery group died during the dosing period

*: Significantly different from the control group ($p<0.05$)

Table 9. Comparison of unequivocally toxic levels and NOAELs in newborn and young rats

	Level (mg/kg/day)	Ratio (young/newborn)
4-Nitrophenol		
Unequivocally toxic level (newborn)	230	2.6 -3.5
Unequivocally toxic level (young)	600 - 800	
NOAEL (newborn)	110	3.6
NOAEL (young)	400	
2,4-Dinitrophenol		
Unequivocally toxic level (newborn)	30	2.7
Unequivocally toxic level (young)	80	
NOAEL (newborn)	10	2.0
NOAEL (young)	20	

mineralization of the corticomedullary junction in kidneys was observed in both sexes at 80 mg/kg in the scheduled-sacrifice and recovery groups, but the change was only statistically significant in males of the scheduled-sacrifice group. On hematological examination, increase in Hb and Ht during the treatment, and decrease in RBC, Hb and Ht in the recovery period were observed, limited to 80 mg/kg males (data not shown). Although blood chlorine levels were slightly decreased in 30 and 80 mg/kg males and total bilirubin was slightly increased in females receiving 10 mg/kg and more (data not shown), no changes in histopathology or organ weights were observed at 30 mg/kg or lower. In the dose-finding study, no significant changes were apparent except for a prone position of a few animals given 60 mg/kg during the early dosing period (Table 7).

As an unequivocally toxic level, 80 mg/kg/day is appropriate, based on the clear toxic signs with deaths at 80 mg/kg and the slight effects at 60 mg/kg in the dose-finding study. The NOAEL is presumed to be 20 mg/kg/day from the dose-finding study because adverse effects at 30 mg/kg were mostly observed only after the first dosing in the main study even though the exposure period of the dose-finding study was shorter than the main study.

DISCUSSION

Several investigations of chemical exposure of perinatal animals through their dams have been reported, but only a few studies have been conducted with direct administration to newborn animals. Unfortunately, detailed analyses of general toxicity have not hitherto been performed in the latter cases. During the newborn period, the infants may orally intake chemicals not only through their mothers' milk but also by mouthing of pacifiers, plastic goods, etc. or eating baby foods containing pollutant chemicals. The present study was therefore conducted to allow comparison of the results for newborn and young rats under the same experimental conditions. For toxicity/safety regulation in general, an NOAEL must first be

established, then the acceptable daily intake (ADI) or tolerable daily intake (TDI) is derived using uncertainty factors. However, the NOAEL does not indicate the precise toxic potential because it is directly derived from doses used in toxicity studies. Therefore, we estimated appropriate NOAELs based on both the dose-finding study and main study, and compared these values. Secondly, we estimated appropriate unequivocally toxic levels from both animal studies and compared them in addition. In this study, these were established as doses inducing severe toxic signs, including death or critical histopathological damage.

Symptoms of the toxicity of 4NP generally include hyperthermia, respiratory depression, methemoglobinemia, CNS depression, and central and peripheral vagal stimulation [4]. As for repeated dose oral toxicity, only a 13 week rat gavage study conducted in Hazleton (1989), not yet published, is described in Toxicological Profiles [1]. However, early death at 40 mg/kg in the Hazleton study was not confirmed by our young rat study (no lethality at 400 mg/kg), and we are not able to speculate on the reason for this big dose difference in lethality because of the lack of detailed information about the Hazleton study. ATSDR [1] also described no severe tissue damage by 4NP in most studies with any exposure route, the results being consistent with our investigation, but the reports did not include newborn toxicity data. Therefore the present study is the first to look into newborn toxicity of 4NP. The unequivocally toxic levels were estimated to be 230 mg/kg/day in newborn rats and 600 to 800 mg/kg/day in young rats, and NOAELs to be 110 mg/kg/day in newborn rats and 400 mg/kg/day in young rats. Based on these estimation, newborn rats are 2.5 to 4 times more susceptible to this chemical than young rats, as shown in Table 9.

Since DNP was used extensively in diet pills during the 1930s, various effects in humans likely due to oxidative phosphorylation uncoupling effects, such as death, hyperthermia, excessive perspiration, elevated respiration and pulse rate and body weight loss are described in Toxicological Profiles [2]. Many animal studies with oral administration were also conducted but mostly in the 1930s and 1940s, and no toxicity study in infants has been reported. The present study provides the newest reliable data, with toxicity level and toxic profile in line with those of the old studies. The unequivocally toxic effect levels were estimated to be 30 and 80 mg/kg/day, and NOAELs to be 10 and 20 mg/kg/day in newborn and young rats, respectively. The differences in sensitivity between newborn and young rats range from 2 to 3 times (Table 9).

As neither chemical induced clear histopathological changes or would be expected to accumulate in the body, based on metabolic experiments [23, 24, 27], any toxicity, including mortality, must be the result of pharmacological actions. Therefore, the toxicity differences between newborn and young rats would be due to variation in metabolic rate including elimination and toxicodynamics. However, there is no supporting information for this speculation in the literature.

This is the first systematic study to look into the direct effects of chemicals in newborn to weaning animals and also the delayed responses without further treatment up to young adult ages. Neither chemical exerted any developmental toxicity in the newborn in terms of external development and reflex ontogeny. This result clearly indicates that there is no infant specific toxicity with the two chemicals.

In the pesticide program by US EPA [28], the counsel group recommended use of an additional uncertainty factor of 10 for the ADI for infant safety in the absence of specific data for developmental toxicity, following the recommendations of the NRC [22], Schilter *et al*. [26] and Dourson *et al*. [6]. For example, an additional uncertainty factor of 10 was applied to Thiamethoxam, on condition that the additional uncertainty factor would be removed when infant safety was confirmed [8]. The results of the present studies seem to support the conclusion that this additional uncertainty factor is appropriate for assessing infant safety.

In Japan, more than 300 toxicity studies, including 28-day repeated dose toxicity studies in young rats and OECD combined repeated dose and reproductive/developmental toxicity screening tests [OECD TG 422] in young adult rats, have been conducted in the existing chemical testing program since 1980. Approximately 500 of the 28-day repeated dose studies in young animals for new industrial chemicals have also been conducted as screening tests in the Japanese Chemical Control Act and the results were submitted to the Japanese Government from the chemical industry. These abundant repeated dose toxicity data for young and young adult animals will become more valuable when the appropriate safety factors for infants are established for general application with full analysis of our series of studies.

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Comparative Toxicity Study of 3-Aminophenol in Newborn and Young Rats

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ABSTRACT. Repeated dose toxicity of 3-aminophenol was examined on oral administration to newborn and young rats, and susceptibility was analyzed in terms of the no observed adverse effect level (NOAEL) and the unequivocally toxic level. In the 18-day newborn rat study, starting at day 4 after birth, tremors and depression of body weight gain were observed, as well as hypertrophy of thyroid follicular epithelial cells and increases of relative liver and kidney weights at 240 mg/kg. Increase of relative liver weights in males and decrease of blood sugar in females without any histopathological changes at 80 mg/kg were not considered to be adverse effects. No chemical-related changes were observed at 24 mg/kg. Abnormalities of external development and reflex ontogeny in the newborn were not observed. In the 28-day study, starting at 5 weeks of age, depression of body weight gain, tremors, anemia, and liver, kidney and thyroid toxicity were observed at 720 mg/kg. Although slight pigmentation in the renal proximal tubular epithelium was observed in females at 240 mg/kg, this was not considered to be an adverse effect because of the lack of changes in related toxicological parameters. It was concluded that the NOAEL is 80 mg/kg/day in newborn rats and 240 mg/kg/day in young rats, with unequivocally toxic levels of 240 mg/kg/day and 720 mg/kg/day, respectively. Based on these two endpoints, the susceptibility of newborn rats to the chemical was approx. 3 times higher than that of young rats, consistent with our previous results for 4-nitrophenol and 2,4-dinitrophenol. — Key words: toxicity in newborn rats, 3-aminophenol

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INTRODUCTION

The toxicity of chemicals is generally evaluated on the basis of results of repeated dose, developmental and reproductive toxicity studies in adult animals. While unique and/or higher toxicity of some chemicals in infants has recently been reported [12, 13, 17], there is no systematic toxicity study protocol to examine direct toxic effects of chemicals in newborn animals. Although the results of one- and/or two-generation studies might reflect the toxicity of chemicals in infants exposed from the neonatal period to weaning, the exposure occurs only via their maternal milk. As there is a significant possibility that infants are exposed to chemicals directly via mouthing toys and household materials, or having chemical-contaminated milk and baby food, and so on, we should examine toxicity levels and profiles of chemicals in infant animals by direct exposure.

For this purpose, we have established a protocol for infant repeated dose toxicity studies to allow comparison of results with these of the routine repeated dose toxicity study in young animals [14]. We have already examined the toxicity of 14 phenolic chemicals in newborn rats using this protocol and reported comparative toxicity evaluation of two nitrophenols, 4-nitrophenol and 2,4-dinitrophenol [14, 15, 23, 25]. Comparison of toxicity levels was especially focused on unequivocally toxic levels, defined as doses inducing severe toxic signs including death or critical histological damage, as well as no observed adverse effect levels (NOAELs), in both newborn and young rats. Integral analysis, including dose-finding studies, demonstrated the toxic susceptibility of newborn rats to these chemicals to be 2 to 4 times higher than that of young rats.

In this study, 3-aminophenol was chosen for analysis. This chemical has been widely used as a raw material for generation of heat-sensitive dyes and pesticides, with a production capacity of 1,500 tons in Japan in 2000, as stated in the Chemical Products' Handbook [4]. An earlier feeding study using weanling female Sprague-Dawley rats, exposed for 90 days followed by mating without exposure and the re-exposure during their gestation period, demonstrated hemolytic anemia and morphological change in the thyroid glands at the highest dose, 1% in the diet, but no teratogenic or embryofetal toxicity was observed [21]. Recently, a 28-day repeated dose study in young rats was conducted as part of the Japanese existing chemical safety program [19]. As the latter was evaluated for no observed effect level (NOEL) and toxicity profile, we re-evaluated the same study for the NOAEL and the unequivocally toxic level to compare with those from a newborn study using the same strain of rats.

MATERIALS AND METHODS

Materials

3-Aminophenol (CAS No. 591-27-5, 99.7% purity) was obtained from Mitsui Chemicals Inc. (Tokyo, Japan) and suspended in 1.0 w/v% carboxymethylcellulose-Na (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) solution. The suspension was prepared at least once a week and the stability of 3-aminophenol under these conditions was confirmed to be at least 8 days. All other reagents used in this study were specific purity grade.

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Animals

Sprague-Dawley SPF rats [Crj: CD(SD)IGS] were purchased from Charles River Japan Inc. (Atsugi, Japan) and maintained in an environmentally controlled room at 19～24℃ with a relative humidity of 35～69%, a ventilation rate of 10～15 times per hour, and a 12:12 hr light/dark cycle. For the 18-day study of newborn rats, 20 pregnant rats (gestation day 14) were purchased and normally delivered. Among all newborn separated from each dam at the age of 3 days, 4 males and 4 females were randomly selected and assigned to 4 dose groups, including controls. Twelve foster mothers suckled the 4 males and 4 females assigned to each group up to weaning on day 21 after birth (termination of dosing). After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks. In the 28-day study of young rats, 4 week-old male and female rats were obtained and used at ages of 5 weeks old after a one-week acclimation. All animals were allowed free access to sterilized basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Study Design (Time schedule the same as reported previously [14])

1. 18-Day Repeated Dose Study in Newborn Rats

1) Dose-finding study

Newborn rats (6/sex/dose) were administered the test substance at 0, 30, 80 or 240 mg/kg/day by gastric intubation from days 4 to 21 after birth. Rats were examined for general behavior, body weight and physical development (abdominal fur appearance, incisor eruption, eye opening) during the dosing period, and sacrificed at 22 days of age after overnight starvation for assessment of hematology (red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), white blood cell count, platelet count), blood biochemistry (total protein, glucose, total cholesterol, urea nitrogen (BUN), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase), macro findings and organ weights.

2) Main study

Newborn rats were administered the test substance at 0, 24, 80 or 240 mg/kg/day by gastric intubation daily from days 4 to 21 after birth, based on results of the dose-finding study, and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery-maintenance groups at the same dosages were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age. The number of animals at each sex/dose was 6 for both the scheduled-sacrifice and recovery-maintenance groups

General behavior was observed daily for newborn rats and foster mothers. Body weights were measured twice a week during the dosing period and once a week during the recovery-maintenance period. For food consumption, cumulative values for 2-4 days were measured during the recovery-maintenance period. Abdominal fur appearance was observed at ages of 7 and 11 days, incisor eruption at 11 and 14 days, eye opening at 14 and 17 days, preputial separation at 42 and 49 days, and vaginal opening at 35 and 42 days. At treatment day 18, the pupillary reflex, corneal reflex, surface righting, mid-air righting and auricular reflexes (Preyer reflexes) were examined as parameters of reflex ontogeny. Color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, osmotic pressure and urine volume were examined in the last week of the recovery-maintenance period. For hematology and blood biochemistry, blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for both the scheduled-sacrifice and recovery-maintenance groups. One part of the blood was examined for hematological parameters such as RBC, Hb, Ht, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte ratio and differential leukocyte count. In addition, blood clotting parameters such as prothrombin time and activated thromboplastin time were measured for the recovery-maintenance group. Plasma obtained from the other portion of the blood was analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, BUN, creatinine, GOT, GPT, γ-glutamyl transpeptidase, lactate dehydrogenase, alkaline phosphatase, phospholipid, calcium, inorganic phosphorus, sodium, potassium, chlorine). After recording of macro findings for all organs of animals sacrificed under ether anesthesia, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes, epididymides, ovaries and uterus were removed and weighed. The trachea, pancreas, lymph node, esophagus, submandibular gland, sublingual gland, stomach, intestine, urinary bladder, eyeballs, spinal cord, sciatic nerve, seminal vesicles, prostates, vagina, mammary gland, bone and bone marrow, skeletal muscle, skin and macroscopic abnormal regions as well as the above organs were fixed with 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymis, and following 3% glutaraldehyde/2.5% formalin-fixation for eyeballs), and paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination.

2. 28-Day Repeated Dose Study in Young Rats

1) Dose-finding study (14-day study)

Five-week-old rats (5/sex/dose) were administered the test substance at 0, 80, 200 or 500 mg/kg/day by gastric intubation for 14 days. Rats were examined for general behavior, body weight and food consumption during dosing and sacrificed at day 15 after overnight starvation for assessment of hematology (RBC, Hb, Ht, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count), blood biochemistry (total protein, glucose, total cholesterol, triglyceride, BUN, creatinine, GOT, GPT, alkaline phosphatase, sodium, potassium, chlorine), macro findings and organ weights.

2) Main study

Five-week-old rats were given the test substance by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Referring to the results of the above dose-finding study, 4 doses were established (0, 80, 240 or 720 mg/kg/day).

Recovery groups (0 and 720 mg/kg/day) were maintained without chemical treatment for 2 weeks after the last treatment and fully examined at 11 weeks of age. The number of animals for each sex/dose was 7 for both the scheduled-sacrifice and recovery groups. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology, blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Continuous data were analyzed by the Bartlett's test [2] for homogeneity of distribution. When homogeneity was recognized, Dunnett's test [5] ($p < 0.01$ or 0.05) was conducted for comparison between control and individual treatment groups (after one-way layout analysis of variance [27] for the 28-day young rat study). If not homogenous, the data were analyzed using the Kruskal-Wallis test [16] following the Mann-Whitney's U test [18] ($p < 0.05$) or a mean rank test of Dunnett type [10] ($p < 0.01$ or 0.05). For quantitative urinalysis data, the cumulative chi square test [26] ($p < 0.01$ or 0.05) or the Kruskal-Wallis ranking test [16] following the Mann-Whitney's U test [18] ($p < 0.05$) was performed. In the newborn rat study, data for physical development and reflex ontogeny were analyzed by the chi square test [6] and histopathological results by the Mann-Whitney's U test [18] ($p < 0.01$ or 0.05).

RESULTS

18-day study in newborn rats (including the dose-finding study)

No deaths occurred up to 240 mg/kg in either the dose-finding study or the main study. The toxicity profile, including effects on general behavior, body weight and organ weights, was the same in both cases. In the 240 mg/kg group in the main study, tremors were observed in all animals from dosing days 2 to 12 and then the incidence decreased, with disappearance after dosing day 16 in males and 17 in females. In this group, the body weights were significantly lowered from dosing day 8 in males and from dosing day 4 in females, as shown in Figure 1. The body weights of both sexes at 24 and 80 mg/kg were comparable to the control values. No definitive changes in abdominal fur appearance, incisor eruption, eye opening, preputial separation or vaginal opening, as well as reflex ontogeny parameters were detected in any of the dose groups.

At the scheduled sacrifice in the main study, hematological examination showed only a slight increase in the reticulocyte ratio in 240 mg/kg males (21.7%, compared to 18.0% in controls) but no changes in RBC, Hb and Ht in any groups. On blood chemical examination, total bilirubin was significantly increased in both sexes at 240 mg/kg group and slight lowering of glucose was noted in females at 80 and 240 mg/kg, as shown in Table 1. There were no dose-related changes in GOT, GPT and BUN, major parameters for hepatic and renal toxicity. Significant increases in relative liver weight were found in both sexes at 240 mg/kg and males at 80 mg/kg (Table 2). Although a very slight increase in relative kidney weights was also evident

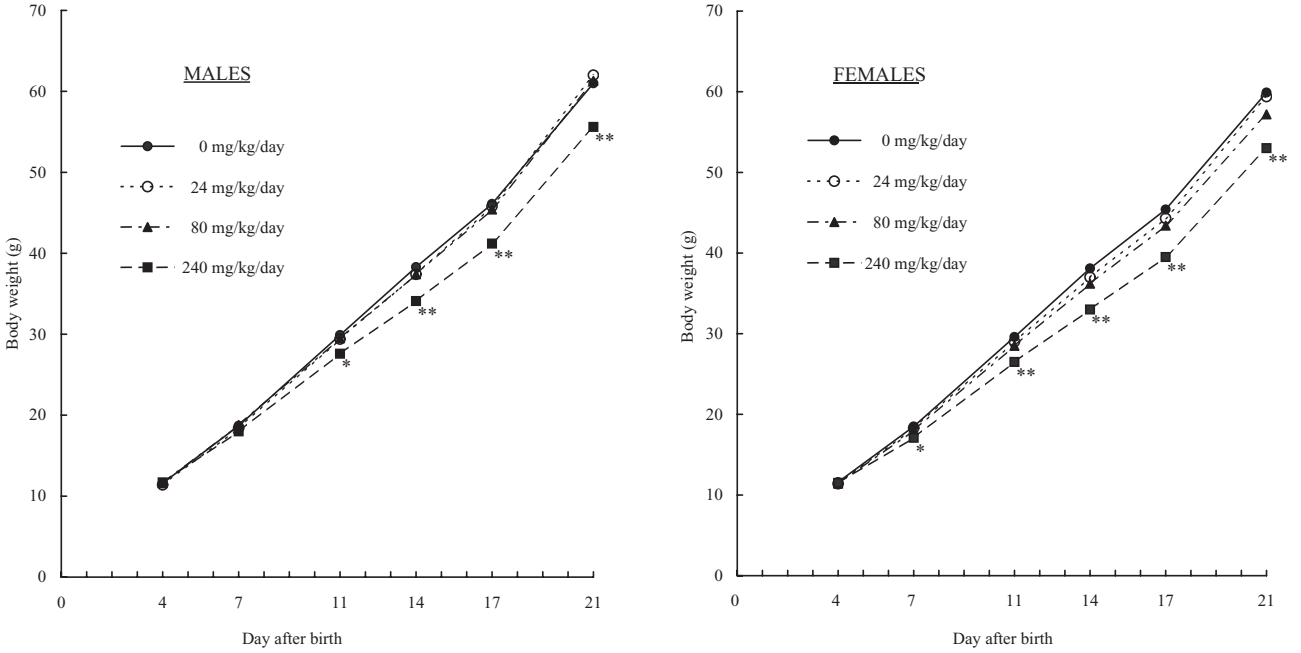

Fig 1. Body weight change during dosing period in the 18-day study of 3-aminophenol in newborn rats (main study)
*: Significantly different from controls ($p < 0.05$), **: Significantly different from controls ($p < 0.01$)

Table 1. Blood chemical findings after dosing period in the 18-day study of 3-aminophenol in newborn rats (main study)

	Dose (mg/kg)			
	0	24	80	240
Males				
No. of animals	6	6	6	6
GOT (IU/L)	93±17	91±4	86±7	81±7
GPT (IU/L)	34±3	35±4	34±3	39±6
Total bilirubin (mg/dL)	0.15±0.03	0.14±0.02	0.16±0.02	0.19±0.02*
Glucose (mg/dL)	126±8	111±14	114±10	114±17
Total cholesterol (mg/dL)	96±17	91±16	95±11	89±10
Triglyceride (mg/dL)	79±20	82±11	80±21	83±26
BUN (mg/dL)	13±3	13±1	13±1	13±3
Females				
No. of animals	6	6	6	6
GOT (IU/L)	86±5	85±7	88±12	78±8
GPT (IU/L)	30±6	33±4	31±4	33±4
Total bilirubin (mg/dL)	0.14±0.03	0.15±0.03	0.15±0.02	0.21±0.05**
Glucose (mg/dL)	119±7	111±7	107±8*	94±8**
Total cholesterol (mg/dL)	87±11	100±13	93±10	93±21
Triglyceride (mg/dL)	66±13	70±15	62±8	78±27
BUN (mg/dL)	16±3	15±3	16±1	12±3*

*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$)

Table 2. Organ weights after dosing period in the 18-day study of 3-aminophenol in newborn rats (main study)

	Dose (mg/kg)			
	0	24	80	240
Males				
No. of animals	6	6	6	6
Body weight [a] (g)	57.5±6.7	58.4±5.0	58.3±3.9	51.7±4.9
Brain (g)	1.53±0.06[b] (2.69±0.27[c])	1.55±0.03 (2.68±0.21)	1.55±0.04 (2.66±0.18)	1.48±0.05 (2.87±0.22)
Thyroid gland (g)	6.0±1.0 (10.5±2.2)	7.1±1.5 (12.3±3.4)	6.6±0.9 (11.4±2.0)	6.8±1.6 (13.1±2.1)
Liver (g)	1.69±0.25 (2.93±0.24)	1.79±0.15 (3.07±0.09)	1.90±0.10 (3.27±0.07**)	1.76±0.19 (3.40±0.15**)
Spleen (g)	0.27±0.04 (0.46±0.03)	0.27±0.06 (0.46±0.07)	0.28±0.05 (0.48±0.07)	0.23±0.05 (0.43±0.08)
Kidney (g)	0.67±0.07 (1.17±0.15)	0.67±0.06 (1.14±0.06)	0.70±0.08 (1.20±0.14)	0.64±0.08 (1.23±0.10)
Testis (g)	0.27±0.06 (0.46±0.07)	0.28±0.02 (0.49±0.03)	0.28±0.03 (0.49±0.04)	0.24±0.03 (0.45±0.03)
Epididymis (mg)	41±7 (72±7)	42±6 (72±6)	42±5 (72±8)	34±3 (67±3)
Females				
No. of animals	6	6	6	6
Body weight [a] (g)	55.2±6.1	54.6±2.3	53.1±5.3	48.1±5.3
Brain (g)	1.47±0.06 (2.68±0.24)	1.46±0.04 (2.68±0.09)	1.51±0.02 (2.86±0.25)	1.46±0.04 (3.06±0.30*)
Thyroid gland (g)	6.6±1.1 (12.1±2.1)	7.4±0.8 (13.6±1.3)	7.4±1.6 (13.9±2.7)	6.9±0.4 (14.5±1.7)
Liver (g)	1.65±0.15 (2.99±0.09)	1.65±0.11 (3.01±0.09)	1.65±0.20 (3.10±0.08)	1.59±0.17 (3.30±0.11**)
Spleen (g)	0.27±0.06 (0.50±0.13)	0.22±0.03 (0.40±0.04)	0.21±0.03* (0.40±0.04)	0.17±0.03** (0.35±0.04*)
Kidney (g)	0.63±0.07 (1.14±0.07)	0.66±0.04 (1.21±0.04)	0.65±0.08 (1.22±0.04)	0.65±0.08 (1.35±0.10**)
Ovary (mg)	11.5±4.6 (20.4±7.3)	10.5±2.4 (19.1±3.7)	8.8±1.7 (16.5±2.3)	10.2±2.1 (21.2±3.8)
Uterus (mg)	43±4 (79±12)	44±7 (80±13)	38±6 (72±13)	37±8 (77±12)

[a]: Body weights after overnight starvation following the last dosing, b): Absolute weights, c): Relative weights (g or mg/100 g body weight)
*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$)

in females at 240 mg/kg, no changes were observed in thyroid gland and reproductive organ weights. On histopathological examination, slight hypertrophy of follicular cells in the thyroid glands was observed in 4/6 males and 2/6 females at 240 mg/kg. There were no dose-related changes in histopathology of other organs, including the liver, kidneys and spleen.

There were no chemical-related changes in the recovery-maintenance study except a slight increase in RBC ($852 \times 10^4/\mu L$, compared to $802 \times 10^4/\mu L$ in controls), Hb (16.3 g/dL, 15.5 g/dL) and Ht (49%, 46%) in 240 mg/kg females at the end of the period.

The NOAEL for newborn rats was concluded to be 80 mg/kg/day, although a slight increase in relative liver weight in males with no histopathological change and a slight low of blood glucose in females were found at this dose. The unequivocally toxic level was 240 mg/kg/day, based on clear toxic signs such as tremors, depression of body weight gain and histopathological change in thyroid glands.

28-day study in young rats (including the dose-finding study)

No deaths occurred up to 500 mg/kg in the dose-finding study and 720 mg/kg in the main study. Tremors and salivation were observed only on the first dosing day in a few females of the 500 mg/kg group in the dose-finding study and sporadically during the dosing period in both sexes of the 720 mg/kg group in the main study (Table 3). Body weights were comparable to control values at 500 mg/kg in the dose-finding study, but in the main study significant lowering from dosing day 2 to recovery day 7 was evident, but at most 10% in both sexes at 720 mg/kg. Food consumption was decreased transiently during the dosing period in both sexes at 720 mg/kg in the main study. Urinalysis at dosing week 4 revealed significant increases in urine volume and water consumption, with decrease in urine specific gravity only noted in females at 720 mg/kg in the main study.

At the scheduled sacrifice, anemia with decrease in RBC and Hb, and an increased reticulocyte ratio were observed in females receiving 720 mg/kg in the main study (Table 4) but not in the dose-finding study at any dose. Blood chemical examination showed a slight increase in GPT (26.0 IU/L, compared to 22.8 in controls) at 500 mg/kg in the dose-finding study, and increases in GPT and total bilirubin in both sexes, increase in total cholesterol and decrease in triglyceride in males, and increase in BUN in females at 720 mg/kg in the main study, as shown in Table 5. There were significant increases in absolute or relative weights of thyroid glands and relative weights of liver and kidneys in both sexes, as well as absolute and relative spleen weights in females given 720 mg/kg (Table 6). On histopathological examination in the main study, slight to mild changes related to hemolytic action were observed in liver, kidneys and spleen in both sexes at 720 mg/kg, as shown in Table 7. At 240 mg/kg, slight deposition of pigment in the renal proximal tubular epithelium was only observed in females. As for other changes, hypertrophy of thyroid follicular cells in both sexes and of basophilic cells in the pituitary glands in two of seven males was noted at 720 mg/kg. In kidneys, the incidence of hyaline droplets in proximal epithelium was increased in males at 720 mg/kg.

In the recovery group (720 mg/kg), a significant decrease of RBC and Hb and increase of the reticulocyte ratio in males, and increase of Ht in females were found (Table 4). Deposition of hemosiderin in various tissues had greatly reduced, but still remained in the spleen of males and kidneys and spleen of females (Table 7). Hypertrophy of follicular cells in thyroid glands had completely disappeared, although increased relative weights still remained. None of the other changes observed in the dosing period and at the scheduled sacrifice persisted in males and females of the recovery groups.

The NOAEL was concluded to be 240 mg/kg/day, because slight pigmentation in the renal proximal tubular epithelium observed in females at this dose was not considered an adverse effect owing to no accompanying hematological changes. As for the unequivocally toxic level, 720 mg/kg/day is appropriate, based on anemia, hepatotoxicity and renal toxicity, in addition to tremors, depression of body weight gain and histopathological changes in thyroid glands.

DISCUSSION

Neonates exhibit many differences in characteristics compared to young adults. As physiological differences relative to the body weight of neonates, there is a greater % of body water and a lower % of body fat, greater cardiac output and respiratory rate, larger liver and brain, and higher blood flow to the brain but less to kidneys. Functional immaturities in neonates are found in phase I and phase II drug metabolic enzymes, renal function, and the blood-brain barrier. Furthermore, all organs of neonates develop quickly, especially in terms of increase in organ size and function, including the neuron network in the brain. Considering

Table 3. Clinical signs in repeated dose studies of 3-aminophenol in young rats

	14-day dose-finding study			28-day study		
Dose (mg/kg)	80	200	500	80	240	720
Males						
No. of animals	5	5	5	14	14	14
-Salivation	-	-	-	-	-	5[b)]
-Tremor	-	-	-	-	-	9[b)]
Females						
No. of animals	5	5	5	14	14	14
-Salivation	-	-	1[a)]	-	-	4[b)]
-Tremor	-	-	2[a)]	-	-	10[b)]

[a)]: Observed only on the first dosing day, [b)]: Observed sporadically during the dosing period

Table 4. Hematological findings for 28-day repeated dose study of 3-aminophenol in young rats (main study)

	Scheduled-sacrifice group				Recovery group	
Dose (mg/kg)	0	80	240	720	0	720
Males						
No. of animals	7	7	7	7	7	7
RBC ($10^4/\mu$ L)	787.0±60.8	765.0±45.4	795.1±23.7	775.4±38.4	891.3±11.6	852.0±15.9**
Ht (%)	47.99±2.74	46.50±1.77	47.93±1.61	47.13±1.90	50.41±1.21	49.80±1.16
Hb (g/dL)	15.94±1.04	15.46±0.37	15.71±0.48	15.44±0.52	16.99±0.37	16.41±0.38*
MCV (fL)	61.09±2.23	60.91±3.07	60.31±2.22	60.80±1.45	56.56±1.14	58.46±0.97**
MCH (pg)	20.30±1.04	20.23±0.90	19.79±0.76	19.93±0.48	19.07±0.45	19.26±0.29
MCHC (g/dL)	33.24±0.91	33.26±0.88	32.77±0.39	32.76±0.39	33.70±0.45	32.99±0.23**
Reticulocyte ratio (%)	37.6±5.1	29.9±8.1	30.6±6.7	38.9±7.4	14.4±1.7	20.3±4.8**
Females						
No. of animals	7	7	7	7	7	7
RBC ($10^4/\mu$ L)	768.4±48.9	788.1±51.5	754.7±52.5	691.4±35.0*	842.9±38.0	858.1±41.0
Ht (%)	45.89±2.33	46.27±2.82	43.23±3.65	42.63±2.81	46.41±2.08	49.90±2.97*
Hb (g/dL)	16.07±0.83	16.04±0.78	15.41±0.99	14.70±0.91*	15.99±0.68	16.89±0.97
MCV (fL)	59.79±1.52	58.71±1.17	57.26±1.50*	61.63±1.99	55.10±1.54	58.13±1.43**
MCH (pg)	20.94±0.52	20.39±0.58	20.43±0.59	21.26±0.78	18.99±0.38	19.67±0.57*
MCHC (g/dL)	35.03±0.53	34.69±0.78	35.71±0.91	34.50±0.63	34.46±0.30	33.83±0.35**
Reticulocyte ratio (%)	30.1±6.3	26.0±5.0	27.1±7.4	53.3±19.6*	17.7±3.9	17.9±1.8

*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$)

Table 5. Blood chemical findings after dosing period in 28-day repeated dose study of 3-aminophenol in young rats (main study)

	Dose (mg/kg)			
	0	80	240	720
Males				
No. of animals	7	7	7	7
GOT (IU/L)	64.9±6.9	66.0±5.2	66.9±6.4	77.1±16.9
GPT (IU/L)	26.4±4.4	23.3±3.1	27.1±3.8	41.9±10.2*
Total bilirubin (mg/dL)	0.046±0.008	0.050±0.014	0.047±0.011	0.101±0.023**
Glucose (mg/dL)	157.3±21.1	148.7±10.6	133.6±20.4*	153.9±16.8
Total cholesterol (mg/dL)	64.1±7.8	58.1±6.8	60.0±7.1	77.3±12.3*
Triglyceride (mg/dL)	49.1±18.2	50.6±21.9	35.9±12.5	26.1±5.9*
BUN (mg/dL)	15.77±1.44	14.99±1.38	17.33±1.65	15.47±1.74
Females				
No. of animals	7	7	7	7
GOT (IU/L)	65.0±5.9	59.1±2.9	58.9±4.1	64.3±7.3
GPT (IU/L)	21.3±2.4	19.1±3.7	22.4±1.4	31.1±3.9**
Total bilirubin (mg/dL)	0.057±0.017	0.057±0.008	0.063±0.010	0.093±0.010**
Glucose (mg/dL)	135.7±7.1	135.7±14.9	129.1±10.4	124.9±15.3
Total cholesterol (mg/dL)	79.6±14.0	73.7±17.2	69.6±13.0	72.6±8.1
Triglyceride (mg/dL)	16.4±8.1	15.0±6.2	15.3±5.1	13.3±1.7
BUN (mg/dL)	17.41±0.98	17.31±1.36	18.17±3.75	21.64±4.37*

*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$)

these differences, it might be expected that chemical toxicity in neonates may be higher or having a different profile. In our first comparative toxicity analysis of two chemicals, 4-nitrophenol and 2,4-dinitrophenol, the toxic response to these chemicals in newborn rats was at most 4 times higher than that in young rats, based on the NOAEL and the unequivocally toxic level (Koizumi *et al*., 2001). This latter was defined as a dose inducing severe toxicity, including death or critical histopathological changes. In the previous analysis, it was estimated as the dose inducing death, because no other clear signs were observed. Although the definition of the unequivocally toxic level seems not uniform, it should be valuable if it is estimated according to appropriate toxicity endpoints. As the chemicals in question caused neither developmental toxicity nor morphological changes, the higher toxicity in newborn rats seems to be due to physiological and functional differences. In the first case, using both toxicity endpoints enables us to draw a more reliable conclusion of toxicity differences between newborn and young animals.

In this study, 3-aminophenol was selected for the second trial comparative toxicity analysis between newborn and young

Table 6. Organ weights after dosing period in 28-day study of 3-aminophenol in young rats (main study)

	Dose (mg/kg)			
	0	80	240	720
Males				
No. of animals	7	7	7	7
Body weight [a] (g)	328.6±14.1	337.4±22.8	324.0±15.0	288.7±27.2**
Brain (g)	2.03±0.09[b]	2.09±0.07	2.09±0.13	2.08±0.09
	(0.62±0.04[c])	(0.62±0.05)	(0.65±0.03)	(0.73±0.05**)
Thyroid gland (mg)	18.13±3.27	21.37±2.53	21.33±4.01	27.86±4.25**
	(5.50±0.84)	(6.34±0.75)	(6.56±1.03)	(9.72±1.78**)
Liver (g)	10.36±0.84	10.67±1.45	10.54±0.87	11.13±1.26
	(3.15±0.23)	(3.15±0.23)	(3.25±0.24)	(3.85±0.19**)
Spleen (g)	0.63±0.07	0.74±0.09	0.74±0.11	0.65±0.11
	(0.19±0.02)	(0.22±0.01)	(0.23±0.03)	(0.22±0.03)
Kidney (g)	2.67±0.25	2.76±0.23	2.80±0.18	2.73±0.36
	(0.81±0.05)	(0.82±0.04)	(0.86±0.06)	(0.94±0.07**)
Testis (g)	3.09±0.09	3.05±0.34	3.03±0.25	3.21±0.31
	(0.94±0.05)	(0.90±0.09)	(0.94±0.09)	(1.12±0.09**)
Epididymis (mg)	727±47	697±66	757±44	694±33
	(223±17)	(209±20)	(234±21)	(241±17)
Females				
No. of animals	7	7	7	7
Body weight (g)	203.1±19.1	195.3±5.3	196.6±8.7	183.1±14.9
Brain (g)	1.93±0.08	1.89±0.05	1.87±0.05	1.86±0.11
	(0.95±0.05)	(0.97±0.03)	(0.95±0.06)	(1.02±0.08)
Thyroid gland (mg)	14.23±2.66	18.06±3.22	17.57±2.67	23.84±6.77**
	(7.00±1.04)	(9.24±1.55)	(9.00±1.74)	(12.94±2.85**)
Liver (g)	6.27±0.78	6.08±0.57	6.42±0.37	6.63±0.95
	(3.08±0.14)	(3.11±0.23)	(3.26±0.14)	(3.61±0.27**)
Spleen (g)	0.44±0.06	0.45±0.09	0.43±0.05	0.61±0.09**
	(0.22±0.01)	(0.23±0.05)	(0.22±0.03)	(0.33±0.04**)
Kidney (g)	1.72±0.20	1.73±0.11	1.80±0.11	1.81±0.21
	(0.84±0.06)	(0.88±0.04)	(0.92±0.07)	(0.99±0.05**)
Ovary (mg)	83.1±13.3	96.3±18.2	83.0±8.8	83.3±22.1
	(40.88±4.49)	(49.19±8.59)	(42.25±4.38)	(45.03±8.72)

[a]: Body weights after overnight starvation following the last dosing, [b]: Absolute weights, [c]: Relative weights (g or mg/100 g body weight)

*: Significantly different from control group ($p<0.05$), **: Significantly different from control group ($p<0.01$)

rats. The NOAELs were relatively simply concluded to be 80 mg/kg/day for newborn rats and 240 mg/kg/day for young rats. The unequivocally toxic levels for both were based on low body weights, tremors and histopathological changes in the thyroid glands, being 240 mg/kg/day for newborn rats and 720 mg/kg/day for young rats. These results indicate that the toxic sensitivity of newborn rats to 3-aminophenol is three times higher than that in young rats (NOAEL 240/80 = 3, unequivocally toxic level 720/240 = 3). As anemia, hepatotoxicity and renal toxicity appeared in young rats, but not in newborn rats (although slightly suggestive data were also obtained for the latter), all these changes might have been due to hemolytic toxicity. Although methemoglobin levels were not determined in this study, it is well known that aromatic amines induce methemoglobinemia, leading to hemolytic anemia. As 2-aminophenol, 4-aminophenol and phenylhydroxyamine induce methemoglobinemia at least *in vitro*, and the potency of phenylhydroxyamine is the highest [1, 9], hydroxylation of aminophenols *in vivo* might be a key point. If so, lower susceptibility regarding anemia than for other parameters of toxicity in the newborn might be due to the lower hepatic hydroxylation potential.

Only one toxicity study of 3-aminophenol has been reported to our knowledge, a 90-day female rat study at doses of 0.1, 0.25 and 1.0% in the diet [21]. In the 0.25% group, a significant reduction in body weight was noted, and in the 1.0% group significant reduction in body weights and deposition of iron-positive pigments in the spleen, liver, and kidney, combined with decreased RBC and Hb, indicating a hemolytic effect, were observed. There were also morphologic changes in the thyroid gland, consistent with hyperactivity. This observed toxicity is entirely in agreement with our 28-day study results, although the actual daily intake in the diet study was not given. A structural isomer, 4-aminophenol is a major metabolite of the analgesic and antipyretic drug, acetaminophen, and is known to exert renal toxicity [3, 8]. Mechanistic studies in rats suggest that 4-aminophenol may be oxidized to benzoquinoneimine and then conjugated with glutathione in the liver to be excreted in

Table 7. Histological findings for 28-day study of 3-aminophenol in young rats (main study)

		Scheduled-sacrifice group				Recovery group	
Dose (mg/kg)	Grade	0	80	240	720	0	720
Males							
No. of animals examined		7	7	7	7	7	7
Liver							
-Deposition of brown pigment in Kupffer cells	+	0	0	0	7	0	1
Kidneys							
-Deposition of brown pigment in proximal epithelium	+	0	0	0	7	0	0
-Hyaline droplet in proximal epithelium	+	2	1	2	7	0	1
Spleen							
-Deposition of hemosiderin	+	0	0	0	2	0	5
	++	0	0	0	4	0	1
Total observed number		0	0	0	6	0	6
Pituitary gland							
-Hypertrophy in basophilic cells	+	0	0	0	2	0	0
Thyroid							
-Hypertrophy in follicular cells	+	0	0	0	3	0	0
Females							
No. of animals examined		7	7	7	7	7	7
Liver							
-Deposition of brown pigment in Kupffer cells	+	0	0	0	7	0	2
Kidneys							
-Deposition of brown pigment in proximal epithelium	+	0	0	6	7	0	7
Spleen							
-Deposition of hemosiderin	+	0	0	1	0	0	0
	++	0	0	0	7	0	7
Total observed number		0	0	1	7	0	7
Thyroid							
- Hypertrophy in follicular cells	+	0	0	0	5	0	0

+: Slight, ++: Mild

the bile, and subsequently reabsorbed and transported via the systemic circulation to the kidney, where toxic effects occur [7]. Relatively recent studies have also indicated a contribution of glutathione to renal toxicity in rats and mice [11, 22]. However, this pathway may not be important for 3-aminophenol, because of the structural difficulty in formation of quinoneimine derivatives in this case. In rat renal slices, 4-aminophenol proved more toxic than 2-aminophenol, the authors drawing conclusions as to a rule for the position of the amino group for this kind of renal toxicity [24]. However, it remains to be elucidated whether oxidation-glutathione conjugation contributed to the cytotoxicity in renal slices. Based on our results, the renal toxicity of 3-aminophenol seems to be completely different from that of 4-aminophenol.

In conclusion, the susceptibility of the newborn rats to 3-aminophenol based on tremors, lowering of body weights and thyroid toxicity appears to be approx. 3 times higher than that of young rats, consistent with our previous results for two other chemicals, 4-nitrophenol and 2,4-dinitrophenol. The analytical results support the recommendation by the NRC [20] that an additional uncertainty factor of 10 for the ADI is appropriate for infant safety in the absence of specific data for developmental toxicity.

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Higher Susceptibility of Newborn Than Young Rats to 3-Methylphenol

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ABSTRACT. To determine susceptibility of infants to 3-methylphenol, a repeated dose toxicity study was conducted with oral administration to newborn and young rats. In an 18-day newborn study from postnatal days 4 to 21 at doses of 30, 100 and 300 mg/kg/day, various clinical signs including deep respiration, hypersensitivity on handling and tremors under contact stimulus, and depressed body weight gain were observed at 300 mg/kg. At 100 mg/kg, hypersensitivity and tremors were also noted in a small number of males only on single days during the dosing period. No adverse effects were observed in the 30 mg/kg group. There were no abnormalities of physical development, sexual maturation and reflex ontogeny. The no observed adverse effect level (NOAEL) for newborn rats was considered to be 30 mg/kg/day and the unequivocally toxic level 300 mg/kg/day. In a 28-day study starting at 5 weeks of age, clinical signs and depression of body weight gain, as observed in the newborn rats, appeared in both sexes at 1000 mg/kg but not 300 mg/kg. The NOAEL and the unequivocally toxic level were 300 mg/kg/day and 1,000 mg/kg/day, respectively. From these results, newborn rats were concluded to be 3 to 10 times more susceptible to 3-methylphenol than young rats. However, the realistic no adverse effect dose for the newborn must be slightly lower than 100 mg/kg/day, at which the toxicity incidence was very low, rather than 30 mg/kg/day. Based on this speculation and the equal toxicity at unequivocally toxic levels, the differences in the susceptibility to 3-methylphenol could be concluded to be 3 to 4 times. This is consistent with the results of our previous comparative studies on 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol, which showed 2 to 4 times differences in the susceptibility between newborn and young rats. —Key words: toxicity in newborn rats, 3-methylphenol

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INTRODUCTION

It is known that neonates have specific physiological characteristics with regard to water volume per body, weight of liver and brain relative to body size, cardiac output, respiratory rate, and blood flow to brain and kidney, for example. In fact, the toxicokinetic ability of infants seems to differ from that of adults with respect to their metabolism, clearance, protein binding and volume of distribution, based on data obtained with therapeutic drugs [2, 14, 21], although there is very little information regarding environmental chemicals. Furthermore, the sensitivity of rapidly developing tissues/systems in neonates may also differ from that in adults [10, 24, 33]. Since infants are always exposed to various chemicals by putting fingers, toys and other objects into their mouths as well as via mother's milk, there is growing concern about effects on infant health. Unfortunately, there is no generally accepted Test Guideline for newborn toxicity studies. Therefore, we established a new protocol [16] in order to investigate the differences of susceptibility between the newborn and young rats. This protocol includes a detailed examination of physical development and sexual maturation, and a complete toxicity analysis after the 9-week recovery-maintenance period, the age of the young adult. Furthermore, a unique feature is that the same lot number of chemical and the same rat strain from the same supplier are used as in young rat studies.

Using this protocol, we have already tested 14 phenolic derivatives as a part of an existing chemical testing program of Japan in 1999. So far, we have reported three comparative analyses of 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol [16-18, 22, 28, 35]. In these studies, for more precise / appropriate comparison, we estimated both the no observed effect levels (NOAELs) and unequivocally toxic levels, defined as doses inducing severe toxic signs including death or critical histological damage, based on the results of both the main studies and the dose-finding studies for each case. In consequence, it was concluded that the susceptibility of newborn rats to the toxicity of these chemicals ranged from 2 to 4 times that of young rats.

In the present study, we selected 3-methylphenol, widely known as m-cresol and used in synthetic resins, disinfectants and pharmaceutical raw materials [8]. Several reviews on the toxicity of this chemical or cresols, including three isomers, have been published [1, 9, 12]. For 3-methylphenol, although various clinical signs, growth inhibition and some developmental effects have been reported [5-7, 20, 23, 32], there are no data to our knowledge on its direct effects in newborn animals. In this study, we estimated the NOAELs and unequivocally toxic levels of 3-methylphenol, and compared them between newborn and young rats employing the previously described protocol.

MATERIALS AND METHODS

Materials

3-Methylphenol (CAS No. 108-39-4, purity: 99.13%) was obtained from Honshu Chemical Industry Co., Ltd. (Wakayama, Japan), and dissolved in olive oil. The test solution was prepared at least once a week and kept cool and in the dark until dosing. The stability was confirmed to be at least 8 days under these conditions. All other reagents used in this study were specific purity grade.

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Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 20-26℃ with a relative humidity of 45-65%, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. In the 18-day main study of newborn rats, 21 pregnant rats (gestation day 15) were purchased and allowed to deliver spontaneously. Among all newborns separated from dams at postnatal day 3 (the date of birth was defined as postnatal day 0), 48 males and 48 females were selected by stratified random sampling based on the body weight and assigned to 4 dose groups, including controls. Twelve foster mothers suckled the 4 males and 4 females assigned to each group up to weaning on postnatal day 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks. In the 28-day study of young rats, 4-week-old male and female rats were obtained and used at ages of 5 weeks after acclimation. All animals were allowed free access to basal diet (newborn rat study: LABO MR stock, Nihon Nosan Kogyo Inc., Yokohama, Japan; young rat study: CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Study Design (Time schedule as reported previously [16])

1. 18-Day Repeated Dose Study in Newborn Rats

1) Dose-finding study

Newborn rats (5/sex/dose) were administered the test substance at 0, 100, 300 or 1,000 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21. They were examined for general behavior and body weights during the dosing period, and sacrificed at postnatal day 22 after overnight starvation, for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Newborn rats (12/sex/dose) were administered 3-methylphenol at 0, 30, 100 or 300 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21, based on results of the dose-finding study, and 6 males and 6 females in each group were sacrificed on postnatal day 22, after overnight starvation. Recovery-maintenance groups (rest of animals in all groups) were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age. General behavior was noted at least once a day for newborn rats (separated from each foster mother) and foster mothers. Body weight was measured at postnatal days 4, 7, 10, 13, 16, 19 and 21, and then at 7-day intervals, and food consumption (for 24 hr from the day before) at the same days after weaning. At postnatal day 20 for males and day 21 for females, gait condition, pupillary reflex, auricular reflex, corneal reflex, visual placing reflex, surface and mid-air righting reflexes, and ipsilateral flexor reflex were examined. Furthermore, fur appearance, incisor eruption and eye opening were observed in all animals from postnatal days 7, 9 and 11, respectively, and testes descent and vaginal opening were examined from postnatal days 17 and 29, respectively. Color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity and volume of the urine were examined only at the end of the recovery-maintenance period. Blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for scheduled-sacrifice and recovery-maintenance groups. One part was treated with EDTA-2K or 3.8% sodium citrate and examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count, and leukocyte analysis percentage, as well as blood clotting parameters such as prothrombin time and activated thromboplastin time. Serum obtained from another portion of the blood was analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase, cholinesterase, calcium, inorganic phosphorus, sodium, potassium, chlorine). After gross examination of the body surface, orificial mucosa and internal organs of animals sacrificed by exsanguination following collection of blood, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, prostates, seminal vesicle, ovaries and uterus were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The trachea, stomach, intestine, pancreas, lymph glands, urinary bladder, spinal cord, bone marrow and sciatic nerve as well as the above organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopic examination. For other groups, the organs with macroscopically abnormal findings or in which dose-related effects were evident on microscopic examination for the highest dose group, were examined.

2. 28-Day Repeated Dose Study in Young Rats

1) Dose-finding study (14-day study)

Five-week-old rats (5/sex/dose) were administered the test substance at 0, 125, 250, 500 or 1,000 mg/kg/day in olive oil by gastric intubation for 14 days. They were examined for general behavior, body weight and food consumption during dosing and sacrificed after overnight starvation following the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Five-week-old rats were given the test substance in olive oil by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment. Referring to the results of the dose-finding study, 4 doses, including the control, were established (0, 100, 300, 1,000 mg/kg/day). Recovery groups (0, 1,000 mg/kg) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The number of animals for each sex/dose was 7 for both scheduled-sacrifice and recovery cases. Rats were examined for general behavior, body weight, food consumption, urinalysis,

hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Data were statistically analyzed as follows [26, 27, 36]. Continuous data were analyzed by Bartlett's test for distribution. When homogeneity was recognized, one-way analysis of variance was performed. When a significant difference was observed, Dunnett's or Scheffe's tests were conducted for comparisons between control and 3-methylphenol-treated groups. If not homogenous or in the case of quantitative urinalysis data, analysis was performed using the Kruskal-Wallis test. In consequence, if a significant difference was detected, Dunnett type, Scheffe type or Mann-Whitney's U tests [19] were conducted. In the newborn rat study, categorical data for general appearance, reflex ontogeny, necropsy and histopathology were analyzed by Fisher's exact probability test. A probability less than 5% was considered statistically significant.

RESULTS

18-day study in newborn rats (including the dose-finding study)

In a dose-finding study at doses of 100, 300 and 1,000 mg/kg, all animals at 1,000 mg/kg died within two days after the first treatment (Table 1). These rats showed deep respiration, decrease in spontaneous activity and pale skin. At 300 mg/kg, deep respiration and tremors under contact stimulus were noted during the dosing period in all animals, but no deaths occurred. No clinical signs were observed at 100 mg/kg. Body weight gain was depressed in females at 300 mg/kg. Blood biochemical examination showed a slight increase in total bilirubin in both sexes receiving 300 mg/kg (males; 0.36 mg/dL, compared with 0.32 mg/dL in controls, females; 0.37 mg/dL, 0.32 mg/dL). Organ weights, shown in Table 2, demonstrated a significant increase in relative liver weight in males at 100 and 300 mg/kg, and in females at 300 mg/kg but not absolute liver weights. No dose-related changes in hematology or gross findings were observed. Based on these results, the clearly toxic dose of 300 mg/kg was selected as the top dose in the main study, and 30 and 100 mg/kg were derived by approximately one-third divisions.

Table 1. Clinical signs and mortality in 18-day studies of 3-methylphenol in newborn rats

	Dose-finding study			Main study		
Dose (mg/kg)	100	300	1,000	30	100	300
Males						
No. of animals	5	5	5	12	12	12
No. of dead animals	-	-	5[a)]	-	-	-
No. of animals with clinical signs						
Deep respiration	-	5	3	-	-	5
Increase in motor activity	-	-	-	-	-	12
Decrease in spontaneous activity	-	-	5	-	-	1
Hypersensitivity on handling	-	-	-	-	1[b)]	7
Tremors under contact stimulus	-	5	-	-	3[c)]	12
Pale skin	-	-	1	-	-	-
Females						
No. of animals	5	5	5	12	12	12
No. of dead animals	-	-	5[a)]	-	-	-
No. of animals with clinical sign						
Deep respiration	-	5	2	-	-	3
Increase in motor activity	-	-	-	-	-	12
Decrease in spontaneous activity	-	-	5	-	-	1
Hypersensitivity on handling	-	-	-	-	-	10
Tremors under contact stimulus	-	5	-	-	-	12
Pale skin	-	-	2	-	-	-

- : No animals with clinical sign
a): All animals died within 2 days after first treatment.
b): Observed only at dosing day 18.
c): Observed only at dosing day 4 in one rat and at dosing day 11 in another two.

In the main study, various toxic signs such as deep respiration, increase in motor activity and tremors under contact stimulus were noted during the dosing period in all animals receiving 300 mg/kg, but no deaths occurred (Table 1). With 100 mg/kg, although no clinical signs were observed in the dose-finding study, three males showed tremors under contact stimulus only on single days, dosing days 4, 11 and 11, respectively, and another male showed hypersensitivity on handling on a single day, dosing day 18. No change in general behavior was observed at 30 mg/kg. Body weights of both sexes given 300 mg/kg were lowered during the dosing period, but at 100 and 30 mg/kg were comparable to control values (Fig.1). No definitive changes in developmental parameters, including sexual maturation, as well as reflex ontogeny were detected in any dose group. At the scheduled sacrifice, blood biochemical examination of the 300 mg/kg group showed increases in γ-GTP, total bilirubin and BUN in males (Table 3). Significant increase of relative liver weight but not absolute liver weight was noted in both sexes given this highest dose (Table 2). In addition, there was a decrease in absolute brain weight in both sexes, but this change was not noted in the dose-finding study. On histopathological examination, basophilic tubules in kidneys showed a tendency to increase in 300 mg/kg males (slight and moderate changes in 2/6 and 3/6, respectively, compared with only slight change in 5~6/6 animals in other groups). After the recovery-maintenance period, there were no dose-related changes in body weight, blood biochemistry and histopathology, but low absolute brain weights remained in males (1.90 g, compared with 2.08 g in controls). No dose-related changes in food consumption, urinalysis, hematology and gross finding were observed throughout this study, including the recovery-maintenance period.

Since the hypersensitivity on handling and tremors under contact stimulus observed in a small number of males of the 100 mg/kg group were considered as dose-related adverse effects, the NOAEL in the main study was concluded to be 30 mg/kg/day. However, these clinical signs at 100 mg/kg were observed only on single days during the dosing period in the main study and not in the dose-finding study. Therefore, as for the unequivocally toxic level, 300 mg/kg/day was concluded to be appropriate because significant toxic effects in the central nervous system were observed at this dose, along with decrease in body weight gain.

28-day study in young rats (including the dose-finding study)

In the dose-finding study for 14 days at doses of 125, 250, 500 and 1,000 mg/kg, no deaths occurred at any dose (Table 4). Salivation, tremors and prone/lateral position were observed during the dosing period in both sexes at 1,000 mg/kg. Body weights and food consumption were lowered in males receiving 1,000 mg/kg. At 500 mg/kg and less, no changes in clinical signs, body weight and food consumption were observed. Blood biochemical examination showed increase in total cholesterol in females at 1,000 mg/kg (76.0 mg/dL, compared with 53.4 mg/dL at controls) and 500 mg/kg (72.2 mg/dL). Increase in relative liver weights in both sexes at 1,000 mg/kg (males: 3.86 g/100 g body weight, compared with 3.32 g/100 g body weight at controls, females: 3.70 g/100 g body weight, 3.34 g/100 g body weight) and 500 mg/kg (males: 3.60 g/100 g body weight, females: 3.68 g/100 g body weight), and in relative kidney weight in males at 1,000 mg/kg (0.47 g/100 g body weight, compared with 0.43 g/100 g body weight) was also observed. There were no dose-related changes evident on hematological and gross examination. Based on the results, the upper limit

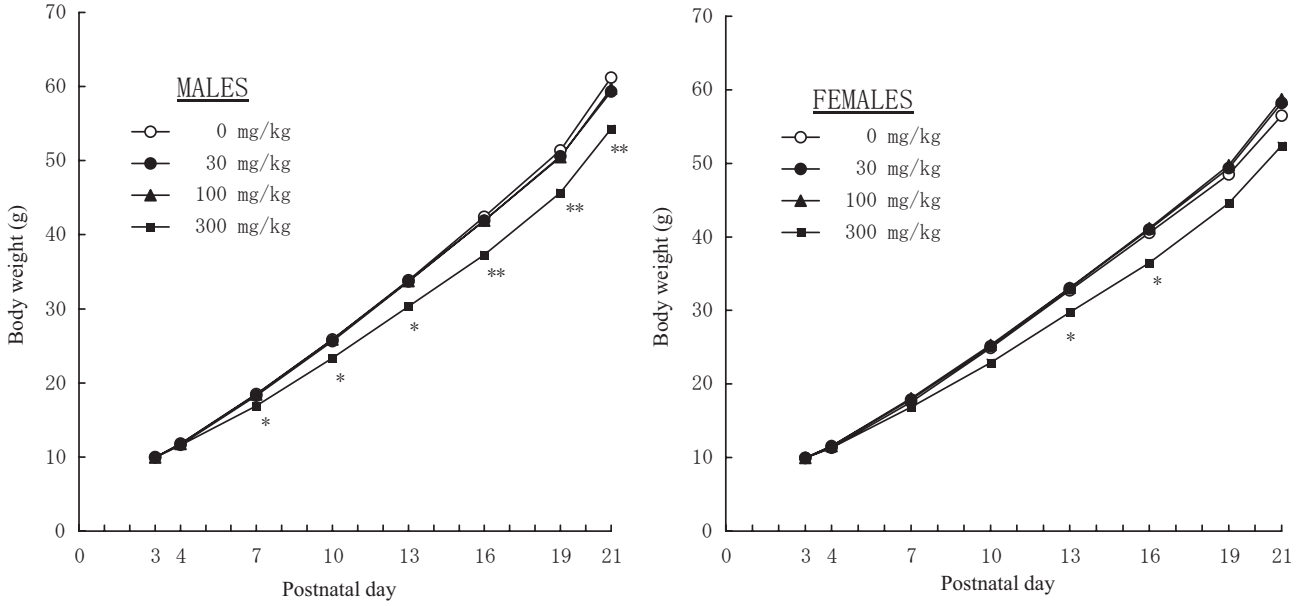

Fig. 1. Body weight change in 18-day study of 3-methylphenol in newborn rats (main study).
*: Significantly different from controls ($p < 0.05$).
**: Significantly different from controls ($p < 0.01$).

Table 2. Organ weights after 18-day repeat dosing of 3-methylphenol in newborn rats

	Dose-finding study[a)]			Main study			
Dose (mg/kg)	0	100	300	0	30	100	300
Males							
No. of animals	5	5	5	6	6	6	6
Body weight [b)] (g)	62±5	62±6	59±7	53.1±3.3	52.7±3.5	51.4±3.5	46.7±4.3*
Brain (g)	1.53±0.05[c)]	1.57±0.06	1.52±0.06	1.55±0.04	1.58±0.06	1.51±0.06	1.47±0.02*
	(2.47±0.19[d)])	(2.55±0.18)	(2.61±0.23)	(2.93±0.18)	(3.00±0.11)	(2.94±0.13)	(3.16±0.28)
Liver (g)	1.81±0.13	1.91±0.19	1.94±0.24	1.74±0.15	1.71±0.13	1.75±0.24	1.75±0.20
	(2.90±0.04)	(3.08±0.19**)	(3.29±0.05**)	(3.27±0.12)	(3.24±0.14)	(3.39±0.25)	(3.74±0.13**)
Kidney (g)	0.69±0.06	0.72±0.07	0.68±0.05	0.64±0.04	0.66±0.06	0.62±0.04	0.58±0.02
	(1.11±0.03)	(1.16±0.03)	(1.16±0.07)	(1.21±0.05)	(1.26±0.05)	(1.20±0.06)	(1.25±0.10)
Testis (mg)	310±20	310±30	320±40	300±28	293±36	282±14	270±27
	(500±20)	(500±40)	(540±20)	(566±43)	(555±52)	(549±15)	(581±51)
Females							
No. of animals	5	5	5	6	6	6	6
Body weight (g)	61±4	59±6	51±6*	49.4±3.8	50.5±4.1	51.6±3.3	45.5±1.4
Brain (g)	1.50±0.05	1.44±0.03	1.43±0.10	1.52±0.05	1.48±0.06	1.48±0.05	1.42±0.05*
	(2.46±0.18)	(2.45±0.24)	(2.81±0.16*)	(3.09±0.27)	(2.94±0.27)	(2.88±0.13)	(3.13±0.10)
Liver (g)	1.77±0.13	1.77±0.12	1.67±0.15	1.59±0.18	1.59±0.13	1.72±0.08	1.61±0.05
	(2.91±0.08)	(3.01±0.10)	(3.29±0.15**)	(3.21±0.13)	(3.16±0.04)	(3.34±0.11)	(3.54±0.12**)
Kidney (g)	0.71±0.05	0.70±0.04	0.63±0.06	0.63±0.04	0.63±0.04	0.65±0.04	0.61±0.04
	(1.19±0.07)	(1.19±0.10)	(1.24±0.09)	(1.27±0.03)	(1.25±0.04)	(1.27±0.09)	(1.34±0.07)
Ovary (mg)	14.9±2.8	14.4±1.1	15.7±2.4	15.6±4.0	15.4±3.0	13.8±2.1	12.6±2.3
	(24.4±5.1)	(24.5±1.5)	(30.9±4.8)	(31.7±8.9)	(30.5±5.5)	(26.8±4.7)	(27.8±5.1)

Data are mean ± SD values.
a): In the 1,000 mg/kg group of the dose-finding study, since all animals died by dosing day 2, measurement of organ weights was not conducted.
b): Body weight after overnight starvation following the last dosing.
c): Absolute weight.
d): Relative weight (g or mg/100 g body weight).
* : Significantly different from the control group ($p<0.05$).
** : Significantly different from the control group ($p<0.01$).

Table 3. Blood chemical findings after dosing period in 18-day study of 3-methylphenol in newborn rats (main study)

Dose (mg/kg)	0	30	100	300
Males				
No. of animals	6	6	6	6
GOT (IU/L)	127±13	121±7	121±11	132±22
GPT (IU/L)	24±4	21±4	21±3	21±3
γ -GTP (IU/L)	0.84±0.24	0.90±0.15	1.07±0.11	1.19±0.15**
Total bilirubin (mg/dL)	0.40±0.03	0.41±0.04	0.41±0.03	0.47±0.02**
Total cholesterol (mg/dL)	74±11	78±9	81±7	85±9
Triglyceride (mg/dL)	29±10	25±6	32±3	28±7
BUN (mg/dL)	13.5±1.8	11.8±2.1	13.0±2.1	17.9±3.6*
Females				
No. of animals	6	6	6	6
GOT (IU/L)	122±15	119±12	131±9	116±10
GPT (IU/L)	16±2	19±4	19±4	17±2
γ -GTP (IU/L)	0.93±0.21	0.85±0.10	0.98±0.26	1.20±0.14
Total bilirubin (mg/dL)	0.41±0.04	0.40±0.03	0.40±0.02	0.45±0.03
Total cholesterol (mg/dL)	77±11	77±10	75±8	78±12
Triglyceride (mg/dL)	24±5	26±2	25±3	23±3
BUN (mg/dL)	13.5±2.3	13.5±2.5	13.2±2.3	14.2±2.8

Data are mean ± SD values.
* : Significantly different from control group ($p<0.05$).
** : Significantly different from the control group ($p<0.01$).

Table 4. Clinical signs and mortality in repeated dose studies of 3-methylphenol in young rats

	Dose-finding study (14-day)				Main study		
Dose (mg/kg)	125	250	500	1,000	100	300	1,000
Males							
No. of animals	5	5	5	5	7	7	14
No. of dead animals	-	-	-	-	-	-	-
No. of animals with clinical signs							
Salivation	-	-	-	3	-	-	11
Tremors	-	-	-	3	-	-	12
Prone/lateral position	-	-	-	1	-	-	1
Soiled perigenital fur	-	-	-	-	-	-	-
Females							
No. of animals	5	5	5	5	7	7	14
No. of dead animals	-	-	-	-	-	-	-
No. of animals with clinical signs							
Salivation	-	-	-	-	-	-	8
Tremors	-	-	-	4	-	-	13
Prone/lateral position	-	-	-	2	-	-	2
Soiled perigenital fur	-	-	-	-	-	-	2

- : No animals with clinical sign.

dose in the Test Guideline of 1,000 mg/kg was selected as the top dose for the main study, and 300 and 100 mg/kg were derived by division.

In the main study, deaths did not occur even at 1,000 mg/kg (Table 4). Salivation and tremors were observed throughout the dosing period at only 1,000 mg/kg in most males and females. At this dose, body weights were significantly lowered (finally 9% lower than controls for males and 11% for females) throughout the dosing period in males and from dosing day 14 in females, and food consumption was transiently lowered during the early dosing period in both sexes. At dosing week 4, increases in water consumption and urine volume were found in males and lowering of urinary pH in both sexes in the 1,000 mg/kg group. At 100 and 300 mg/kg, no changes in clinical signs, body weight, food consumption and urinalysis data were observed. Blood biochemical examination showed only slight increases in total cholesterol and BUN in males with a tendency for increase in total cholesterol in females receiving 1,000 mg/kg (Table 5). No dose-related changes in hematological findings were observed in any 3-methylphenol-treated group. There were significant increases in relative liver weights of both sexes at 1,000 mg/kg and of females at 300 mg/kg and in relative kidney weights of females at 1,000 mg/kg (Table 6). However, there was no change in absolute organ weights in any 3-methylphenol-treated group. On histopathological examination, no dose-related changes were observed in any of the 3-methylphenol-treated groups. At the end of the recovery period, no significant changes in any parameters were observed.

Based on clinical signs of neurotoxicity with lowering of body weights, the unequivocally toxic level was concluded to be 1,000 mg/kg/day. Increase in relative liver weight without related changes at 300 mg/kg in the main study was not considered as an adverse effect. In the dose-finding study, effects on liver were noted at 500 mg/kg but no dose-related changes were evident at 250 mg/kg, which could not be taken into consideration of the estimation of the NOAEL because of the insufficient dosing period (14 days). Therefore, the NOAEL was concluded to be 300 mg/kg/day.

DISCUSSION

Concerning health of infants exposed to chemicals, our testing project has provided the following benefits. First, detailed examination of physical development and sexual maturation during the early postnatal period provides specific information on chemical toxicity towards newborn animals. Second, because the same experimental conditions, as much as possible, are set between newborn and young rat studies, this facilitates comparisons of toxicity. Furthermore, for toxicity levels, two additional analyses (estimation of unequivocally toxic levels in addition to NOAELs and careful incorporation of the dose-finding study) allow more precise / appropriate comparisons. So far, we have reported three comparative analyses of 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol [16-18, 22, 28, 35]. As results, the toxicity profiles of these chemicals were similar in both ages, the susceptibility of newborn rats was 2 to 4 times higher than that of young rats, and no effects on physical development, sexual maturation and reflex ontogeny were observed.

In the present study, 3-methylphenol was selected as a fourth chemical. Clinical signs, indications of neurotoxicity to the central nervous system, were observed in both ages but not at the same dose level. Decrease in body weight gain also occurred in both ages but at a 3 times lower dose in

Table 5. Blood chemical findings after dosing period in repeated dose studies of 3-methylphenol in young rats (main study)

Dose (mg/kg)	0	100	300	1,000
Males				
No. of animals	7	7	7	7
GOT (IU/L)	68.6±4.8	65.4±5.4	62.7±3.2	59.4±5.4**
GPT (IU/L)	24.7±2.9	25.4±3.7	27.0±2.9	28.0±3.7
γ -GTP (IU/L)	0.17±0.24	0.21±0.13	0.60±1.15	0.36±0.23
Total bilirubin (mg/dL)	0.056±0.005	0.049±0.007	0.054±0.010	0.050±0.008
Total cholesterol (mg/dL)	52.7±15.1	58.1±11.8	58.3±5.8	69.0±9.4*
Triglyceride (mg/dL)	43.7±19.8	54.7±22.4	37.6±3.1	50.0±26.9
BUN (mg/dL)	13.89±1.46	14.10±0.85	14.56±1.17	16.23±2.14*
Females				
No. of animals	7	7	7	7
GOT (IU/L)	57.1±4.3	65.9±3.6	62.0±5.7	59.1±3.1
GPT (IU/L)	20.6±2.2	21.4±2.9	18.9±3.1	20.1±4.2
γ -GTP (IU/L)	0.83±0.20	0.90±0.16	1.00±0.29	1.06±0.10
Total bilirubin (mg/dL)	0.053±0.011	0.056±0.011	0.043±0.008	0.054±0.008
Total cholesterol (mg/dL)	63.4±14.0	58.7±10.6	61.4±10.3	78.7±13.7
Triglyceride (mg/dL)	15.4±8.2	11.3±4.8	9.9±1.8	16.1±5.2
BUN (mg/dL)	17.71±1.96	16.63±1.11	17.30±2.14	18.03±2.00

Data are mean ± SD values.
* : Significantly different from control group ($p<0.05$).
** : Significantly different from the control group ($p<0.01$).

Table 6. Organ weights after dosing period in repeated dose studies of 3-methylphenol in young rats (main study)

Dose (mg/kg)	0	100	300	1,000
Males				
No. of animals	7	7	7	7
Body weight[a)] (g)	325.0±23.5	345.6±23.5	335.9±16.7	298.3±31.8
Brain (g)	2.04±0.06[b)]	2.11±0.09	2.03±0.08	2.05±0.06
	(0.63±0.05[c)])	(0.61±0.03)	(0.60±0.02)	(0.69±0.06*)
Liver (g)	10.55±1.30	11.28±1.08	11.29±0.68	10.94±2.01
	(3.24±0.22)	(3.26±0.19)	(3.36±0.11)	(3.65±0.34**)
Kidney (g)	2.65±0.24	2.82±0.24	2.78±0.19	2.61±0.23
	(0.82±0.04)	(0.82±0.03)	(0.83±0.06)	(0.88±0.05)
Testis (g)	2.96±0.31	3.06±0.21	2.95±0.30	2.93±0.22
	(0.91±0.11)	(0.89±0.08)	(0.88±0.10)	(0.99±0.14)
Females				
No. of animals	7	7	7	7
Body weight (g)	210.1±15.4	207.6±13.0	197.3±19.3	186.4±17.4*
Brain (g)	1.96±0.06	1.90±0.07	1.89±0.07	1.88±0.06
	(0.93±0.06)	(0.92±0.05)	(0.96±0.08)	(1.01±0.09)
Liver (g)	6.39±0.68	6.59±0.56	6.60±0.67	6.51±0.45
	(3.04±0.17)	(3.17±0.08)	(3.35±0.13**)	(3.50±0.20**)
Kidney (g)	1.66±0.18	1.73±0.11	1.65±0.17	1.72±0.14
	(0.79±0.06)	(0.84±0.05)	(0.84±0.06)	(0.92±0.03**)
Ovary (mg)	81.4±13.7	82.0±14.3	85.0±14.0	78.9±13.3
	(38.8±6.3)	(39.5±6.1)	(43.4±8.3)	(42.4±6.8)

Data are mean ± SD values.
a): Body weight after overnight starvation following last dosing.
b): Absolute weight.
c): Relative weight (g or mg/100 g body weight).
* : Significantly different from control group ($p<0.05$).
** : Significantly different from control group ($p<0.01$).

newborn animals. In the newborn study, significant decrease in absolute brain weight was also evident at the highest dose, but no abnormalities on histopathology in the brain or in terms of functional development (reflex ontogeny) were observed. Brain weight changes were observed only in the groups showing 10% and more lowering of body weight and were not noted in the dose-finding study. Brain weight might be affected by decrease in body weight gain. As unequivocally toxic levels were clearly judged to be 300 mg/kg/day and 1,000 mg/kg/day for newborn and young studies, respectively, based on neurotoxic effects and decrease in body weight gain, newborn rats were considered to be approx. 3 times more susceptible to this chemical than young rats. NOAELs were concluded to be 30 mg/kg/day and 300 mg/kg/day for newborn and young rats, respectively, indicating a 10 times higher susceptibility in the newborn. However, tremors under contact stimulus were observed in only three males on single days and hypersensitivity on handling was noted only in one male on a single day in the 100 mg/kg newborns. Furthermore, no such toxic clinical signs were noted at 100 mg/kg in the dose-finding study under the same experimental conditions. It appears that the realistic no adverse effect dose for the newborn is slightly lower than 100 mg/kg/day rather than around 30 mg/kg/day. Based on this speculation and equal toxicity at the unequivocally toxic levels, the difference in the sensitivity to 3-methylphenol between newborn and young rats could be considered to be 3- to 4-fold.

As for the toxicity of 3-methylphenol, much information is available including unpublished data reported in reviews on this chemical or cresols [1, 9, 12]. In a 28-day feeding study [23], F344 rats were given diet containing 3-methylphenol at 0, 300, 1,000, 3,000, 10,000, 30,000 mg/kg diet. Depression of body weight gain, increase in relative liver and kidney weight and uterus atrophy were observed at 30,000 mg/kg diet (about 2,390 mg/kg/day). Increase in relative liver weight was also noted at 10,000 mg/kg diet (866 mg/kg/day). These results are consistent with our present results for young rats. However, clinical signs observed in our young rat study (daily administration by gavage) were not found at any doses in this NTP study, which might be due to the lower blood concentration with dietary application than in our gavage study. In a 90-day study [20], SD rats were administered 3-methylphenol by gavage at 50, 150, 450 mg/kg. In addition to depression of body weight gain at 150 mg/kg and more, a pronounced increase in the incidence of salivation, tremors and urination was observed at 450 mg/kg. In another 90-day study under the same test conditions with more detailed neurotoxic analysis, hypoactivity, rapid labored respiration and excessive salivation were observed sporadically in all treated groups, although few significant changes were found in performance on neurobehavioral test batteries, and no brain weight changes and no gross or histopathological changes in the brain or other nervous tissues [32]. These clinical signs observed at lower doses than our young rat study might be due to the longer dosing period, but no information was provided on the incidence or dose-relationship. As for developmental toxicity, no effects on fetuses were observed in rats treated with 3-methylphenol by gavage at 450 mg/kg or less on days 6-15 of gestation [5]. However, in a 2-generation reproductive toxicity study on rats by gavage [7], some effects on pup body weights and survival (no details on the incidence and the degree) were evident with 450 mg/kg, which caused severe maternal toxicity including death and various clinical signs. There were occasional body weight changes in lower dose groups, but it is not clear whether these changes were treatment-related.

Some causes of differences in susceptibility of newborn and young rats to 3-methylphenol can be considered, such as specific physiological characteristics and immaturity of the brain-blood barrier and metabolism in the newborn. It is reported that 3-methylphenol is mainly eliminated as glucuronides in urine [4]. UDP-glucuronyltransferase activity in rat liver is known to be substrate-specific and generally low in neonates, and the activity against phenolic substances, p-nitrophenol and 1-naphthol, at birth has been shown to be comparable to adults but nearly 50% lower during the suckling period (exposure period in our newborn study) [25, 34]. Therefore, the low capacity of glucuronidation might be one of the major causes for higher susceptibility of newborn rats to 3-methylphenol. In the case of humans, hepatic glucuronidation at birth is known to be relatively immature [11], and it has been shown that in vitro bilirubin glucuronidation activity at birth is much lower than that of mature-phase values [13]. These data suggest that human infants may be more susceptible to chemicals that are detoxified by this pathway.

The effects on the central nervous system, leading to death, are a major toxicological outcome characteristic of some phenolic compounds [16, 18]; however, the mechanism(s) responsible for eliciting neurotoxicity is unknown. As for hepatotoxicity, several studies on the mechanism and the structure activity relationship, using hepatocytes or liver slices, have been reported for three isomers of methylphenols and some para-alkylphenols [3, 15, 29-31]. In these studies, it has been shown that the quinone intermediates are most likely to be the causative agents for hepatotoxicity, possibly via mitochondrial toxicity, and the hepatotoxicity of alkylphenols depends on the position and the kind of alkyl groups with 4-methylphenol exerting the greatest degree of hepatotoxicity. In the case of 3-methylphenol, the neurotoxicity seems to be the most sensitive endpoint in both newborn and young animals, since only minor increases in relative liver weight have been observed without any histopathological changes.

In conclusion, 3-methylphenol showed the same toxicity profile -that is neurological symptoms and growth inhibition- in both newborn and young rats. However, the susceptibility of the newborn rats was 3 to 4 times higher than that of young rats, consistent with our previous results for three chemicals, 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol, which showed 2 to 4 times differences in susceptibility between newborn and young rats.

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CHAPTER 3

Reproduction Toxicology Related To

Comparison of Reproductive Parameters between Crj:CD(SD)and Crj:CD(SD)IGS Rats (II)

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ABSTRACT. A reproductive study (breeding study) using international gold standard rats was conducted under the environmental and breeding conditions at The Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd., to collect data on reproductive functions and the development (body weight, necropsy findings, estrous cycle test results, mating results, sperm examination results, cesarean section examination results) of embryos and fetuses. The results showed that the Crj:CD(SD)IGS rats used in this study exhibited almost the same values as the background data (BG) for Crj:CD(SD) rats in all examinations. —Key words: reproductive parameters, Crj:CD(SD), Crj:CD(SD)IGS

CD(SD)IGS-2002/2003 : 135-140

INTRODUCTION

A reproductive study (breeding study) using international gold standard rats was conducted under the environmental and breeding conditions at the Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd., to collect data on reproductive functions and development of embryos and fetuses.

MATERIALS AND METHODS

1. *Animals*

Male and female Crj:CD(SD)IGS rats (Charles River Japan, Inc.) were purchased at 6 weeks of age and used at 8 weeks of age for the males and 10 weeks of age for the females. The animals were kept in a breeding room with a barrier system set at a temperature of 24±2℃ and relative humidity of 55±10%, with lighting for 12 hours (7:00 a.m. to 7:00 p.m.) and ventilation 13 to 15 times/hour. As food, rat chow (MF, Oriental Yeast, Co., Ltd.) was given *ad libitum*, and as a drinking water, well water was given *ad libitum* through an automatic water feed system.

2. *Observation*

For both the males and females, clinical observation and confirmation of life or death were conducted every day, and body weight was determined twice a week from the day following group assignment to the start of mating and on Days 0, 4, 7, 10, 14, 17 and 20 of gestation during pregnancy. As concerns, for the females, a vaginal smear was collected at a set time every morning from the day following group assignment to establishment of copulation to conduct a estrous cycle test, but the results during the mating period were used as reference data.

Mating was conducted by allowing a male and a female (aged 12 weeks) to mate overnight, and the females in which sperm or a vaginal plug were identified in the vaginal smear on the next day were considered to have copulated, and that day was defined as Day 0 of gestation. In addition, mating was conducted within the same group, and the mating period was set at 2 weeks at the longest. After the end of the mating period, the number of days required for copulation, the copulation rate and the fertility rate were calculated.

The animals for which copulation was confirmed were exsanguinated to death by cutting down the lateral iliac artery under anesthesia with ether after the end of the mating period for males and on Day 20 of gestation for females, and the organs and tissues were then observed macroscopically.

As concerns the males, the tail of the extracted right epididymis was minced in Hank's Balanced Salt Solution (containing 0.5% BSA) to allow the sperm to migrate (stock sperm solution), and the motile sperms and non- motile sperms were then counted to calculate the sperm motility rate. Subsequently, the stock sperm solution was diluted with 0.5% formalin-physiological saline, and the number of sperm was calculated using Thoma's cytometer to calculate the number of sperms per 1 g of the tail of epididymis. A smear specimen was then prepared from the stock sperm solution, the shape of the sperm was observed using an optical microscope, and the sperm form anomalies index was calculated.

For the females, the ovaries and uterus were extracted, the number of corpus lutea, the number of implantations, the number of early resorbed embryos, the number of late resorbed embryos, the number of dead fetuses and the number of live fetuses were determined, and the pre-implant loss rate, the early resorption rate, the late resorption rate, the rate of dead fetuses and the total fetal mortality were calculated.

As concerns the sexes of the live fetuses were differentiated to calculate the sex ratio (male/female), and the body weights of the live male and female fetuses were determined. Furthermore, visceral examination was conducted on half of the live fetuses to calculate the incidence of fetuses with visceral abnormalities and the incidence of fetuses with differential visceral abnormalities. As concerns the remaining live fetuses, a skeletal examination was conducted to calculate the incidence of fetuses with skeletal anomalies, the incidence of fetuses with skeletal variations, the incidence of the fetuses with differential skeletal anomalies, the incidence of the fetuses with differential skeletal variations, the progress of sternal ossification and the progress of phalangeal ossification.

3. *Statistical Analysis*

The mean value and standard deviation were determined for each group for body weight, estrous cycle, count of estruses, number of days required for copulation, number of sperms, the sperm motility rate, number of corpus lutea, number of

implantations, count of live fetuses, body weights of male and female fetuses (the mean value for each dam was used) and number of vertebral bodies and vertebral arches (the mean value for each dam was used). In addition, the measured values with respect to the fetuses were treated for each litter.

RESULTS AND CONCLUSIONS

The changes in body weight are shown in Table 1. The weight gain for males from 4 weeks before the start of mating to the day of the start of mating was 143.2 to 147.9 g, which was slightly larger than the BG (86.7 to 135.7 g). The weight gain for females was 28.7 to 29.1 g from 2 weeks before the start of mating to the day before the start of mating and 146.5 to 148.3 g from Days 0 to 20 of gestation, which were almost within the range of the BG.

The necropsy findings are shown in Table 2. Diaphragmatic hernia in liver and light yellow nodule in the epididymis were observed in 1 male each and accessory spleen and atrophy in the eye ball were observed in 1 female each. The incidence of this change was almost the same as that of the BG.

The results of the estrous cycle test are shown in Table 3. The mean count of estruses was 3.3 to 3.4 times, which was slightly lower than the BG (3.73 to 3.81 times). In addition, the estrous cycle was 4.08 to 4.72 days, which was within the range of the BG.

The results of the fertility study (results of mating) are shown in Table 4. The copulation rate and the fertility rate were 100% and 95% respectively. And the number of days required for copulation was 1.75 to 2.55 days. These values were almost within the range of the BG.

The results of the sperm examination are shown in Table 5. The number of sperm was 447.7×10^6 to 486.4×10^6, the sperm motility rate was 89.1 to 92.5%, and the sperm form anomalies index was 1.65 to 6.37%. The value of sperm form anomalies index indicated the high value to be to 6.37%, but the value except one animal is 1.53%, and showed the almost same range of the BG.

The results of the examination at cesarean section are shown in Table 6. The number of corpora lutea and the number of implantations were 16.26 to 16.47 and 15.47 to 16.16, respectively, both of which were within the range of the BG. In addition, the pre-implant loss rate was 1.92 to 4.85%, which tended to show a slightly lower value than the BG (3.34 to 16.37%). Total fetal mortality was 3.40 to 4.23%, which was almost the same as that of the BG. The early resorption rate, the late resorption rate and the number of live fetuses were 3.40 to 4.23%, 0% and 0%, respectively, which were the same as those of the BG. The mean sex ratio was 0.79 to 0.86, and the mean F1 body weight was 3.55 to 3.70 g for males and 3.39 to 3.50 g for females, both of which were within the range of the BG.

The results of the external examination are shown in Table 7. There were no significant differences in external examination.

The results of the visceral examination are shown in Table 8. Ventricular septal defect occurred in 0 to 0.66%, dilatation of the ureter in 0.66 to 3.36%, and thymic remnant in the neck in 0 to 1.32%. The incidences of visceral anomalies were within the range of the BG.

The results of the skeletal examination are shown in Tables 9 and 10. No skeletal anomaly was observed in any examination. As skeletal variations, lumbar rib, splitting of the sternebra, splitting of the vertebral body and shortened 13th rib were observed, but the incidences of these mutations were within the range of the BG.

In the examination of the progress of ossification, the number of ossified cervical vertebral bodies was 0.50 to 1.03, which showed earlier ossification than the BG (0.10 to 0.49). In the sternebra, the degrees of ossification of the fifth and sixth sternebra were 69.01 to 84.44% and 94.81 to 98.59%, respectively, and the degrees of ossification tended to be slightly earlier than the BG (55.26% to 85.27% and 81.12% to 98.63%, respectively) for this strain. In the phalanxes of the hind limbs, the degrees of ossification were 0 to 0.30% for the left distal phalanx and 0 to 0.89% for the right distal phalanx, which tended to be slightly delayed as compared with the BG (0.75% to 34.23% and 0.67% to 31.70%, respectively). The degree of ossification for other test items was within the range of the BG.

Based on the above, the Crj:CD(SD)IGS rats used in this study showed almost the same results as the BG for all test items.

Table 1. Body weight gains (g) in rats

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of animals	20	20	5～36
Days of pre-mating			
male（4w）	143.2	147.9	86.7～135.7
female（2w）	28.7	29.1	16.6～32.7
Days of gestation			
	146.5	148.3	122.9～149.1

Table 2. Necropsy findings in rats

	Crj:CD(SD)IGS				Crj:CD(SD)
Organs and findings	Male		Female		−
Group	1	2	1	2	−
Number of animals	20	20	20	20	5〜36
Digestive system					
Liver					
Diaphragmatic hernia	0	1(5.00)	0	0	(0〜10.0)
Hematopoietic system					
Spleen					
Accessory spleen	0	0	0	1(5.00)	0
Genital system					
Epididymis					
nodule, light yellow	1(5.00)	0	0	0	0
Nervous system					
Eye ball					
atrophy	0	0	1(5.00)	0	0

( ): %

Table 3. Estrous cycle in female rats

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of animals examined	20	20	5〜36
Count of estrus	3.30±0.47[a)]	3.40±0.82	3.73〜3.81
Estrous cycle (days)	4.08±0.28	4.72±2.31	3.98〜5.72

a) Mean±S.D.

Table 4. Mating and pregnancy in rats

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of mated animals			
Male	20	20	11〜25
Female	20	20	11〜25
No. of copulated animals			
Male	20(100)	20(100)	(83.33〜100)
Female	20(100)	20(100)	(83.33〜100)
No. of impregnated	19(95.00)	19(95.00)	(89.47〜100)
No. of pregnant animals	19(95.00)	19(95.00)	(85.1〜100)
Duration of mating	1.75±1.41	2.55±1.28	2.07〜2.95

( ): %

Table 5. Examination of sperm in male rats

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of animals examined	20	20	6～25
Count of sperm($\times 10^6$/g)	486.4±75.5[a)]	447.7±108.7	495.5～557
Sperm motility(%)	89.1±20.7	92.5±3.8	88.0～95.3
Sperm form anomalies index(%)	6.37±21.70	1.65±1.34	1.41

a) Mean±S.D.

Table 6. Findings at cesarean section in F_0 dams and fetuses

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of dams	19	19	6～25
No. of corpora lutea	309(16.26±1.97)[a)b)]	313(16.47±1.54)	(15.32～18.84)
No. of implants	294(15.47±2.01)[a)b)]	307(16.16±1.30)	(13.5～17.57)
No. of pre-implant loss	15(4.85)[c)]	6(1.92)	(3.34～16.37)
No. of total dead fetuses	10(3.40)[d)]	13(4.23)	(3.50～8.48)
Early resorptions	10(3.40)[d)]	13(4.23)	(3.50～8.80)
Late resorptions	0	0	(0～0.27)[d)]
Dead fetuses	0	0	(0～0.27)[d)]
No. of live fetuses	284(14.95±1.96)[a)b)]	294(15.47±1.54)	(12.82～16.61)
Sex ratio of live fetuses	0.86(131/153)[e)]	0.79(130/164)	0.71～1.36
Body weight of live fetuses (g)			
Male	3.70±0.29[a)]	3.55±0.27	3.28～3.85
Female	3.50±0.27	3.39±0.25	3.10～3.65

a) Mean±S.D.
b) No. per dam
c) % for No. of corpora lutea
d) % for No. of implants
e) ( ): male/female

Table 7. External examination of F_1 fetuses

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of fetuses examined	284	294	80～374
No. of fetuses with external anomalies	0	0	(0～0.95)

( ): % for No. of fetuses examined

Table 8. Visceral examination in F_1 fetuses

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of fetuses examined	149	152	33～151
No. of fetuses with visceral anomalies	5(3.36)	4(2.63)	(0.79～11.11)
Ventricular septal defect	0	1(0.66)	(0～0.79)
Dilatation of the ureter	5(3.36)	1(0.66)	(0～8.66)
Dilatation of the renal pelvis and ureter	0	0	(0～1.68)
Thymic remnant in the neck	0	2(1.32)	(0～6.30)

( ): % for No. of fetuses examined

Table 9. Skeletal examination in F_1 fetuses

	Crj:CD(SD)IGS		Crj:CD(SD)
Group	1	2	
No. of fetuses examined	135	142	59～245
No. of fetuses with skeletal anomalies	0	0	(0～1.40)
No. of fetuses with skeletal variations	18(13.33)	10(7.04)	(1.69～22.07)
Lumbar rib	16(11.85)	7(4.93)	(0.70～22.07)
Unilateral	11(8.15)	4(2.82)	(0.43～10.39)
Bilateral	5(3.70)	3(2.11)	(0～12.21)
Splitting of the sternebra	1(0.74)	0	(1～2.08)
Splitting of the vertebral body	1(0.74)	1(0.70)	(0～3.23)
Shortened 13th rib	0	1(0.70)	(0～2.80)
Lumbar rib and splitting of the vertebral body	0	1(0.70)	(0～1.33)

( ): % for No. of fetuses examined

Table 10. Skeletal examination(progress of ossification) in F_1 fetuses

		Crj:CD(SD)IGS		Crj:CD(SD)
Group		1	2	
No. of fetuses examined		135	142	58～245
Vertebrae				
Cervical	arch (R)	7.00±0.00[a)]	7.00±0.00	7.00
	arch (L)	7.00±0.00	7.00±0.00	7.00
	body	1.03±0.75	0.50±0.46	0.10～0.49
Thoracic	arch (R)	13.00±0.00	13.00±0.00	12.98～13.00
	arch (L)	13.00±0.00	13.00±0.00	12.98～13.00
	body	13.00±0.00	13.00±0.00	12.92～13.00
Lumbar	arch (R)	6.00±0.00	6.00±0.00	5.99～6.03
	arch (L)	6.00±0.00	6.00±0.00	6.00～6.03
	body	6.00±0.00	6.00±0.00	5.99～6.03
Sacrocaudal	arch (R)	6.00±0.00	5.89±0.17	6.00～6.03
	arch (L)	5.97±0.21	5.88±0.17	5.50～5.99
	body	8.01±0.41	7.90±0.30	7.64～8.04
Sternebrae				
1st		135(100)[b)]	142(100)	(99.56～100)
2nd		135(100)	142(100)	(97.10～100)
3rd		135(100)	142(100)	(99.51～100)
4th		135(100)	142(100)	(98.76～100)
5th		114(84.44)	98(69.01)	(55.26～85.27)
6th		128(94.81)	140(98.59)	(81.12～98.63)
Metacarpus	(R)	528(78.22)	551(77.61)	(69.78～79.96)
Metacarpus	(L)	534(79.11)	554(78.03)	(69.41～79.91)
Phalanges of forelimb				
Proximal	(R)	109(16.15)	80(11.27)	(0.67～31.70)
Proximal	(L)	98(14.52)	66(9.30)	(0.75～34.23)
Middle	(R)	0	0	(0～0.11)
Middle	(L)	0	0	(0)
Distal	(R)	671(99.41)	710(100)	(97.04～100)
Distal	(L)	674(99.85)	710(100)	(97.97～100)
Metatarsus	(R)	546(80.89)	571(80.42)	(79.66～86.17)
Metatarsus	(L)	547(81.04)	572(80.56)	(79.74～86.87)
Phalanges of hindlimb				
Proximal	(R)	2(0.30)	0	(0.75～34.23)
Proximal	(L)	6(0.89)	0	(0.67～31.70)
Middle	(R)	0	0	(0)
Middle	(L)	0	0	(0)
Distal	(R)	668(98.96)	708(99.72)	(96.10～98.96)
Distal	(L)	664(99.10)[c)]	710(100)	(99.10～100)

a) Mean±S.D.
b) ( ): % for No. of fetuses examined
c) Technical error

Comparison of Background Data Between Crj: CD (SD) IGS Rats and Crj: WI (Glx/BRL/Han) IGS Rats in Reproductive/Developmental Toxicity Studies

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ABSTRACT. Background data obtained in reproductive/developmental toxicity studies in Crj: WI (Glx/BRL/Han) IGS rats were compared with those in Crj: CD (SD) IGS rats.

Examination of Offspring: The number of implantation scars and number of live offspring in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the sex ratio, number of stillbirths, birth index, viability index, or weaning index between these strains of rats. No offspring with external abnormalities were noted in either strain of rats. Body weights of offspring of both sexes in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. Compared with Crj: CD (SD) IGS rats, separation of the eyelids and cleavage of the balanopreputial gland were delayed about 1 day in Crj: WI (Glx/BRL/Han) IGS rats. No differences were seen in reflexes, emotionality, or learning ability between these strains of rats.

Examination for Copulation and Fertility: No differences were seen in the copulation index or fertility index between Crj: WI (Glx/BRL/Han) IGS rats and Crj: CD (SD) IGS rats.

Sperm Analysis: The progressive sperm rate and survivability rate in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the motile sperm rate, path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, abnormal sperm rate, or number of sperms between these strains of rats.

Observation of Fetuses: The number of corpora lutea, number of implantation sites, and number of live fetuses in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the pre-implantation loss rate, post-implantation loss rate, or sex ratio between these strains of rats. Fetal body weights of both sexes in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. In the morphological observation, the incidence of supernumerary lumbar vertebra, which is a skeletal abnormality, and the incidences of full supernumerary rib, short supernumerary rib, and cervical rib, which are skeletal variations, in Crj: WI (Glx/BRL/Han) IGS rats were higher than those in Crj: CD (SD) IGS rats. No differences were seen in the incidence of external abnormalities, incidence of visceral abnormalities, or degree of ossification between these strains of rats.

Since any abnormalities which deserve special mention were not noted in the results of the reproductive/developmental toxicity studies in either strain of rats, as described above, it is considered that both Crj: CD (SD) IGS rats and Crj: WI (Glx/BRL/Han) IGS rats are suited to be used in reproductive/developmental toxicity studies. —Key words: Crj: CD (SD) IGS rats, Crj: WI (Glx/BRL/Han) IGS rats, development, reproduction, sperm analysis

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INTRODUCTION

Crj: CD (SD) IGS rats are commonly used in reproductive/developmental toxicity studies performed in the testing facility, and the background data obtained from the studies have already been reported [1] [2] [3]. Recently, Charles River Japan Inc. has started reproduction of Crj: WI (Glx/BRL/Han) IGS rats. To use this strain of rats in reproductive/developmental toxicity studies performed in the testing facility, we collected background data on Crj: WI (Glx/BRL/Han) IGS rats and compared our data obtained in the examination of offspring, examination for copulation and fertility, sperm analysis, and examination of fetuses between Crj: CD (SD) IGS rats (1999 - 2003) and Crj: WI (Glx/BRL/Han) IGS rats (2002 - 2003).

MATERIALS AND METHODS

1. *Examination of Offspring*

For the examination, we used 212 pregnant female Crj: CD (SD) IGS rats which had been used as control animals in 13 studies (1999 - 2003) and 23 pregnant female Crj: WI (Glx/BRL/Han) IGS rats which had been used as control animals in 1 study (2002 - 2003).

The animals were housed in stainless steel cages in an animal room with a 12-hour light and dark cycle (lighting: 6:00 a.m. - 6:00 p.m.), a temperature range of 20 - 26℃, a relative humidity range of 40 - 70%, and air changes of 12 times per hour. From Day 20 of pregnancy onward, dams were housed in plastic cages floored with a substrate of autoclaved wood chips (Sun Flake, Charles River Japan Inc.), where they were allowed to deliver spontaneously and rear their offspring. The animals were given free access to pellet feed (CRF-1 or CR-LPF, Oriental Yeast Co., Ltd.) and to tap water.

Males (12 - 16 weeks old) and females (12 - 14 weeks old) were paired and put together on a one-to-one basis from the evening to the next morning. The day when sperm was found in the vaginal smear was defined as Day 0 of pregnancy.

Delivery conditions were observed 3 times a day (9:00 a.m., 1:30 p.m., and 3:00 p.m.) from Day 21 of pregnancy onward, and nursing conditions were observed once a day until 21 days after delivery. Dams were sacrificed 21 days after delivery by bleeding from the abdominal aorta under diethyl ether anesthesia and necropsied to count the number of implantation scars.

The numbers of live offspring and stillbirths were counted after completion of delivery, and the live offspring were sexed and observed for external abnormalities. Offspring were weaned 21 days after birth. Adjustment of the number of offspring per litter was performed 4 days after birth by random selection normally of 4 offspring of each sex in each litter.

Offspring were weighed 0 days after birth (day of birth) and 4, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days after birth. Morphological differentiation was observed for separation

of the auricles 4 days after birth, for eruption of the incisors and separation of the eyelids 14 days after birth, for opening of the vagina 33 days after birth, and for cleavage of the balanopreputial gland 41 days after birth. Reflexes were observed for righting reflex 6 days after birth, for negative geotaxis (a slanting board used) 10 days after birth, and for free-fall reflex, pinna reflex, and corneal reflex 17 days after birth.

Offspring were examined for emotionality 4 weeks after birth by the open-field test [4], in which frequency of ambulation, frequency of rearing, frequency of face-washing, frequency of grooming, number of fecal pellets, and frequency of urination during 3 minutes and latency of first movement were counted.

Offspring were examined for learning ability from 6 to 7 weeks after birth by the water multiple T-maze method, in which the animals were trained in a straight waterway 3 times on the 1st day and tested in a water maze 4 times a day for the next 3 days. Each trial was continued for 3 minutes at the longest. The time necessary to reach the goal, selection errors, backing errors, and intra-zonal errors were recorded, and the total errors were calculated for each day.

2. *Examination for Copulation and Fertility*

For the examination, we used 172 Crj: CD (SD) IGS rats of each sex which had been used as control animals in 9 studies (1999 - 2003) and 46 Crj: WI (Glx/BRL/Han) IGS rats of each sex which had been used as control animals in 1 study (2002 - 2003).

The animals were housed in stainless steel cages in an animal room with a 12-hour light and dark cycle (lighting: 6:00 a.m. - 6:00 p.m.), a temperature range of 20 - 26℃, a relative humidity range of 40 - 70%, and filter-sterilized fresh air changes of 12 times per hour. The animals were given free access to solid feed (CRF-1 or CR-LPF, Oriental Yeast Co., Ltd.) and to tap water.

The females were observed for estrous cycles by vaginal smear test daily for 14 days from 10 or 12 weeks after birth onward. Males (12 - 16 weeks old) and females (12 - 14 weeks old) were paired and put together on a one-to-one basis from the evening to the next morning. The day when sperm was found in the vaginal smear was defined as Day 0 of pregnancy. Females which had copulated were sacrificed on Day 13 or 20 of pregnancy by bleeding from the abdominal aorta under diethyl ether anesthesia and necropsied to confirm pregnancy by the presence of implantation.

3. *Sperm Analysis*

For the analysis, we used 124 male Crj: CD (SD) IGS rats which had been used as control animals in 8 studies (1999 - 2003) and 46 male Crj: WI (Glx/BRL/Han) IGS rats which had been used as control animals in 1 study (2002 - 2003).

The animals were housed in stainless steel cages in an animal room with a 12-hour light and dark cycle (lighting: 6:00 a.m. - 6:00 p.m.), a temperature range of 20 - 26℃, a relative humidity range of 40 - 70%, and filter-sterilized fresh air changes of 12 times per hour. The animals were given free access to solid feed (CRF-1 or CR-LPF, Oriental Yeast Co., Ltd.) and to tap water.

The animals were sacrificed at the age of 17 - 18 weeks by bleeding from the abdominal aorta under diethyl ether anesthesia, necropsied, and then subjected to the sperm analysis. Semen samples were prepared by cutting the right cauda epididymis in culture fluid for sperm (Medium 199 with 0.5% bovine serum albumin) warmed to 37℃. The prepared semen samples were subjected to examine sperm motility, survivability, and morphology.

To examine sperm motility, the semen sample was diluted with culture fluid for sperm, and the diluted semen sample was incubated for about 30 minutes (incubation conditions: at 37℃ and aerated with a mixture of 5% CO_2 and 95% air). Using TOX IVOS (Hamilton Thorne Research), the motile sperm rate was calculated, and then the progressive sperm rate, path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, and beat cross frequency were calculated for moving sperms [3].

To examine sperm survivability following Kato et al.'s method [5], the semen sample was diluted with culture fluid for sperm 2 - 3 times in micro-sell plates, the diluted semen sample was incubated for about 2 hours (incubation conditions: at 37℃ and aerated with a mixture of 5% CO_2 and 95% air) and stained with calcein acetoxy methyl ester and ethidium homodimer-1. Sperms were classified into live sperms, sperms which died in the course of sperm analysis, and dead sperms under a fluorescence microscope to obtain the viability rate and survivability rate. Sperms whose head to tail emitted green fluorescence were regarded as live sperms. Sperms whose head and tail emitted red fluorescence and green fluorescence, respectively, were regarded as sperms which died in the course of sperm analysis. Sperms whose head emitted red fluorescence but whose tail did not emit green fluorescence were regarded as dead sperms.

To examine sperm morphology, the semen sample was smeared on a glass slide, fixed in 10 vol% neutral buffered formalin, and stained with 1% eosin solution. Morphology of sperms was observed under a microscope [3].

The number of sperms was calculated using TOX IVOS (Hamilton Thorne Research) by homogenizing the left cauda epididymis in 0.1% Triton X-100 [3]. The number of sperms per gram of left cauda epididymis was also calculated.

4. *Examination of Fetuses*

For the examination, we used 365 pregnant female Crj: CD (SD) IGS rats which had been used as control animals in 25 studies (1999 - 2003) and 44 pregnant female Crj: WI (Glx/BRL/Han) IGS rats which had been used as control animals in 1 study (2002 - 2003).

The animals were housed in stainless steel cages in an animal room with a 12-hour light and dark cycle (lighting: 6:00 a.m. - 6:00 p.m.), a temperature range of 20 - 26℃, a relative humidity range of 40 - 70%, and air changes of 12 times per hour. The animals were given free access to solid feed (CRF-1 or CR-LPF, Oriental Yeast Co., Ltd.) and to tap water.

Males (12 - 16 weeks old) and females (12 - 14 weeks old) were paired and put together on a one-to-one basis from the evening to the next morning. The day when sperm was found in the vaginal smear was defined as Day 0 of pregnancy.

Pregnant females were sacrificed on Day 20 of pregnancy by bleeding from the abdominal aorta under diethyl ether

anesthesia and necropsied to examine the numbers of corpora lutea, implantation sites, number of post-implantation losses, and number of live fetuses. Live fetuses were observed for external abnormalities, including those in the oral cavity, sexed, and weighed.

About half of the live fetuses from each dam were fixed in ethanol. The remaining live fetuses were fixed in Bouin's solution or 10 vol% neutral buffered formalin. Regarding the fetuses fixed in ethanol, cleared and stained skeletal specimens were prepared by Dawson's method [6] and examined for skeleton. Regarding the fetuses fixed in Bouin's solution or 10 vol% neutral buffered formalin, the thoracic region was examined by Nishimura's method [7], and the visceral organs were examined by Barrow's method [8].

RESULTS

1. *Examination of Offspring*

Viability from the day of birth to the day of weaning is shown in Table 1. The number of implantation scars and number of live offspring at birth in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the gestation length, sex ratio, number of stillbirths, birth index, viability index, or weaning index between these strains of rats. No external abnormalities were noted at birth in either strain of rats.

Body weights of both sexes are shown in Table 2. Body weights of both sexes of offspring in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats throughout the observation period.

Morphological differentiation is shown in Table 3. Compared with Crj: CD (SD) IGS rats, separation of the eyelids and cleavage of the balanopreputial gland were delayed about 1 day in Crj: WI (Glx/BRL/Han) IGS rats.

Reflexes are shown in Table 4. No differences were seen in the righting reflex, negative geotaxis, free-fall reflex, pinna reflex, or corneal reflex between these strains of rats.

Emotionality is shown in Table 5. No differences were seen in the frequency of ambulation, frequency of rearing, frequency of face-washing, frequency of grooming, number of fecal pellets, frequency of urination, or latency of first movement between these strains of rats.

Learning ability is shown in Tables 6 and 7. No differences were seen in the time necessary to reach the goal, selection errors, backing errors, intra-zonal errors, or the total errors observed in the water maze from the second to fourth days between these strains of rats.

2. *Examination for Copulation and Fertility*

Results of the examination for copulation and fertility are shown in Table 8. No differences were seen in the number of estrous cases before pairing, copulation index, or fertility index between Crj: WI (Glx/BRL/Han) IGS rats and Crj: CD (SD) IGS rats.

3. *Sperm Analysis*

Results of the sperm analysis are shown in Table 9. The progressive sperm rate and survivability rate in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the motile sperm rate, path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, abnormal sperm rate, number of sperms, or number of sperms per gram of left cauda epididymis between these strains of rats.

4. *Observation of Fetuses*

Results of the examination at Caesarean section are shown in Table 10. The number of corpora lutea, number of implantation sites, and number of live fetuses in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. Fetal body weights of both sexes in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the implantation rate, number of pre-implantation losses, pre-implantation loss rate, number of post-implantation losses, post-implantation loss rate, or sex ratio between these strains of rats. External abnormalities were noted at lower frequency in Crj: CD (SD) IGS rats, but none of the Crj: WI (Glx/BRL/Han) IGS rat fetuses had external abnormalities.

Results of the skeletal examination are shown in Table 11. Supernumerary lumbar vertebra, a skeletal abnormality, was noted in Crj: WI (Glx/BRL/Han) IGS rats more frequently than in Crj: CD (SD) IGS rats. Fetuses with skeletal variations and some skeletal variations, namely full supernumerary rib, short supernumerary rib, and cervical rib, were noted in Crj: WI (Glx/BRL/Han) IGS rats more frequently than in Crj: CD (SD) IGS rats. No differences were seen in degree of ossification between these strains of rats.

Results of the visceral examination are shown in Table 12. No differences were seen in the incidence of visceral abnormalities between these strains of rats.

DISCUSSION

Background data obtained in reproductive/developmental toxicity studies in Crj: WI (Glx/BRL/Han) IGS rats were compared with those in Crj: CD (SD) IGS rats.

In the examination of offspring, the number of implantation scars and number of live offspring in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the sex ratio, number of stillbirths, birth index, viability index, or weaning index between these strains of rats. No offspring with external abnormalities were noted in Crj: CD (SD) IGS rats or Crj: WI (Glx/BRL/Han) IGS rats. Body weights of both sexes of offspring in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. Compared with Crj: CD (SD) IGS rats, separation of the eyelids and cleavage of the balanopreputial gland were delayed about 1 day in Crj: WI (Glx/BRL/Han) IGS rats. No differences were seen in reflexes, emotionality, or learning ability between these strains of rats. It can be concluded that there are no problems with viability or growth of offspring of Crj: WI (Glx/BRL/Han) IGS rats, although, compared with Crj: CD (SD) IGS rats, Crj: WI (Glx/BRL/Han) IGS rats had smaller litter size, lower body weight of offspring, and delayed separation of the eyelids and cleavage of the balanopreputial gland, as described above.

In the examination for copulation and fertility, no differences were seen between Crj: WI (Glx/BRL/Han) IGS rats and Crj: CD (SD) IGS rats.

In the sperm analysis, the progressive sperm rate and survivability rate in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the motile sperm rate, path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, abnormal sperm rate, or number of sperms between these strains of rats. No differences were seen between the results of the sperm analysis in Crj: CD (SD) IGS rats obtained in the present study (1999 - 2003) and those obtained in 1997 - 1999 [3]. It can be concluded that there is no difference in copulation or fertility between Crj: CD (SD) IGS rats and Crj: WI (Glx/BRL/Han) IGS rats, although sperm motility in Crj: WI (Glx/BRL/Han) IGS rats is considered to have been slightly lower than that in Crj: CD (SD) IGS rats, as described above.

In the examination of fetuses, the number of corpora lutea, number of implantation sites, and number of live fetuses in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats. No differences were seen in the pre-implantation loss rate, post-implantation loss rate, or sex ratio between these strains of rats. Fetal body weights of both sexes in Crj: WI (Glx/BRL/Han) IGS rats were lower than those in Crj: CD (SD) IGS rats.

In the morphological observation of fetuses, the incidence of supernumerary lumbar vertebra, which is a skeletal abnormality, and the incidences of full supernumerary rib, short supernumerary rib, and cervical rib, which are skeletal variations, in Crj: WI (Glx/BRL/Han) IGS rats were higher than those in Crj: CD (SD) IGS rats. No differences were seen in the incidence of external abnormalities, incidence of visceral abnormalities, or degree of ossification between these strains of rats. Since no marked differences were seen between the results of the examination of fetuses in Crj: CD (SD) IGS rats obtained in the present study (1999 - 2003) and those reported previously [2], it is considered that results of examinations of Crj: CD (SD) IGS rat fetuses do not vary according to the years when examinations are performed. The external abnormalities, skeletal abnormalities and variations, and visceral abnormalities noted in the present study are known to occur spontaneously in Crj: CD (SD) rats [9].

Compared with Crj: CD (SD) IGS rats, smaller litter size, lower fetal body weight, higher incidence of supernumerary lumbar vertebra, which is a skeletal abnormality, and higher incidences of full supernumerary rib, short supernumerary rib, and cervical rib, which are skeletal variations, were noted in Crj: WI (Glx/BRL/Han) IGS rats, as described above. However, the occurrence of such abnormalities and variations in Crj: WI (Glx/BRL/Han) IGS rats is not considered to interfere with the assessment of the results of reproductive/developmental toxicity studies. It is considered, therefore, that both Crj: CD (SD) IGS rats and Crj: WI (Glx/BRL/Han) IGS rats are suited to be used in reproductive/developmental toxicity studies.

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Table 1. Observation of offspring

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams	212	23
Gestation length (days)	22.0±0.4	21.8±0.3
No. of implantation scars	14.6±2.2	13.5±1.3
Gestation index (%)[a)]	99.5	100.0
No. of live offspring at birth	13.5±2.5	12.6±1.5
Sex ratio[b)]	0.50±0.1	0.50±0.11
No. of stillbirths	0.2±0.5	0.0±0.0
Birth index (%)[c)]	92.4±9.8	93.0±7.2
Viability index (%)[d)]	98.4±7.5	99.3±2.3
Weaning index (%)[e)]	99.9±0.9	100.0±0.0
External abnormalities (%)[f)]	0.0±0.0	0.0±0.0

Each value shows mean±S.D.
a) (No. of pregnant dams with live offspring/No. of pregnant dams)×100.
b) No. of male offspring/(No. of male offspring + No. of female offspring).
c) (No. of live offspring at birth/No. of implantation scars)×100.
d) (No. of live offspring 4 days after birth/No. of live offspring at birth)×100.
e) (No. of live offspring 21 days after birth/No. of live offspring after culling)×100.
f) No. of external abnormalities in live offspring at birth.

Table 2. Body weights of offspring

Strain		Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams		212	23
Males			
Days after birth			
	0	6.5±0.5	5.8±0.3
	4	10.3±1.2	9.5±1.0
	7	16.9±2.0	15.9±1.4
	14	34.6±3.8	33.6±2.6
	21	55.6±6.1	44.3±3.1
	28	95±9	75±6
	35	150±13	120±9
	42	216±18	169±13
	49	281±23	214±15
	56	343±27	261±18
	63	394±33	300±20
	70	439±38	328±21
No. of dams		212	23
Females			
Days after birth			
	0	6.2±0.5	5.5±0.3
	4	9.8±1.1	9.1±0.9
	7	16.1±1.9	15.4±1.2
	14	33.6±3.5	32.8±2.4
	21	53.8±5.5	42.9±3.0
	28	87±8	71±5
	35	130±11	108±6
	42	168±13	139±7
	49	196±16	159±8
	56	223±18	179±10
	63	244±21	194±12
	70	262±24	207±14

Each value shows mean (g)±S.D.

Table 3. Morphological differentiation of offspring

Strain		Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams		199	23
Separation of auricles	Day 4	100±1	100±0
Eruption of incisors	Day 14	100±0	100±0
Separation of eyelids	Day 14	50±40	4±18
	Day 15	91±21	60±43
	Day 16	100±3	100±0
Opening of vagina	Day 33	51±43	45±30
	Day 34	70±39	50±30
	Day 35	87±29	76±36
	Day 36	97±14	95±21
	Day 37	98±12	100±0
Cleavage of the	Day 41	47±45	26±34
balanopreputical gland	Day 42	66±42	52±37
	Day 43	79±35	78±31
	Day 44	90±26	89±20
	Day 45	96±16	96±9

Each value shows mean (%)±S.D.

Table 4. Reflexes of offspring

Strain		Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams		155	23
Righting reflex	Day 6	90±20	89±13
	Day 7	98±8	100±0
Negative geotaxis	Day 10	55±28	57±36
	Day 11	79±24	84±18
	Day 12	94±11	91±12
	Day 13	87±21	99±5
	Day 14	98±9	100±0
Free-fall reflex	Day 17	87±19	92±16
	Day 18	100±0	100±0
Pinna reflex	Day 17	100±0	100±0
Corneal reflex	Day 17	100±0	100±0

Each value shows mean (%)±S.D.

Table 5. Emotionality of offspring

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of male offspring	153	46
Ambulation	25.9±21.2	31.8±21.1
Rearing	5.0±4.1	8.0±5.7
Face-washing	0.5±0.9	0.8±0.8
Grooming	0.2±0.6	0.3±0.5
Defecation	2.3±2.1	4.7±2.1
Urination	0.5±0.6	0.5±0.7
Latency (sec.)	12.3±22.9	5.8±3.4
No. of female offspring	153	46
Ambulation	32.2±23.7	33.9±20.8
Rearing	6.2±6.5	10.7±8.1
Face-washing	0.6±0.9	0.8±0.8
Grooming	0.1±0.4	0.2±0.4
Defecation	2.3±2.1	4.3±2.2
Urination	0.5±0.8	1.6±1.1
Latency (sec.)	11.4±18.3	4.6±3.1

Each value shows mean±S.D.

Table 6. Learning ability of male offspring

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of male offspring	152	47
Maze trial		
Time (sec.)		
2nd day	174±54	221±94
3rd day	91±38	114±44
4th day	81±32	98±45
Selection error		
2nd day	10.5±3.8	8.6±2.4
3rd day	4.0±3.6	3.6±2.3
4th day	2.3±2.8	2.3±2.7
Backing error		
2nd day	3.5±2.7	1.9±1.7
3rd day	1.3±1.9	1.5±1.8
4th day	0.9±1.4	0.9±1.5
Intra-zonal error		
2nd day	14.5±5.9	13.2±4.7
3rd day	4.1±4.7	3.3±2.9
4th day	1.9±2.8	1.8±2.5
Total error		
2nd day	28.5±10.9	23.6±7.8
3rd day	8.8±9.4	8.3±6.5
4th day	4.9±6.2	5.1±6.2

Each value shows mean±S.D.

Table 7. Learning ability of female offspring

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of female offspring	153	45
Maze trial		
Time (sec.)		
2nd day	185±55	234±79
3rd day	115±50	99±51
4th day	103±43	76±34
Selection error		
2nd day	11.2±4.5	10.2±4.2
3rd day	5.4±3.9	4.0±3.3
4th day	3.6±3.1	2.5±2.1
Backing error		
2nd day	3.9±3.0	3.2±2.7
3rd day	2.4±2.8	1.2±2.0
4th day	1.8±2.1	0.8±1.1
Intra-zonal error		
2nd day	14.7±6.2	15.3±6.5
3rd day	5.3±4.5	4.1±3.7
4th day	3.0±3.5	2.1±2.2
Total error		
2nd day	29.8±12.4	28.7±12.4
3rd day	12.4±10.3	9.2±8.3
4th day	7.9±7.9	5.4±4.8

Each value shows mean±S.D.

Table 8. Number of estrous cases and reproductive function of rats

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of females	172	46
No. of estrous cases before pairing (14 days) (Mean±S.D.)	3.4±0.6	3.5±0.5
No. of females	172	46
No. of females with copulation	166	45
Copulation index (%)[a)]	96.5	97.8
No. of days till copulation after pairing (Mean±S.D.)	2.6±1.6	2.5±1.3
No. of pregnant females	155	44
Fertility index (%)[b)]	93.4	97.8

a) (No. of females with copulation/No. of females)×100.
b) (No. of pregnant females/No. of females with copulation)×100.

Table 9. Sperm analysis in male rats

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of males	124	46
Sperm motility		
Motile sperm rate (%)	83.1±6.7	84.7±3.8
Progressive sperm rate (%)	38.2±11.0	23.3±6.3
Path velocity (μ m/sec)	154.1±9.6	148.1±7.8
Straight line velocity (μ m/sec)	106.6±8.6	93.3±4.0
Curvilinear velocity (μ m/sec)	353.7±22.4	349.4±18.3
Amplitude of lateral head displacement (μ m/sec)	20.8±1.0	22.7±1.0
Beat cross frequency (Hz)	29.7±1.4	30.2±0.9
Viability rate (%)[a)]	98.9±1.8	99.5±0.4
Survivability rate (%)[b)]	80.4±7.7	75.6±6.4
Sperm morphology		
Abnormal sperm rate (%)[c)]	3.9±3.6	1.6±1.0
Abnormal head rate (%)[c)]	3.6±3.4	1.5±0.9
Abnormal tail rate (%)[c)]	0.3±0.4	0.2±0.3
No. of sperms in left cauda epididymis ($\times 10^6$)	311.9±57.3	273.4±42.0
No. of sperms/g weight of left cauda epididymis ($\times 10^6$)	1082.6±162.8	1072.0±127.1

Each value shows mean±S.D.
a) ((No. of live sperms + No. of sperms which died during incubation)/No. of sperms examined)×100.
b) (No. of live sperms/No. of sperms examined)×100.
c) (No. of abnormal sperms/No. of sperms examined)×100.

Table 10. Observation of fetuses

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams	365	44
No. of corpora lutea	15.6±1.9	13.4±1.6
No. of implantation sites	14.5±2.3	12.0±3.2
Implantation rate (%)[a)]	92.6±9.7	88.6±20.7
No. of pre-implantation losses[b)]	1.1±1.3	1.4±2.4
Pre-implantation loss rate (%)[c)]	7.4±9.7	11.4±20.7
No. of post-implantation losses	0.7±1.1	0.4±0.6
Post-implantation loss rate (%)[d)]	5.0±8.1	2.9±4.7
No. of live fetuses	13.8±2.5	11.6±3.2
Sex ratio[e)]	0.50±0.14	0.49±0.17
Fetal body weight (g)		
Male	3.90±0.26	3.63±0.18
Female	3.69±0.25	3.46±0.23
Fetuses with external abnormalities (%)[f)]	0.15±1.32	0.00±0.00
Anasarca (%)[f)]	0.04±0.49	0.00±0.00
Gastroschisis (%)[f)]	0.02±0.35	0.00±0.00
Umbilical hernia (%)[f)]	0.10±1.21	0.00±0.00
Acaudate (%)[f)]	0.02±0.37	0.00±0.00
Short tail (%)[f)]	0.02±0.35	0.00±0.00
Abnormal placentae (%)[g)]	0.00±0.00	0.00±0.00

Each value shows mean±S.D.
a) (No. of implantation sites/No. of corpora lutea)×100.
b) No. of corpora lutea - No. of implantation sites.
c) (No. of pre-implantation losses/No. of corpora lutea)×100.
d) (No. of post-implantation losses/No. of implantation sites)×100.
e) No. of males/(No. of males + No. of females).
f) (No. of fetuses with external abnormalities/No. of live fetuses)×100.
g) (No. of abnormal placentae/No. of placentae)×100.

Table 11. Skeletal examination of fetuses

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams	187	44
Fetuses with abnormalities (%)	0.37±2.23	3.70±15.55
Misshapen basisphenoid (%)	0.00±0.00	0.38±2.52
Misshapen mandibula (%)	0.07±0.91	0.00±0.00
Supernumerary lumbar vertebra (%)	0.07±0.91	2.94±15.29
Absent sacral vertebra (%)	0.08±1.04	0.00±0.00
Absent caudal vertebra (%)	0.08±1.04	0.00±0.00
Fused sternebra (%)	0.07±0.91	0.00±0.00
Sternoschisis (%)	0.07±0.91	0.00±0.00
Wavy rib (%)	0.00±0.00	0.38±2.52
Short rib (%)	0.16±1.52	0.00±0.00
Fetuses with variations (%)	10.46±16.45	50.51±31.15
Bipartite ossification of thoracic centrum (%)	1.11±4.85	0.00±0.00
Bipartite ossification of lumbar centrum (%)	0.11±1.46	0.00±0.00
Full supernumerary rib (%)	0.14±1.38	6.75±18.07
Short supernumerary rib (%)	8.81±15.92	46.78±30.33
Cervical rib (%)	0.42±3.95	3.64±9.45
Ossifications		
Fetuses with unossifications (%)	5.47±12.56	2.50±6.66
Unossified hyoid bone (%)	5.25±12.36	2.50±6.66
Unossified thoracic centrum (%)	0.14±1.38	0.00±0.00
Unossified pubis (%)	0.08±1.04	0.00±0.00
No. of ossified bones		
Sternebrae	5.7±0.4	5.9±0.2
Metacarpal bone of forepaw	7.4±0.6	7.4±0.6
Proximal phalanx of forepaw	0.6±0.8	1.0±0.9
Middle phalanx of forepaw	0.0±0.0	0.0±0.0
Metatarsal bone of hindpaw	7.9±1.0	8.0±0.1
Proximal phalanx of hindpaw	0.0±0.0	0.0±0.0
Middle phalanx of hindpaw	0.0±0.0	0.0±0.0
Sacrococcygeal centrum	8.0±0.9	7.7±0.5

Each value shows mean ± S.D.

Table 12. Visceral examination of fetuses

Strain	Crj: CD (SD) IGS	Crj: WI (G1x/BRL/Han) IGS
No. of dams	232	42
Fetuses with abnormalities	4.78±8.74	1.73±5.55
Absent lung lobe	0.05±0.82	0.00±0.00
Abnormal lung lobation	0.05±0.82	0.00±0.00
Membranous ventricular septum defect	1.33±4.67	0.00±0.00
Muscular ventricular septum defect	0.05±0.82	0.00±0.00
Right-sided aortic arch	0.05±0.82	0.00±0.00
Malpositioned carotid branch	0.14±1.54	0.00±0.00
Narrowed brachiocephalic artery	0.06±0.94	0.00±0.00
Short brachiocephalic artery	0.18±1.55	0.00±0.00
Malpositioned subclavian artery	0.05±0.82	0.00±0.00
Abnormal liver lobation	0.37±2.32	0.00±0.00
Supernumerary liver lobe	2.15±5.98	1.33±5.02
Diverticulum of intestine	0.67±3.19	0.00±0.00
Short intestine	0.00±0.00	0.40±2.58
Absent kidney	0.06±0.94	0.00±0.00
Absent adrenal	0.05±0.82	0.00±0.00
Dilated ureter	0.06±0.94	0.00±0.00
Convoluted ureter	0.11±1.64	0.00±0.00

Each value shows mean (%)±S.D.

Historical Control Data of Skeletal Findings in Crj:CD(SD)IGS Rat Fetuses - Bone and Cartilage Alterations -

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ABSTRACT. Several authors have reported historical control data on reproductive parameters and fetal external and skeletal abnormalities in Crj:CD(SD)IGS rats which are used to assess reproductive and developmental toxicity, but there have been few reports regarding morphological cartilage alterations in fetuses. In this report, we examined the spontaneous alterations of cartilage in control fetuses observed with a double staining procedure, alcian blue and alizarin red S, as well as the strain-differences in reproductive parameters and fetal external and skeletal abnormalities in Crj:CD(SD)IGS rats and Slc:SD rats. There were no strain-differences between Crj:CD(SD)IGS rats and Slc:SD rats in reproductive parameters nor in bone abnormalities. The cartilaginous variation of bipartite ossification and dumbbell-shaped ossification on thoracic vertebrae occurred more frequently in Slc:SD fetuses in accordance with lower fetal body weight and what of the transverse process on lumbar vertebrae occurred at a higher frequency in Crj:CD(SD)IGS fetuses. The low frequency of split of thoracic centrum, which could not be distinguished from delayed ossification by alizarin red S staining, was observed as a cartilaginous abnormality in Crj:CD(SD)IGS and Slc:SD fetuses. Fusion of cartilaginous caudal vertebrae, which could not be defined in fetuses by alizarin red S staining, was also observed as a cartilaginous abnormality at low frequency in Slc:SD fetuses. In conclusion, this historical control data of cartilaginous abnormalities in Crj:CD(SD)IGS and Slc:SD rat fetuses indicate that the frequency of cartilaginous abnormalities in control fetuses is low. —Key words: Crj:CD(SD)IGS rat fetuses, historical control, cartilage, double stain

CD(SD)IGS-2002/2003 : 152-155

INTRODUCTION

The double staining procedure using alcian blue and alizarin red S for fetal skeletal evaluation was reported by Peters in 1977 [1]. Since then, some reports concerning cartilaginous staining procedures were intermittently published [2, 3, 4, 5, 6, 7] and the methods of cartilaginous evaluation have been widely known; however, fetal cartilage observation has not been established as an evaluation parameter in reproductive and developmental toxicity studies. Specifically, when skeletal abnormalities were observed in fetuses in teratology studies there was no way to know whether cartilage alterations were included among skeletal abnormalities or not. The reasons for preventing the implementation of a double staining procedure in teratology studies are considered to be as follows; a time-consuming work, insufficient historical control data, and the indistinct significance of cartilaginous abnormalities on toxicological assessment. Accordingly, accumulation of historical control data regarding skeletal and cartilaginous abnormalities in fetuses has an important meaning for teratology and toxicological assessment.

MATERIALS AND METHODS

The historical data was generated using fetuses from control Slc:SD dams used in our laboratory in teratology studies (1995-2001).

Crj:CD(SD)IGS rats were obtained from Charles River Japan, Inc. Animals had free access to tap water and a pelleted commercial laboratory animal diet (CE-2, CLEA Japan Inc.). Room environmental conditions were controlled as follows: minimum of 12 air changes per hour with air through HEPA filters, relative humidity of 55%, temperature of 23℃, and a 12-hour light/dark cycle. Animals were housed individually in stainless steel cages, except for the mating period where males and females were housed in a cage on a one-to-one basis. The day on which sperms were observed in vaginal smears was designed as gestation Day 0.

An aqueous solution of 0.5% methylcellulose or distilled water was administered orally by gavage using disposable syringe and a Teflon sonde from gestation Day 6 to 17 in a volume of 10 mg/kg body weight. Dosage volumes were calculated based on their daily body weight. Cesarean sections were performed on gestation Day 21. The number of corpora lutea, implantation sites, late and early resorptions, viable and dead fetuses was recorded for each dam. Viable fetuses were weighed individually, their sex was determined, and there were examined for external and buccal abnormalities. Placental weights were recorded individually.

Fetal skeletons were visualized by staining with alcian blue and alizarin red S using a method adapted from the one described by Young et al. [8] after removal of the epidermis and soft tissue. The stained skeletons were then examined under a dissecting microscope for any morphological alterations and were classified into abnormalities and variations.

RESULTS AND DISCUSSION

The reproductive and fetal parameters are shown in Table 1. There were no strain-differences in number of corpora lutea, implantation sites, and viable fetuses and embryomortality in Crj:CD(SD)IGS and Slc:SD rats. The fetal body weight and placental weight of the Slc:SD rats were lower than those of the Crj:CD(SD)IGS rats. External abnormality as designated proboscis was observed in 1 fetus Slc:SD rats.

The ossified bone findings are shown in Table 2. As classified skeletal variations, bipartite ossification of thoracic vertebrae

was observed in 1 fetus (0.3%) of Crj:CD(SD)IGS rats and in 22 fetuses (4.3%) of Slc:SD rats. Dumbbell-shaped ossification of thoracic vertebrae was observed in 36 females (11.5%) of Crj:CD(SD)IGS rats and in 131 fetuses (25.6%) of Slc:SD rats. The incidence of these findings was higher in Slc:SD fetuses than in Crj:CD(SD)IGS fetuses, and was considered to correlate with delayed ossification due to their lower body weight. Cervical ribs were observed in 5 fetuses (1.0%) of Slc:SD rats. As classified skeletal abnormalities, thickened rib was observed in 1 fetus (0.3%) of Crj:CD(SD)IGS rats, and wavy ribs were observed in 1 fetus each (0.3% in Crj:CD(SD)IGS rats and 0.2% in Slc:SD rats). In sternebrae, agenesis of the 2nd sternebra was observed in 1 fetus (0.2%) of Slc:SD rats. Thus, there were no strain-differences in ossified bone findings in Crj:CD(SD)IGS and Slc:SD rats with the exception for the skeletal variations in Slc:SD rats fetuses what were correlated with their lower body weight.

The cartilage findings are shown in Table 3. In thoracic vertebrae, split of the cartilaginous centrum (Fig. 1) was detected as a skeletal abnormality in 1 fetus (0.3%) of Crj:CD(SD)IGS rats and 2 fetuses (0.4%) of Slc:SD rats when their skeletons were visualized by alizarin red S in ossified bone and by alcian blue in cartilage. The split of vertebrae centrum could not discriminate from the bipartite ossification of vertebral centrum by single staining procedure, alizarin red S, widely used in teratology studies. The bipartite ossification of the vertebral centrum was generally classified as a skeletal variation due to the delayed ossification, while the split of the vertebral centrum was defined as a morphological abnormality because the vertebra would not recover its normal shape [9]. Thus, cartilaginous staining is considered to be a useful procedure to distinguish the abnormalities and variations from morphological alterations which include the delayed ossification of the vertebral centrum. In lumbar vertebrae, morphological alteration of the cartilaginous transverse process, especially noted in the 1st lumbar vertebra, was observed in 65 fetuses (20.8%) of Crj:CD(SD)IGS rats and in 24 fetuses (4.7%) of Slc:SD rats, and the incidence of this finding in Crj:CD(SD)IGS fetuses showed approximately 5-fold increase than that in Slc:SD fetuses. Supernumerary ribs, which were sometimes observed in fetuses, were generally not observed in adulthood due to complement by an increase in the proportion of fetuses with a fully developed transverse process on the 1st lumbar vertebra [10]. Specifically, the transverse process of lumbar vertebrae would not be fully developed on gestation Day 21, and the alteration of this cartilaginous transverse process that indicates a variation based on their skeletal development. In sacral and caudal vertebrae, fusion of cartilaginous vertebrae (Fig. 2) was observed as a skeletal abnormality in 3 fetuses (0.6%) of Slc:SD rats but not in Crj:CD(SD)IGS fetuses. The fusion of cartilaginous caudal vertebrae observed in fetuses would be ossified along with their growth without recovering the normal shape, and cartilaginous staining, is therefore considered as a useful procedure to detect abnormalities which can not be examined in fetuses stained with alizarin red S. There were no cartilaginous alterations in cervical vertebrae, ribs, and phalanxes.

In conclusion, this historical control data of fetal cartilaginous abnormalities in Crj:CD(SD)IGS and Slc:SD rats indicate that control fetuses have a low frequency of cartilaginous abnormalities and that staining of cartilaginous tissue is a useful procedure for toxicological evaluation in teratology studies. This historical control data provides a useful information on reproductive and developmental assessment.

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Table 1. Reproductive and fetal parameters in Crj:CD(SD)IGS and Slc:SD rats

Strain	Crj:CD(SD)IGS	Slc:SD
No. of dams	33	73
No. of corpora lutea		
Total	542	1186
Mean±S.D.	16.4±2.0	16.2±2.0
No. of implantation sites		
Total	484	1055
Mean±S.D.	14.7±2.9	14.5±2.7
Embryomortality (%)		
Mean±S.D.	5.9±8.1	7.7±14.3
No. of viable fetuses		
Total (males/females)	455 (230/225)	977 (503/474)
Mean±S.D.	13.8±2.9	13.4±3.3
Fetal body weight (g)		
Males Mean±S.D.	5.56±0.27	5.09±0.38
Females Mean±S.D.	5.30±0.25	4.82±0.32
Placental weight (g)		
Mean±S.D.	0.498±0.05	0.448±0.11
No. of fetuses with external abnormalities		
Total (%)	0 (0.0)	1[a)] (0.1)

a) Proboscis
Figures in parentheses represent mean percent by total number of fetuses

Table 2. Ossified bone findings in fetuses stained with alizarin red S

Strain	Crj:CD(SD)IGS	Slc:SD
No. of dams	33	73
No. of fetuses examined	312	511
Skeletal variations		
No. of fetuses with bone variations		
Total (%)	37 (11.9)	158 (30.9)
Thoracic vertebra		
Bipartite ossification of centrum (%)	1 (0.3)	22 (4.3)
Dumbbell-shaped ossification of centrum (%)	36 (11.5)	131 (25.6)
Ribs		
Cervical ribs (%)	0 (0.0)	5 (1.0)
Skeletal abnormalities		
No. of fetuses with bone abnormalities		
Total (%)	2 (0.6)	2 (0.4)
Ribs		
Wavy ribs (%)	1 (0.3)	1 (0.2)
Thickened rib (%)	1 (0.3)	0 (0.0)
Sternebrae		
Agenesis of 2nd sternebra (%)	0 (0.0)	1 (0.2)

Figures in parentheses represent mean percent by total number of fetuses

Table 3. Cartilage findings in fetuses stained with alizarin red S and alcian blue

Strain	Crj:CD(SD)IGS	Slc:SD
No. of dams	33	73
No. of fetuses examined	312	511
No. of fetuses with cartilaginous alterations		
Total (%)	66 (21.2)	29 (5.7)
Cervical vertebra		
Cartilaginous alterations (%)	0 (0.0)	0 (0.0)
Thoracic vertebra		
Split of cartilaginous thoracic centrum (%)	1 (0.3)	2 (0.4)
Lumber vertebra		
Alterations of cartilaginous transverse process of lumber vertebra (%)	65 (20.8)	24 (4.7)
Sacral/Caudal vertebra		
Fusion of cartilaginous caudal vertebrae (%)	0 (0.0)	3 (0.6)
Ribs		
Cartilaginous alterations (%)	0 (0.0)	0 (0.0)
Phalanxes		
Cartilaginous alterations (%)	0 (0.0)	0 (0.0)

Figures in parentheses represent mean percent by total number of fetuses

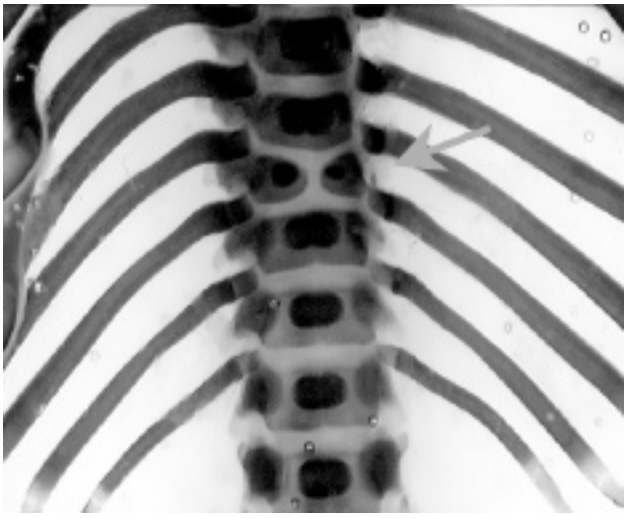

Figure. 1. Split of cartilaginous thoracic centrum

Figure. 2. Fusion of cartilaginous caudal vertebrae

Arrows indicate the cartilage abnormalities.

Morphological Variability and Frequency of Spontaneous Seminiferous Tubular Atrophy in Crj:CD(SD)IGS Rats

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ABSTRACT. Morphological variability and frequency of seminiferous tubular atrophy occurred spontaneously in Crj:CD(SD)IGS rats were described. A total of 976 rats, 8 to 110 week-old, were examined histopathologically. Seminiferous tubular atrophy was noted as a focal and diffuse atrophy, and a minimal change, partial loss of germ cells in a cross section of tubules. Focal atrophy was divided into the following types: 1) the lesion located near rete testis, 2) the lesion appeared linearly along with tunica albuginea, and 3) the lesion was present in no specific area. Similarly, diffuse atrophy was divided into the following types: 1) various stage atrophic tubules were intermingled, 2) Sertoli cell only tubules and normal tubules were intermingled, 3) all atrophic tubules showed uniform morphological picture, and 4) the testis was occupied with Sertoli cell only tubules. The frequency of atrophy was 0, 4.7, 4.8, 5.8 and 20.4% at age of 7-8, 10-14, 19-23, 32 and 110 weeks, respectively. Bilateral atrophy was observed in 4.7% of all animals examined and in 50% of animals bearing atrophy. Moreover, 50% and more in animals with any type of diffuse atrophy showed the same type in both sides. －Key words: Crj:CD(SD)IGS rat, testis, histopathology, background data

CD(SD)IGS-2002/2003 : 156-158

INTRODUCTION

Crj:CD(SD)IGS rats have been produced by the international genetic standard system in Charles River Inc. to supply an experimental animal minimizing genetic ramifications, and it is becoming to be widely used in various studies including general toxicology, reproduction and carcinogenicity studies.

Generally, histopathology is acknowledged as a sensitive endpoint for detection of toxicity, and therefore, pathologists have to pay an attention not only to a chemical induced lesion but also to a spontaneous lesion and a physiological change. Although plenty of background data have been collected by vigorous activities of the CD:(SD)IGS Rat Study Group, there is still a need to collect much data including minimal changes. The purpose of this paper is to provide an information about seminiferous tubular atrophy of the testes observed spontaneously in Crj:CD(SD)IGS rats.

MATERIALS AND METHODS

Animals and Husbandry: A total of 976 male Crj:CD(SD)IGS rats from control groups of toxicological studies or studies for background data collection which were conducted in our laboratories were surveyed. All animals were obtained from Charles River Japan Inc. (Hino, Japan) at 4 weeks of age, and were housed individually in hanging stainless-steel wire mesh cages in an animal room where was controlled at 23±3℃, 50 ± 20% relative humidity, 10 to 15 times/hr air ventilation and 12-hour illumination (7:00 a.m. to 7:00 p.m.), and were supplied commercial diets, CR-LPF or CRF-1 (Oriental Yeast Co., Japan), and tap water *ad libitum*. All animals were sacrificed at 8, 10-14, 19-23, 32 and 110 weeks old, which consisted of 33, 275, 290, 172 and 206 rats, respectively.

Histopathology: After complete necropsy, the testes were fixed with Bouin's fixative. Both testes from 662 rats and the left testes from 314 rats were trimmed with a cross section of the middle portion, embedded in paraffin, stained with hematoxylin and eosin, and were examined histopathologically.

RESULTS AND DISCCUSSION

The incidence of each type of seminiferous tubular atrophy is shown in Table 1. Atrophic change was noted at 10 weeks old and more. Atrophic tubules observed consisted of various histological changes such as vacuolation of Sertoli cells, degeneration/necrosis of germ cells, sloughing and depletion of spermatogenic cells, formation of multinucleated giant cells and retention of spermatids. These findings provide quite important information for detection of a target type of cell, especially in short term studies. However, in the present paper, the changes evaluated were as atrophy of seminiferous tubules that included focal atrophy, diffuse atrophy and a minimal change of atrophy, which were characterized by the histological pictures and the location/distribution as follows:

1. Intratubular segmental atrophy: Partial/patchy depletion of germ cells was seen in a cross section of the seminiferous tubule.
2. Focal atrophy: Atrophic seminiferous tubules were seen focally or regionally in a cut surface of the testes. This type of atrophy was subdivided into the following 3 types.
   1) Focal atrophy type-1: Atrophic tubules located near rete testis.
   2) Focal atrophy type-2: Atrophic tubules appeared linearly along with tunica albuginea, marginal area of the testis.
   3) Focal atrophy type-3: Atrophic tubules were present in no specific area.
3. Diffuse atrophy: Atrophic seminiferous tubules were seen diffusely in a cut surface of the testis. This type of atrophy was subdivided into the following 4 types.
   1) Diffuse atrophy type-1: Various degrees/stages of atrophic tubules, from slight atrophic tubules to Sertoli only tubules were intermingled.
   2) Diffuse atrophy type-2: Sertoli cell only tubules and normal seminiferous tubules were intermingled.
   3) Diffuse atrophy type-3: All seminiferous tubules showed almost same degree/stage of atrophy, excluding Sertoli cell only tubules. This type may be an early stage of

diffuse atrophy of type-4.

4) Diffuse atrophy type-4: The testis was completely occupied by Sertoli cell only tubules.

At 8 weeks old, no animals showed any type of atrophy. At 10-14 weeks old, 13/275 (4.7%) animals showed atrophy, which included intratubular segmental atrophy (2.9%), focal atrophy type-3 (0.7%), and diffuse atrophy type-1 (1.1%). At 19-23 weeks old, 14/290 (4.8%) animals showed atrophy, which included intratubular segmental atrophy (1.7%), focal atrophy type-3 (1.0%), diffuse atrophy type-1(1.4%), diffuse atrophy type-2 (0.3%) and diffuse atrophy type-4 (0.3%). The diffuse atrophy type-1 was the most frequent type of atrophy in this age. At 32 weeks old, 10/172 (5.8%) animals showed atrophy, which included intratubular segmental atrophy (2.3%), focal atrophy type-1 and 3 (0.6% in each), diffuse atrophy type-1 and 4 (1.7 and 0.6%, respectively). At 110 weeks old, 42/206 (20.4%) animals showed atrophy which included intratubular segmental atrophy (1.9%), focal atrophy type-1, 2 and 3 (1.9, 1.5, 1.0%, respectively), diffuse atrophy type-1, 2, 3 and 4 (8.3, 0.5, 2.4 and 1.9%, respectively). The present result suggests that both the frequency and the variability of tubular atrophy may not be increased with aging before 32 weeks old, although it is increased at 110 weeks old, especially in the frequency of diffuse type atrophy. However, intratubular segmental atrophy was observed relatively high incidence at any age excluding 8 weeks old.

It is important to know whether the changes occur bilaterally and the histological picture in each side is comparable, since changes relating to chemical treatment are generally produced in both sides of the testes. The present data shown in Table 2 demonstrated that bilateral atrophy was observed in 4.7% of all animals examined and 50% of animals bearing atrophy. The frequency of bilateral diffuse atrophy was higher than that of bilateral focal atrophy. Moreover, half and more of diffuse atrophy showed the same type of atrophy on both sides.

The present results provide useful background information for various experiments including general toxicology and reproductive studies using Crj:CD(SD)IGS rats. However, it is necessary to accumulate more data from many more animals including a broad range of age, since the number of animals surveyed in the present study is limited.

Table 1. Frequency of seminiferous tubular atrophy in Crj:CD(SD)IGS rats

Age (week)	Number of rats		ITSA[a)]	Focal atrophy				Diffuse atrophy					Total
				Type-1	Type-2	Type-3	Total	Type-1	Type-2	Type-3	Type-4	Total	
8	33	Bearers	0	0	0	0	0	0	0	0	0	0	0
		Incidence (%)	0	0	0	0	0	0	0	0	0	0	0
10-14	275	Bearers	8	0	0	2	2	3	0	0	0	3	13
		Incidence (%)	2.9	0	0	0.7	0.7	1.1	0	0	0	1.1	4.7
19-23	290	Bearers	5	0	0	3	3	4	1	0	1	6	14
		Incidence (%)	1.7	0	0	1.0	1.0	1.4	0.3	0	0.3	2.1	4.8
32	172	Bearers	4	1	0	1	2	3	0	0	1	4	10
		Incidence (%)	2.3	0.6	0	0.6	1.2	1.7	0	0	0.6	2.3	5.8
110	206	Bearers	6	4	3	2	9	17	1	5	4	27	42
		Incidence (%)	2.9	1.9	1.5	1.0	4.4	8.3	0.5	2.4	1.9	13.1	20.4

a): Intratubular segmental atrophy

Table 2. Frequency of bilateral seminiferous tubular atrophy in Crj:CD(SD)IGS rats

	Incidence of bilateral atrophy (%)	Incidence of atrophy of the same type on both sides (%)									
		ITSA[a)]	Focal atrophy				Diffuse atrophy				
			Type-1	Type-2	Type-3	Total	Type-1	Type-2	Type-3	Type-4	Total
Animals examined both sides (N=662)	4.7	0.8	0.2	0	0.2	0.3	2.3	0.2	0.5	0.8	3.6
Animals with atrophy (N=62)	50.0	8.1	1.6	0	1.6	3.2	24.2	1.6	4.8	8.1	38.7
Animals with the same type atrophy	—	5/14 (35.7%)	1/5 (20.0%)	0/4 (0%)	1/4 (25.0%)	—	15/22 (68.2%)	1/2 (50.0%)	3/5 (60.0%)	5/6 (83.3%)	—

a): Intratubular segmental atrophy
b): No. of rats with the same type atrophy on both sides

Comparative Analysis for Effects on Fertility and Early Embryonic Development to Implantation of Rats Using Different Type of Food Pellet

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ABSTRACT. To evaluate the effects of a difference in pellet feed on fertility and early embryonic development to implantation in rats, we conducted a comparative analysis of two studies carried out previously. In the two studies, NMF or Certified Rodent Diet 5002 was used. Some differences were observed in the results of these two studies. Food intake of females in the gestation period showed the greatest difference between the studies. It was found that a difference in the type of pellet feed, where one had a higher protein content than the other, contributed to the increase in food consumption. In conclusion, although different type of pellet food was used the reproductive test parameters evaluated in these two studies were constant and comparable. —Key words: Crj:CD (SD) IGS rat, food, diet, chow, pellet, body weight, food intake, fertility, implantation

CD(SD)IGS-2002/2003 : 159-167

INTRODUCTION

Crj:CD(SD)IGS rats were produced by Charles River Japan Inc. using the international gold standard system, which is a new breeding system, developed for the purpose of supplying experimental animals with minimized genetic ramifications. To evaluate the affects of difference of food pellet, the results from 2 studies investigating the effects on fertility and early embryonic development to implantation, were compiled, compared, and summarized.

MATERIALS AND METHODS

Experimental Animals: Control groups used in 2 studies, study 1 and study 2, were evaluated. Male and female Crj:CD(SD)IGS rats purchased from Charles River Japan, Inc. were obtained at 9 to 8 weeks of age and 5 to 7 weeks of age, respectively. After confirming the health of these animals and that they were without any abnormalities, males and females were used at the age of 11 weeks and 8 weeks, respectively, in both studies.

These animals were housed in an animal room with a barrier system that was controlled at a room temperature of 23 ± 3 ℃ and a relative humidity of 55 ± 15% and ventilated 9 to 15 times/hr with lighting of about 200 luces for 12 hr per day (7:00 to 19:00). During the acclimatization period, one male and 4 females were housed per cage. During the treatment period before mating, males and females were housed individually in animal cages, and during the mating period, males and females were housed in animal cages on a one-to-one basis. After copulation was confirmed, males and females were housed individually in the animal cages again. The animals were allowed free access to pellet food sterilized with a 30 kGy ^{60}Co-γ ray and tap water as drinking water from an automatic watering system. For the pellet food, in study 1, NMF (Oriental Yeast Co., Ltd.) was used and in study 2, Certified Rodent Diet 5002 (PMI Nutrition International, Inc.) was used, respectively.

Administration Method: Animals were administrated 1% methylcellulose (MC: Nacalai Tesque, Inc.) solution for study 1 or 0.5% tragacanth (Wako Pure Chemical Industries, Ltd.) suspension for study 2. The dosing volume was set at 0.5 mL/100 g per body weight in both studies, and the dosing volume was calculated on the basis of the body weight measured on the day most recent to each administration. Then, each vehicle was administered orally once a day using a glass syringe and a metallic oral stomach tube for rats.

The 1%MC solution or 0.5% tragacanth suspension was administered to males for a total of 6 weeks, 2 weeks before mating, 2 weeks during the mating period and for 2 weeks after the mating period. The 1%MC solution or 0.5% tragacanth suspension was administered to females for 2 weeks before mating, during the mating period and until Day 7 of pregnancy.

Observation and Examinations: Both studies consisted of 20 males and 20 females and observations and examinations were conducted in the same manner. Clinical signs of males and females were examined daily and before and after administration during the treatment period. Males were weighed twice a week from the first day of administration to the day of autopsy and females were weighed 14, 11, 7 and 4 days before mating, on the starting day of mating and on Days 0, 3, 7, 10 and 14 of pregnancy. The residual amount of food for males was measured on the days of body weight measurement except for Day 1 of treatment and 2 weeks of mating period, and the residual amount of food for females were measured on the day of body weight measurement except on Day 1 of treatment and Day 0 of pregnancy. Food intake was calculated by subtracting the residual amount from the supplied amount of food measured on the previous day. Furthermore, in females, a vaginal smear was collected daily until the confirmation day of copulation during the mating period and stained with 4% Giemsa solution. The vaginal smear image was observed under a biological microscope to examine the estrous cycle.

After the administration period before mating, males and females of the same study were mated on a one-to-one basis. Vaginal plug and sperms in the smears were examined every morning from the following day. Copulation was confirmed when a vaginal plug was found or sperm was present in the smears and that day was considered as Day 0 of pregnancy. The maximum mating period was 2 weeks and females in which copulation was confirmed were autopsied on Day 14 of pregnancy to examine the conception. The copulation rate and fertility rate were calculated from these results.

The females that copulated successfully were euthanized under ether (diethyl ether: Wako Pure Chemical Industries, Ltd.) anesthesia on Day 14 of pregnancy. They were autopsied to examine the thoracic and intraperitoneal organs macroscopically and to count the number of corpora lutea, number of implants and number of live and dead embryos. If no implantations were macroscopically observed in the uterus, an implantation site test was conducted by Salewski's1) method to confirm the presence or absence of implantations.

The males were euthanized under ether anesthesia by exsanguination from the abdominal aorta and autopsied on the day after the last day of administration. After macroscopic examination of the abdominal and thoracic organs, the right and left testes and epididymides were removed and weighed. The tail of the left epididymis of animals was punctured with an injection needle filled with Hank's Balanced Salt Solution (Hank's solution: Life Technologies, Inc.) containing 0.5% albumin (Sigma-Aldrich Japan Corporation). The discharged sperm fluid was mixed the Hank's Solution and placed in an automatic sperm analyzer (HTM-IVOS: Hamilton Thorne Research) to measure sperm motility.

STATISTICAL ANALYSIS

The results obtained in the present study were expressed as means and standard deviations or percentages. The differences between the measured values in study 1 and study 2 were analyzed using a program for statistical analysis of the Statistical Analysis System version 5.0 (SAS Institute Inc.).

The statistical analysis methods used to analyze each parameter are as follows:

1) F-t test

Body weight, food intake, number of corpora lutea, number of implants, number of dead and live embryos, organ weight (testes and epididymides), period until copulation and sperm motility

First, these parameters were analyzed by the F test (significance level: 25%). Values that showed statistically significant differences between the results of study 1 and study 2 were assessed by Student's t test when a set of variances was homogeneous and by Aspin - Welch's t test when a set of variances was not homogeneous.

2) Wilcoxon's Rank Test

Rate of implantation, rate of dead embryos and rate of live embryos

3) Chi-square test

Rate of copulation in males and females, male insemination rate and female conception rate

The results of the tests are expressed with significance levels of 1% and 5%.

RESUTL AND DISCUSSION

Clinical Signs, Body Weights and Food Intake in Parent Animals: No abnormal changes in clinical signs were observed in males or females of study 1 throughout the treatment period. In study 2, since a crushed left maxillary incisor was observed in 1 male on Day 3 of mating and loss of the right and left maxillary incisors were observed in 1 male on Day 18 of mating, these males were subjected to an unscheduled autopsied. No other animals in study 2 showed abnormal changes in clinical signs throughout the treatment period. The body weights of males during the treatment period are shown in Figure1 and Table 1. In both studies, the body weights of males increased during the treatment period except for the first measurement after the start of mating for study 1. The body weights from study 2 were higher than those from study 1 on Day 11 and 15 of treatment at a significance level of 5%. Although, at the start of the studies, the body weights of males in study 2 were higher than those in study 1 were significantly different on Day 11 and 15 of treatment, they were almost the same as those in study 1 by the end of the study. Food intake of males during the treatment period is shown in Figure 2 and Table 2. The food intake in study 2 was higher than that in study 1 on Day 11, 15, 39 and 43 of treatment at the significant level of 5, 1, 5, 1% respectively. Food intake was almost constant during the treatment period in both studies even when comparing the before and after mating in study 1 and study 2. Although differences in other factors should be considered, it was found that a difference in pellet food given in these studies must have contributed to the low body weight gain in animals of study 2 since food intake was not lower than that in study 1. The body weights of females during the treatment period are shown in Table 3 and body weight changes in females during the before mating period are shown in Figure 3-a and those during the gestation period are shown in Figure 3-b. Food intake of females during the treatment period is shown in Table 3. Food intake of females during the before mating period is shown in Figure 4-a and those during the gestation period is shown in Figure 4-b. The data of infertile animals in the gestation period were excluded from the figures and tables of body weights and food intake. Before the mating period, there were no differences in body weights and food intake between the studies. During the gestation period, the body weight of animals in study 2 were higher than those in study 1 on Day 14 of the gestation period at a significance level of 5%, and food intake of females in study 2 was also higher than that in study 1 at all points of measurement at a significance level of 1%. It was also found that a difference in the type of pellet food must have contributed to this increase in food consumption, especially since the content of protein in the Certified Rodent Diet (20.0%) was lower than that in the NMF (27.8%).

Reproductive Potential: The results of the mating are shown in Table 5. All the animals of these studies copulated but 2 females in study 1 were infertile. In the observation of the female estrous cycle during the mating period, the females of these studies showed normal estrous cycles except for 1 female in study 2 and the first sign of estrus was observed within 5 days after the start of mating. One female in study 2 showed diestrus signs from 2 days after the start of the observation for the estrous cycle, but showed an estrus sign 14 days after copulation. There were no significant differences between the studies in any of the copulation rate, conception rate and period until mating.

Observation at Autopsy of Males: Organ weights of males at autopsy after the treatment period are shown in Table 6. There were no significant differences between the studies in the absolute or relative weights of the right or left testis. The absolute and relative weights of the right epididymis in study 2 were higher than those in study 1 at a significance level of 1%. The absolute and relative weights of the left epididymis in study 2 were also higher than those in study 1 at a significance level of 5%. The reason of which made difference of the epididymal weights between the studies is unknown. Sperm motility is shown in Table 7. Almost all males showed good sperm motility except for 1 male in study 2. The lowest sperm motility was 44% observed in 1 male of study 2. Although this male showed low sperm motility, copulation with a female within a normal period (1 day) was verified as well as successful impregnation. No abnormal changes were observed in the thoracic or intraperitoneal organs in the observation at autopsy in these studies.

Observation at Autopsy of Females: The results of the embryo observation at autopsy of females on Day 14 of pregnancy are shown in Table 8. There were no significant differences between the studies in the number of corpora lutea, the number of implantations or the number of live or dead embryos. There were also no significant differences between the studies in the rate of implantations or the rate of live or dead embryos. No abnormal changes in embryo observation were observed. Almost all females showed a good implantation rate and fetal survival rate. In the observation of the thoracic or intraperitoneal organs at autopsy, there were no abnormal changes between the studies.

In conclusion, although there were some differences between the two study systems: such as food pellet, administrated vehicle. which might affect body weight gain or food intake, the reproductive parameters of these studies indicated constantly stable values.

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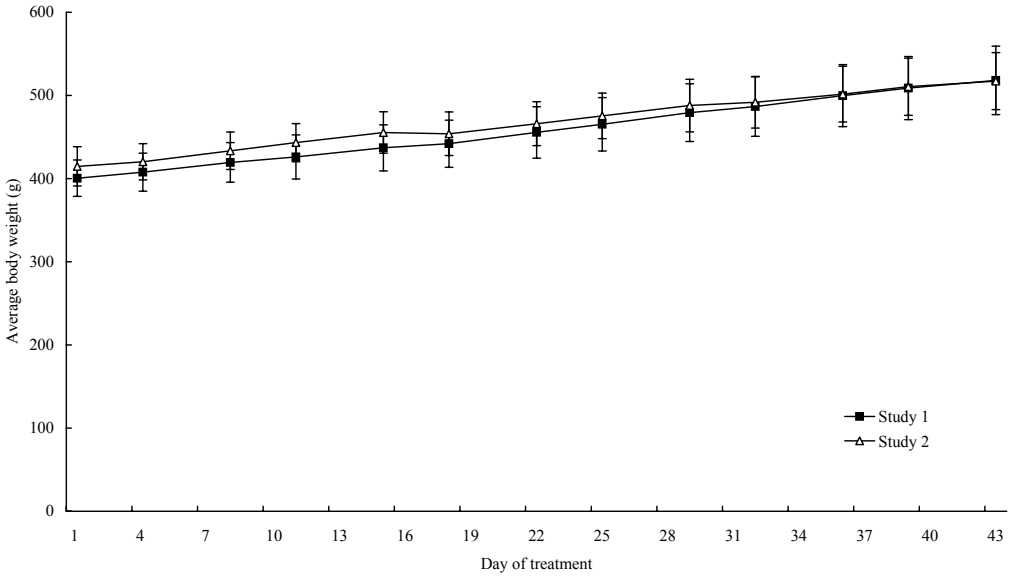

Figure 1. Body weight changes in males

Table 1. Body weght of males

	Day of treatment												
	1	4	8	11	15	18	22	25	29	32	36	39	43
	Before mating period					Mating period				After mating period			
	1	4	8	11	15	3	7	10	14	3	7	10	14
Study 1													
N	20	20	20	20	20	20	20	20	20	20	20	20	20
Body weight (g)	400.5	407.8	419.5	426.1	437.0	441.9	455.5	465.3	479.4	486.6	499.8	508.8	518.1
S.D.	21.9	22.8	23.8	26.4	27.7	28.4	30.9	32.2	34.7	35.7	37.2	37.9	41.2
Study 2													
N	20	20	20	20	20	19	19	19	19	19	18	18	18
Body weight (g)	414.8	420.2	433.5	443.2*	455.5*	454.0	466.0	475.5	487.9	491.8	501.6	510.5	517.3
S.D.	23.5	21.8	22.5	23.0	24.9	26.3	26.4	27.4	31.6	31.0	33.5	34.4	34.3

Significantly different from study 1 * $P < 0.05$ (F-t test)

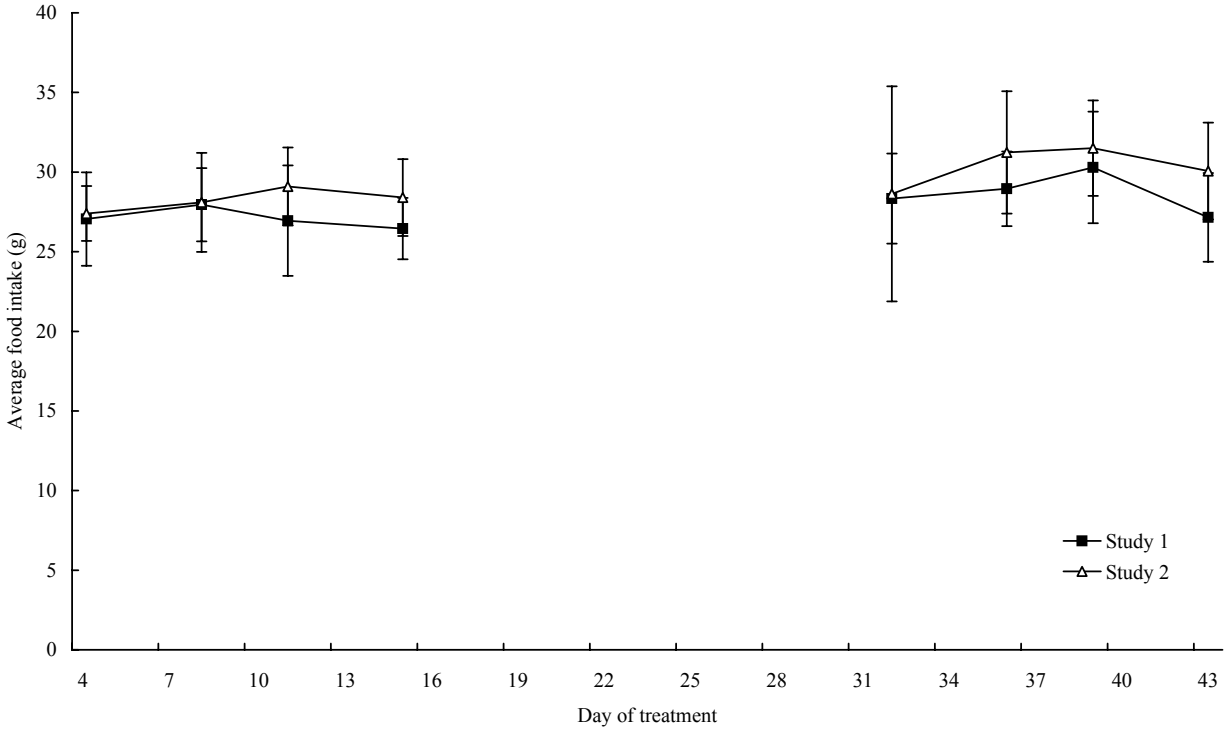

Figure 2. Food intake in males

Table 2. Food intake of males

	Day of treatment											
	4	8	11	15	18	22	25	29	32	36	39	43
	Before mating period				Mating period				After mating period			
	4	8	11	15	3	7	10	14	3	7	10	14
Study 1												
N	20	20	20	20					20	20	20	20
Food intake (g)	27.1	28.0	27.0	26.5					28.3	29.0	30.3	27.2
S. D.	2.9	2.3	3.5	1.9					2.8	2.3	3.5	2.8
Study 2												
N	20	20	20	20					19	18	18	18
Food intake (g)	27.4	28.1	29.1*	28.4**					28.6	31.2	31.5*	30.1**
S. D.	1.7	3.1	2.4	2.4					6.8	3.8	3.0	3.0

Significantly different from study 1 * P < 0.05, ** P < 0.01 (F-t test)

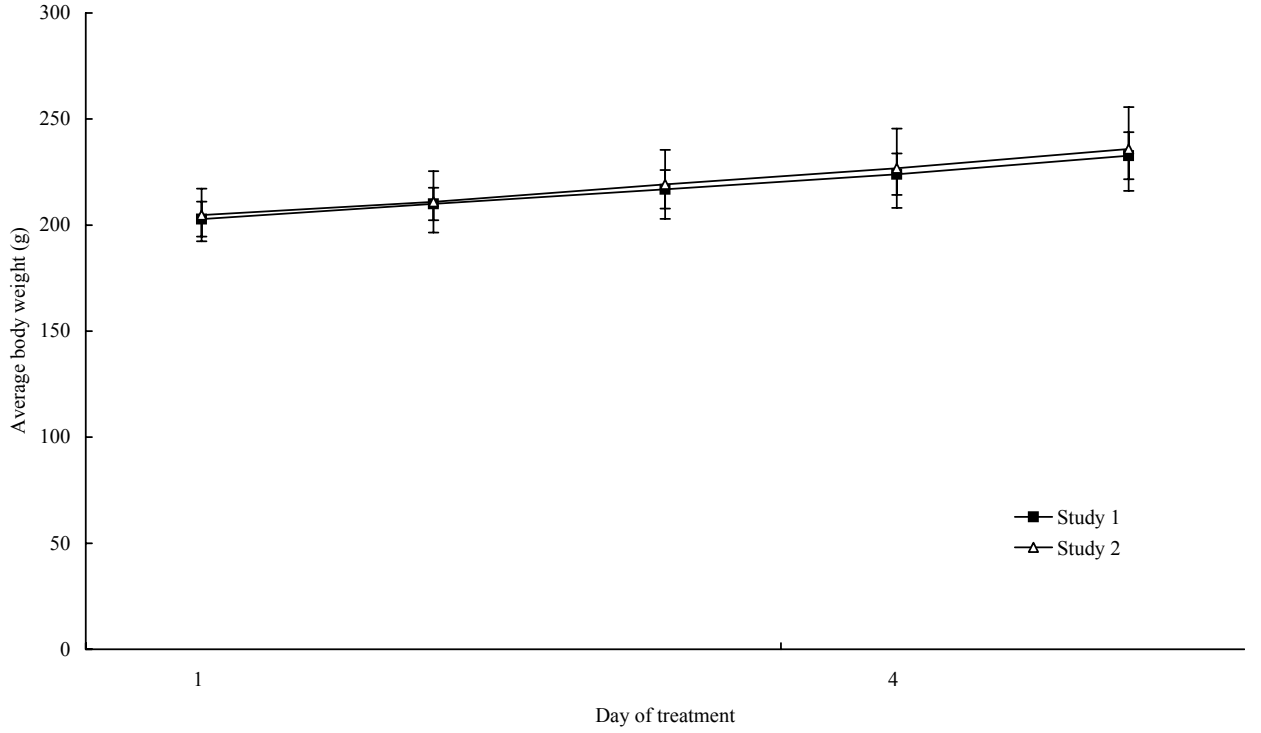

Figure 3-a. Body weight of females during before mating period

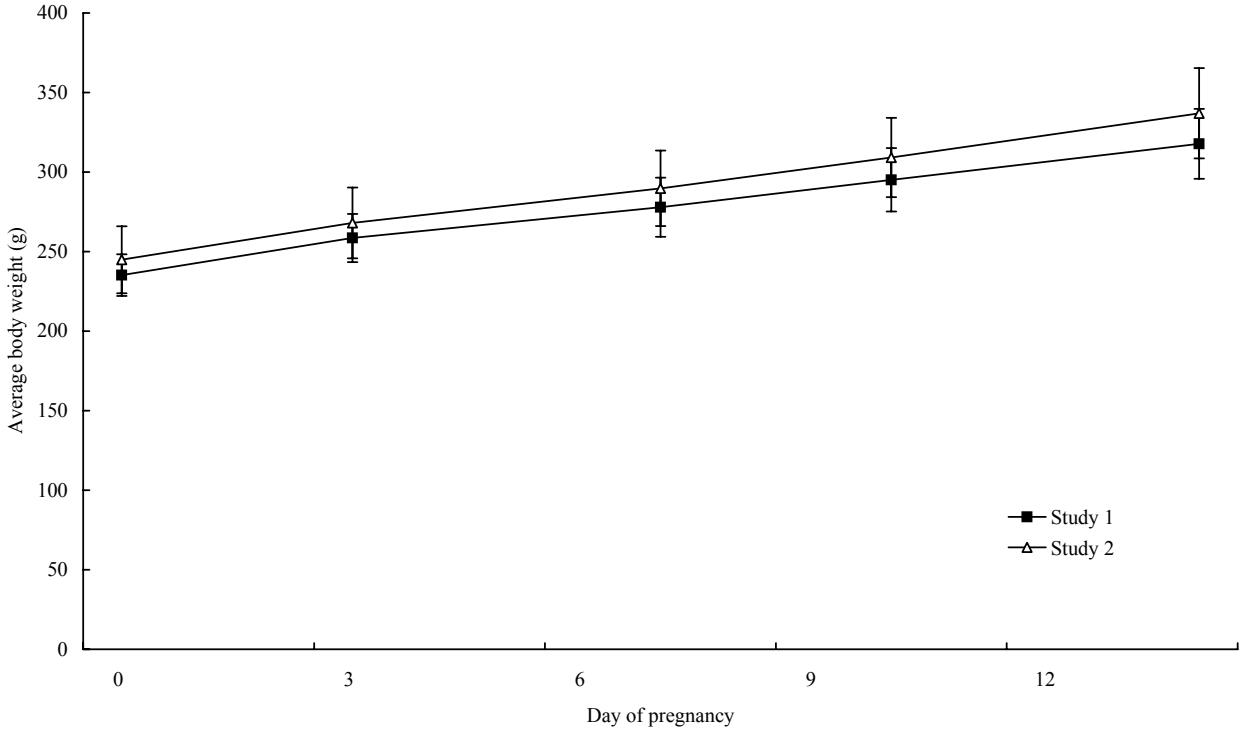

Figure 3-b. Body weight of females during gestation period

Table 3. Body weght of females

	Pre mating period					Gestation period				
	1	4	8	11	15	0	3	7	10	14
Study 1										
N	20	20	20	20	20	18	18	18	18	18
Body weight (g)	202.8	210.0	216.9	224.0	232.7	235.2	258.6	277.9	295.1	317.7
S.D.	8.3	7.7	9.1	9.8	11.1	13.1	15.1	18.5	19.9	21.9
Study 2										
N	20	20	20	20	20	20	20	20	20	20
Body weight (g)	204.8	211.0	219.2	226.8	235.9	244.9	268.1	289.8	309.2	337.0*
S.D.	12.4	14.5	16.3	18.7	19.8	21.1	22.3	23.7	24.9	28.4

Significantly different from study 1 * $P < 0.05$ (F-t test)

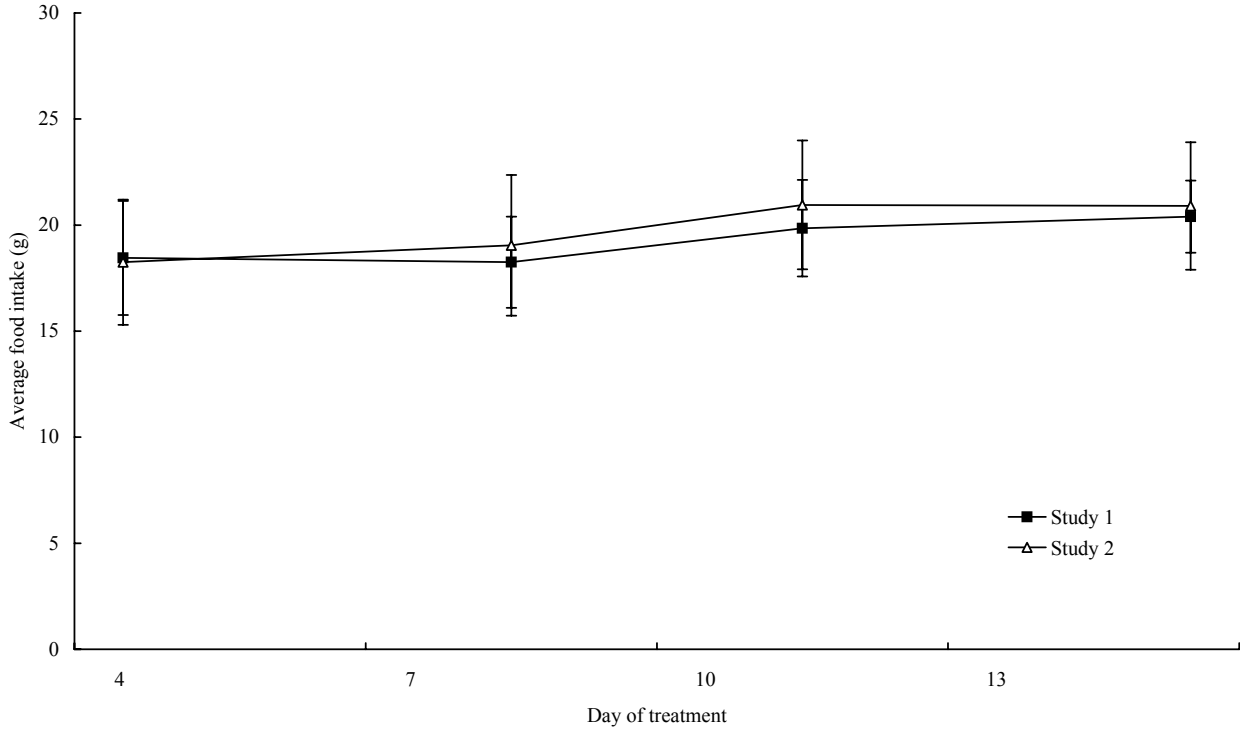

Figure 4-a. Food intake of females during before mating period

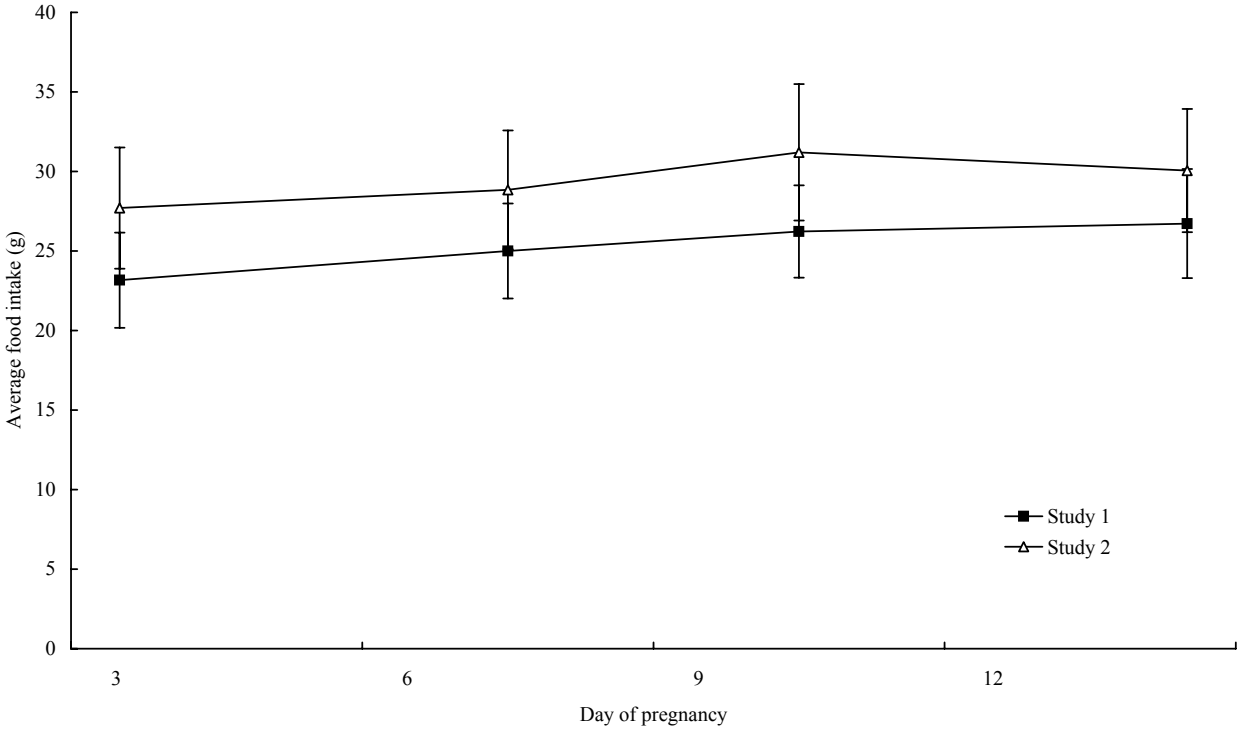

Figure 4-b. Food intake of females during gestation period

Table 4. Food intake of females

	Before mating period				Gestation period			
	4	8	11	15	3	7	10	14
Study 1								
N	20	20	20	20	18	18	18	18
Food intake (g)	18.5	18.3	19.9	20.4	23.2	25.0	26.2	26.7
S. D.	2.7	2.1	2.3	1.7	3.0	3.0	2.9	3.4
Study 2								
N	20	20	20	20	20	20	20	20
Food intake (g)	18.3	19.1	21.0	20.9	27.7**	28.9**	31.2**	30.1**
S. D.	3.0	3.3	3.0	3.0	3.8	3.7	4.3	3.9

Significantly different from study 1 ** $P < 0.01$ (F-t test)

Table 5. Mating result

	Study 1	Study 2
No. of mated animal pairs	20	20
No. of copulated animal pairs (%)	20 (100.0)	20 (100.0)
No. of plegnant females (%) (No. of impregnate males)	18 (90.0)	20 (100.0)
Average mating period (days)	2.1 ± 1.1	2.9 ± 3.1

Mean ± S.D.

Table 6. Organ weights of males

	Study 1	Study 2
N	20	18
Body weight (g)	517.3±34.3	518.1±41.2
Testis, R (g)	1.668±0.127	1.657±0.105
(g%)	0.325±0.036	0.321±0.028
Testis, L (g)	1.669±0.114	1.661±0.110
(g%)	0.325±0.037	0.323±0.029
Epididymis, R (g)	0.623±0.046	0.688±0.044**
(g%)	0.121±0.011	0.134±0.013**
Epididymis, L (g)	0.611±0.044	0.651±0.049*
(g%)	0.118±0.012	0.127±0.013*

Mean ± S. D.
Significantly different from study 1 * $P < 0.05$, ** $P < 0.01$ (F-t test)

Table 7. Sperm motility

	Study 1	Study 2
N	20	18
Sperm motility (%)	79.3 ± 11.87	79.8 ± 4.32

Mean ± S. D.

Table 8. Embryo observation

	Study 1	Study 2
No. of pregnant fetuses	18	20
No. of dams with live fetuses	18	20
(%)	(100.0)	(100.0)
No. of corpora lutea	16.6 ± 2.6	17.1 ± 2.9
No. of implants	15.4 ± 1.3	15.7 ± 3.6
Implantation rate (%)	(94.0)	(91.7)
No. of dead fetuses	0.7 ± 0.8	0.7 ± 0.9
(%)	(4.4)	(5.4)
No. of live fetuses	14.7 ± 1.6	15.1 ± 3.7
(%)	(95.6)	(94.6)

Mean ± S. D.

A Comparison of Incidence of Lumbar Ribs Between Crj:CD(SD)IGS and Crj:CD(SD) Rats in Developmental Studies

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ABSTRACT. Spontaneous incidences of fetal lumbar ribs of CD(SD)IGS rats from five developmental toxicity studies were compared to our historical control data of 11 studies using CD(SD) rats. The incidences of the lumbar ribs were not different between both strains. However, the IGS strain rats seemed to have much more sensitivity in incidence of the lumbar ribs than the SD strain rats when treated with a certain chemical compound (compound A). We conclude that the IGS rats is a useful strain to detect a teratogenic potential in the developmental study. —Key words: CD(SD)IGS, rat, developmental study, lumbar ribs

CD(SD)IGS-2002/2003 : 168-172

INTRODUCTION

Crj:CD(SD)IGS rats (IGS strain) has been developed by Charles River Inc.'s international genetic standard system to provide internationally uniform experimental animals. We performed experiments using IGS strain to collect background data and compared the incidence of the lumbar ribs to our historical control data of the usual SD strain rats. In the field of developmental toxicology, some minor variations, such as lumbar ribs, asymmetric ossification sites and so on, have been suggested to be predictive indicators of teratogenicity of compounds in rodents. We have collected the background data of such fetal variations from the developmental studies including GLP and Non-GLP studies using the IGS or the SD rats to change from old SD to new IGS rats in the developmental study. Furthermore, collecting and comparing both background control data of the SD and IGS rats have been conducted by other researchers. However, the susceptibility to chemical compounds in either strain rats has not been reported. Then, we investigated the difference of the sensitivity in the SD and the IGS rats to a certain chemical in order to know which strain is more proper to use in the developmental studies.

MATERIALS AND METHODS

Crj:CD(SD)IGS rats (Tsukuba Breeding Center, hereafter referred to as "IGS rats") and Crj:CD(SD) rats (Atsugi Breeding Center, hereafter referred to as "SD rats"), which were 10 to 11 weeks of age, were obtained from Charles River Japan Inc.

The SD rat data from the control groups of 11 studies conducted in our laboratory (1982 - 1998), and the IGS rat data from the control groups of five studies conducted in our laboratory (1997 - 1998) were compared especially on the incidence of the lumbar ribs.

In addition, three studies were performed to investigate the incidences of the lumbar ribs when administered with our new pesticide, temporarily named compound A, to investigate whether the compound has a potential of teratogenicity or fetal toxicity. The dosage levels of the compound were selected based on the LD50 value of > 5000 mg/kg. The strain of rat used, number of animals, dosage and treatment period of the studies are as follows.

Study	Strain of rat	Dose (mg/kg/day) of Compound A Number of dams				Treatment period
I	Crj:CD(SD) IGS	0 6			10 6	Day 6 through 15 of gestation
II	Crj:CD(SD) IGS	0 10	0.3 7	3 5		Day 6 through 15 of gestation
III	Crj:CD(SD)	0 24	0.3 24	2 24	10 24	Day 6 through 15 of gestation

For every study, the animals were housed individually in a metal cage in the animal room which was maintained at temperature of 21 ± 1 ℃ and relative humidity of 60 ± 10 %, ventilated approximately 12 times/hour and provided with fluorescent light for 12 hours/day. They were allowed free access to pelleted diet (CA-1, Clea Japan Inc.) and tap water *ad libitum*.

At 12 weeks of age females were paired with males of the same strain. The day on which the presence of vaginal plug or sperm in the vaginal smear was confirmed, was designated as day 0 of gestation.

Females were necropsied on day 21 of gestation. The ovaries and uterus were examined for number of corpora lutea, implantations, live or dead fetuses and resorptions. After measurement of fetal body weights, the live fetuses were sexed and observed for external anomalies. Approximately half of the fetuses in each litter were separated for preparation of skeletal specimens. They were stained by bone-cartilage double staining method with Alizarin-red and Alcian-blue, and observed for skeletal abnormalities and variations.

The normal number of rib pairs in the strain is 13 and presence of extra-ribs was specifically noted. The distribution of supernumerary ribs and transverse processes on the first lumbar vertebra were recorded as lumbar ribs in this report.

Fisher's exact test or Chi-Square test was applied to the frequency for statistical analysis. Values of the treatment groups were compared with those of the concurrent control group of the same strain of rats.

RESULTS AND DISCUSSION

Intrauterine examinations of the IGS and SD rats in our historical control data are shown in Table 1 and 2. There were

no obvious biologically significant differences in the both strains in any parameters. However, there were some tendencies that slightly fewer corpora lutea and preimplantation losses and slightly higher fetal weights were noted in the IGS rats than those in the SD rats.

External findings from our historical control data in SD and IGS rats are shown in Tables 3 and 4. No external anomalies were observed in both strains of rats. External variations such as subcutaneous hemorrhage and immature fetus were observed in both strains. Fusion of placentae was observed only in the IGS rats. For malformations, gastroschisis was observed only in the SD rats. There were no obvious biologically meaningful differences between the two strains of rats in these findings.

Skeletal findings from our historical control data in SD and IGS rats are shown in Tables 5 and 6. Various skeletal variations and malformations were observed between the two strains of rats. The main variations were asymmetry of the sternebrae, bilobed shape of the cervical vertebral body, bilobed shape of the thoracic vertebral body, lumbar ribs and splitting of the cervical vertebral body. The lumbar ribs and splitting of the cervical vertebral body were the most frequent variations. The mean incidence of lumbar ribs was 5.4 ± 5.3 % in SD rats and 6.0 ± 3.6 % in IGS rats. Each of mean incidences of the variations was not significantly between the two strains of rats. We found no increase in the incidence of the lumbar ribs in IGS rats. However, more frequent incidences of the lumbar ribs in IGS rats than SD rats have been previously reported by other investigators (1). It is known that there is a wide range of incidences of the lumbar ribs among rat strains or bleeders (2).

The incidence of the lumbar ribs in fetuses from the SD and IGS rats when administered with compound A on gestation days 6 to 15 are shown in Table 7. Compared to control group, statistically significant and dose-related increase of the lumbar ribs was observed in the fetuses delivered from IGS dams treated compound A at the high dose level of 10 mg/kg. On the other hand, statistically significant decrease of the lumbar ribs was noted in the SD rats treated with compound A at the high dose of 10 mg/kg.

In the maternal toxicity of compound A, there were no treatment-related effects on maternal body weight and food consumption in either strain of rats. Furthermore, in other fetal toxicity of compound A, the number of ossification sites (Table 8) was not affected in any of the treated groups in both strains. Therefore, the increased incidence of the lumbar ribs observed in the IGS rats was not related to the maternal stress caused by treatment of compound A such as depressed body weight gain. The sensibility of the lumbar ribs to compound A in the IGS rats seemed to be higher than the SD rats.

The lumbar ribs in fetuses may be induced by a wide variety of xenobiotics and/or through a general maternal stress (3). The lumbar ribs may be considered to serve as a good indicator of teratogenicity (4). Then using a strain sensitive to chemical compounds in the developmental study is considered to be important to detect possible teratogens.

We conclude that the IGS rats is a useful strain to detect the teratogenic potential in the developmental study.

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Table 1. Intrauterine examinations in SD rats.

Study No.	1	2	3	4	5	6	7	8	9	10	11	Total Mean	S.D.
No. of animals	23	24	23	23	23	24	23	23	24	6	7	223	-
Mean no.of corpora lutea (A)	17.7	17.7	19.0	16.2	19.3	21.5	20.7	19.2	18.0	20.8	17.4	18.9	1.65
Mean no.of implants (B)	15.1	15.0	15.2	14.5	15.0	15.1	15.5	14.7	15.1	15.2	16.0	15.1	0.39
Mean pre-implantation loss (A-B)/(A) (%)	14.7	15.3	20.0	10.5	22.3	29.8	25.1	23.4	16.1	26.9	8.0	19.3	6.93
Mean no.of live fetuses	14.4	14.3	14.3	13.5	14.3	14.1	15.0	14.0	14.6	14.8	15.1	14.4	0.46
Mean weight of fetuses (g)													
Male	5.29	3.76	5.14	5.45	5.19	3.38	5.28	5.17	5.15	5.16	5.11	4.92	0.68
Female	5.04	3.53	4.87	5.11	4.95	3.21	4.95	4.93	4.85	4.74	4.90	4.64	0.64

Table 2. Intrauterine examinations in IGS rats.

Study No.	1	2	3	4	5	Total Mean	S.D.
No. of animals	7	6	6	10	9	38	-
Mean no.of corpora lutea (A)	14.3	19.3	17.7	15.8	17.3	16.9	1.91
Mean no.of implants (B)	13.6	15.7	16.3	13.5	14.9	14.8	1.24
Mean pre-implantation loss (A-B)/(A) (%)	4.9	18.7	7.9	14.6	13.9	12.0	5.51
Mean no.of live fetuses	13.1	15.0	16.3	12.5	14.7	14.3	1.53
Mean weight of fetuses (g)							
Male	5.36	5.39	4.98	5.27	5.55	5.3	0.21
Female	5.10	5.09	4.83	4.99	5.23	5.0	0.15

Table 3. External findings in fetuses in SD rats.

Study No.	1	2	3	4	5	6	7	8	9	10	11	Total Mean	S.D.
No. of animals	23	24	23	23	23	24	23	23	24	6	7	223	-
No. of fetuses examined	332	344	329	311	328	339	345	323	350	89	106	3196	-
Variations													
Subcutaneus hemorrhage	0.3	0.6	0.0	0.0	0.0	0.3	0.6	0.0	0.6	0.0	0.0	0.2	0.27
Immature fetus	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.09
Gastroschisis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.09

Table 4. External findings in fetuses in IGS rats.

Study No.	1	2	3	4	5	Total Mean	S.D.
No. of animals	7	6	6	10	9	38	-
No. of fetuses examined	92	90	98	125	132	537	-
Variations							
Subcutaneus hemorrhage	0.0	0.0	0.0	1.6	0.0	0.3	0.72
Fusion of placentae	0.0	2.2	0.0	0.0	0.0	0.4	0.98
Immature fetus	0.0	0.0	0.0	0.0	0.8	0.2	0.36

Table 5. Skeletal findings in fetuses in SD rats.

Study No.	1	2	3	4	5	6	7	8	9	10	11	Total Mean	S.D.
No. of animals	23	24	23	23	23	24	23	23	24	6	7	223	-
No. of fetuses examined	222	176	166	156	166	170	171	160	174	43	53	1657	-
Variations													
Asymmetry of the sternebrae	0.0	0.0	0.0	0.0	0.6	0.0	6.4	1.3	1.1	7.0	0.0	1.5	2.62
Bilobed shape of sternebrae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.2	0.69
Bilobed shape of the cervical vertebral body	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.9	4.7	0.0	0.7	1.59
Bilobed shape of the thoracic vertebral body	0.0	2.3	0.6	2.6	0.0	3.5	4.1	3.8	1.1	4.7	1.9	2.2	1.67
Cervical rib	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	1.1	0.0	0.0	0.2	0.36
Lumbar rib	10.8	6.3	3.0	9.0	0.6	0.6	4.7	0.6	6.3	0.0	17.0	5.4	5.32
Not ossificated of 1st.thoracic vertebral body	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.1	0.18
Rudimentary of thoracic vertebral body	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.2	0.69
Shortened 13th rib	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00
Shortening of the rib	0.0	0.0	0.6	0.0	1.2	1.2	0.6	1.3	0.0	0.0	0.0	0.4	0.56
Splitting of the cervical vertebral body	0.0	0.0	0.0	0.0	0.0	0.0	5.8	18.1	7.5	30.2	11.3	6.6	9.87
Splitting of the sternebrae	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.6	0.0	0.0	0.1	0.24
Splitting of the thoracic	0.0	0.6	0.0	1.9	0.6	4.7	0.6	0.6	0.0	0.0	0.0	0.8	1.41
Supernumerary vertebral body	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.15
Wavy rib	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.1	0.18
12th rib	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.1	0.36
14th extra rib	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.18
25 presacral vertebrae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.1	0.18

Table 6. Skeletal findings in fetuses in IGS rats.

Study No.	1	2	3	4	5	Total Mean	S.D.
No. of animals	7	6	6	10	9	38	-
No. of fetuses examined	47	90	50	63	66	316	-
Variations							
Asymmetrically cleaved sternebrae	0.0	0.0	0.0	0.0	1.5	0.3	0.67
Asymmetry of sternebrae	0.0	2.2	0.0	0.0	3.0	1.0	1.45
Bifurcation of processus xiphoideus	0.0	0.0	0.0	0.0	3.0	0.6	1.34
Bilobed shape of cervical vertebrae, asymmetry	0.0	0.0	0.0	0.0	1.5	0.3	0.67
Bilobed shape of the cervical vertebral body	4.3	4.4	0.0	1.6	3.0	2.7	1.87
Bilobed shape of the thoracic vertebral body	0.0	2.2	2.0	0.0	1.5	1.1	1.07
Cervical rib	0.0	0.0	0.0	0.0	1.5	0.3	0.67
Lumbar rib	10.6	1.1	6.0	7.9	4.5	6.0	3.57
Rudimentary of the sternebrae	2.1	0.0	14.0	4.8	4.5	5.1	5.35
Shortened 13th rib	0.0	0.0	0.0	1.6	0.0	0.3	0.72
Splitting of the cervical vertebral body	8.5	14.4	10.0	1.6	22.7	11.4	7.80
Splitting of the sternebrae	0.0	0.0	2.0	0.0	0.0	0.4	0.89
Splitting of thoracic vertebral body	0.0	1.1	0.0	0.0	0.0	0.2	0.49
Wavy rib	2.1	0.0	0.0	0.0	0.0	0.4	0.94

Table 7. Incidence of lumbar ribs in fetuses from SD rats and IGS rats treated with compound A.

Incidence of lumbar ribs						
	Crj:CD(SD)IGS				Crj:CD(SD)	
Dose	Exp.No. Ⅰ		Exp.No. Ⅱ		Exp.No.Ⅲ	
(mg/kg)	Litter	Fetuses	Litter	Fetuses	Litter	Fetuses
0	1/6 (16.7%)	1/90 (1.1%)	4/10 (40.0%)	5/63 (7.9%)	6/24 (25.0%)	11/174 (6.3%)
0.3			4/7 (57.1%)	17/106 (16.0%)	1/24 (4.2%)	3/161 (1.9%)
2					7/24 (29.2%)	8/169 (4.7%)
3			4/5 (80.0%)	22/64 $$$,** (34.4%)		
10	5/6 $ (83.3%)	20/86 $$$,**,# (23.3%)			0/24 $ (0.0%)	0/168 ** (0.0%)

$: p<0.05, $$$: p<0.001 (Fisher exact test)
** : p<0.01 (Chi-Square test)

Table 8-1. Number of ossification sites from SD rats and IGS rats treated with compound A.

		Cervical vertebrae			Sacral and caudal vertebrae		
		Crj:CD(SD)IGS		Crj:CD(SD)	Crj:CD(SD)IGS		Crj:CD(SD)
Dose		Exp.No. Ⅰ	Exp.No. Ⅱ	Exp.No.Ⅲ	Exp.No. Ⅰ	Exp.No. Ⅱ	Exp.No.Ⅲ
(mg/kg)		Fetuses	Fetuses	Fetuses	Fetuses	Fetuses	Fetuses
0	Mean	5.70	4.65	5.09	10.53	10.56	10.66
	N	90 (6)	63 (10)	174 (24)	90 (6)	63 (10)	174 (24)
0.3	Mean		5.19			10.36	
	N		106 (7)			106 (7)	
2	Mean			5.55			10.87
	N			169 (24)			169 (24)
3	Mean		4.26			10.70	
	N		64 (5)			64 (5)	
10	Mean	4.70		4.53	10.08		10.46
	N	86 (6)		168 (24)	86 (6)		168 (24)

Table 8-2. Number of ossification sites from SD rats and IGS rats treated with compound A.

		Metatarsus			Hind Proximal Phalanges		
		Crj:CD(SD)IGS		Crj:CD(SD)	Crj:CD(SD)IGS		Crj:CD(SD)
Dose		Exp.No. Ⅰ	Exp.No. Ⅱ	Exp.No.Ⅲ	Exp.No. Ⅰ	Exp.No. Ⅱ	Exp.No.Ⅲ
(mg/kg)		Fetuses	Fetuses	Fetuses	Fetuses	Fetuses	Fetuses
0	Mean	9.70	9.79	9.33	2.23	6.25	2.12
	N	90 (6)	63 (10)	174 (24)	90 (6)	63 (10)	174 (24)
0.3	Mean		9.96			7.51	
	N		106 (7)			106 (7)	
2	Mean			9.34			2.56
	N			169 (24)			169 (24)
3	Mean		10.00			7.42	
	N		64 (5)			64 (5)	
10	Mean	9.42		8.95	1.32		1.20
	N	86 (6)		168 (24)	86 (6)		168 (24)

Age-Related Changes of Genital Systems in the Female Crj: CD® (SD) IGS Rats during Sexual Maturation

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ABSTRACT. The age-related changes of vaginal opening, body weight, the weights of the uterus and ovary, together with histological examination, serum 17β-estradiol (E2) and progesterone levels were examined in intact female Crj: CD® (SD) IGS rats between 21 and 36 days of age to understand the basic biological profile of changes of the female genital system during sexual maturation in the rat for female pubertal assays. With the beginning of the elevation of serum E2 level from 28 days of age, all parameters except body weight started to show drastic change until 31 days of age. The highest incidence of vaginal opening was recorded at 34 days of age. On macroscopic examinations, a number of rats showed uterine imbibition but vaginal opening. Immediately after the confirmation of the vaginal opening, the genital systems of three rats were observed microscopically. Both ovaries already had multiple corpora lutea, and degeneration of endometrial epithelial cells was observed. In conclusion, we obtained essential data on genital tract development of female Crj: CD® (SD) IGS rats for in vivo screening assays that will contribute to detect potential endocrine active chemicals. In addition, it is assumed that the first ovulation precedes or occurs simulutaneously with vaginal opening. — Key words: Crj: CD® (SD) IGS female rat, female pubertal assay, pubertal onset, vaginal opening, first ovulation.

CD(SD)IGS-2002/2003 : 173-177

INTRODUCTION

Currently, there is much concern that certain environmental chemicals may have the potential to disturb normal sexual differentiation and development in wild life and humans [3, 8, 9]. A Uterotrophic assay using immature female rats was proposed by the Organization for Economic Cooperation and Development and Endocrine Disrupter Screening and testing Committee (EDSTAC) of the U.S. Environmental Protection Agency as an *in vivo* screening method to detect the estrogenic or anti-estrogenic activity of the chemicals acting mainly by receptor-mediated mechanisms [6, 7]. EDSTAC has recommended another assay, termed the "female pubertal assay" [5]. The purpose of this assay is to quantify the effects of environmental chemicals on pubertal development and thyroid function in the immature female rat. Focusing on disruption of the sex hormone system, the proposed endpoints of this assay are the age of vaginal opening, reproductive organ weights and sex-related hormone levels. Which strain is best for these assays is currently uncertain, because there are differences in sensitivity among rat strains [12, 13]. Crj: CD® (SD) IGS rat (SD IGS rat) was developed recently under a new breeding system for the purpose of supplying experimental animals with minimal genetic variations. Many researchers have begun to use for regulatory toxicology studies and for various types of biochemical research. However some general biological parameters of this rat have been reported, development of the female genital system in SD IGS rats during peripubertal period has not been well characterized. An understanding of the basic biological profile of changes in the female genital system during sexual maturation in the rat is of importance for analyzing the results of female pubertal assays. In the present study, we determined age-related changes in vaginal opening, body weight, the weights of the uterus and ovary together with gross pathological examination, serum 17 β -estradiol (E2) and progesterone levels of intact non-treated SD IGS female rats from 21 to 36 days of age, i.e., during the pubertal period. In addition, the genital systems of three rats immediately after vaginal opening were examined microscopically to obtain the preliminary data on the relationship between the first ovulation and vaginal opening.

MATERIALS AND METHODS

Animals: Fourteen timed-pregnant specific-pathogen-free female Crj: CD (r) (SD) IGS (SD IGS) rats were purchased from Charles River Japan, Inc. (Hino Breeding Center and Atsugi Breeding Center). Insemination was confirmed by the presence of a sperm-plug in the vagina. The day following overnight mating was designated as pregnant day 0 and the rats were primiparous at 12 weeks of age. They arrived on pregnant day 14. The animals were housed in an animal room with the temperature set at 23±2℃, the relative humidity at 55±10%, the ventilation rate at 10-15 times/h, and lighting for 12 h daily from 7:00 am to 7:00 pm. Dams were housed individually in hanging stainless steel cages with a wire-mesh floor (260 W x 380 D x 180 H mm) from pregnant day 14 to 17. Neonatal rats were delivered in our laboratory (date of birth designated 0 days of age). Dams and their litters were housed in polycabonate cages (280 W x 440 D x 150 H mm) with nesting materials (Sun Flake®, Chiba Animal Material Co., Ltd., Japan) from pregnant day 17 until weaning. At 4 days of age each litter was culled to eight female rats. The rats were weaned at 20 days of age. Then, weanlings were ranked by weight and 20 rats each were randomly assigned into 12 experimental groups using a body-weight stratified randomization method to minimize variation in body weights among the groups. After grouping, the weanlings were housed in stainless steel cages with a wire-mesh floor (165 W x 300 D x 150 H mm) hung in a 6-vertical by 6-horizontal cage allocable stainless steel cage rack (1 animal/cage). The animals were allowed free access to autoclaved solid food (before weaning of offspring, CRF-1, after weaning, MF, Oriental Yeast Co., Ltd., Tokyo, Japan), and

Note: This paper is duly permitted to reprint from original paper (J. Vet. Med. Sci., 64(4):315-319, 2002).

to chlorinated tap water from an automatic dispenser or supply bottles. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *the Japanese Association for Laboratory Animal Science.*

Experimental designs: The twenty intact rats in each group were sacrificed at 21, 23, 24, 28, 29, 30, 31, 32, 33, 34, 35 or 36 days of age. At necropsy, the rats were weighed and blood samples were collected from the abdominal aorta and then euthanized by exanguination under ether deep anesthesia. To reduce the fluctuation of hormonal levels due to stress, the transportation and handling of rats were carried out with great care on the day of necropsy. Macroscopic examinations of the female rat genital system were performed. As 3 rats showed vaginal opening immediately before dissection and corpora lutea-like structures were observed macroscopically, the genital systems of these rats were examined microscopically; the samples were prepared by fixsation in neutral-buffered formalin after embedding in paraffin, followed by sectioning and hematoxylin and eosin staining to confirm that they had corpora lutea. The ovaries and uterus of each rat were removed and weighed. The blotted uterine weights, i.e., weight without excess fluid, were also determined by excising the uterine horns and blotting the excess fluid onto filter paper. The sera were stored at -80℃ until measurement of 17 β -estradiol (E2) and progesterone levels. Until sacrifice, general condition and evidence of vaginal opening were recorded daily and body weight was determined once a week from 21 days of age. The serum E2 level was measured using a commercial radioimmunoassay kit (DPC estradiol double antibody kit, lot No. 300, Diagnostic Products Co., Los Angels, CA, USA), and serum progesterone was measured using a commercial immunoassay kit (Progesterone Enzyme Immunoassay Kit, lot No. 13153A, ©Cayman Chemical Company, Ann Arbor, MI, USA).

RESULTS

General conditions and body weights: During daily clinical observations, no abnormalities were noted and mean body weight gradually increased (Fig. 1). The mean value ± standard deviation (SD) of the body weight at weanling 20 days of age was 48.5 grams ± 2.9.

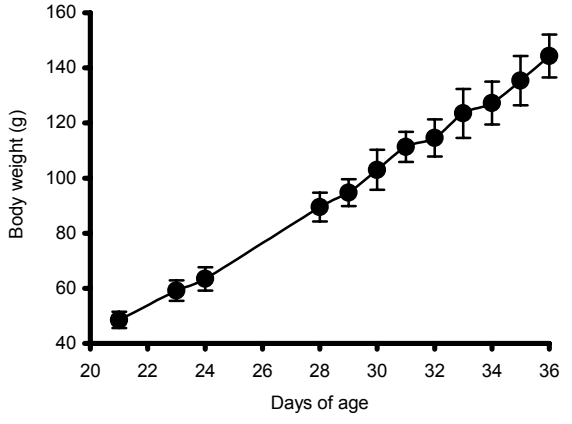

Fig. 1. Body weights of female SD IGS rats during the pubertal period. Points are means and vertical lines represent SD.

Vaginal opening: The earliest incidence of vaginal opening occurred at 30 days of age, the latest at 35 and the highest at 34 (Table 1).

Organ weights and findings: In general, weight of uterus increased slightly until 29 days of age and then increased rapidly, first peaking at 31 days of age, and was associated with the weight fluctuation (Fig. 2A). The profiles of the absolute uterine weights were similar to relative weights (Data not shown). On macroscopic examinations, watery contents in the uterine lumen, i.e., uterine imbibition, was observed in rats older than 30 day. These findings were considered to be identical to those of the proestrus stage. Many rats showed uterine

Table 1. Vaginal Opening in SD IGS Rats during Pubertal Period

	Days of age					
	30	31	32	33	34	35
Number of vaginal opening / examined	1/140	8/120	12/100	3/80	23/60	10/40
Percentage (%)	0.7	6.7	12.0	3.8	38.3	25.0

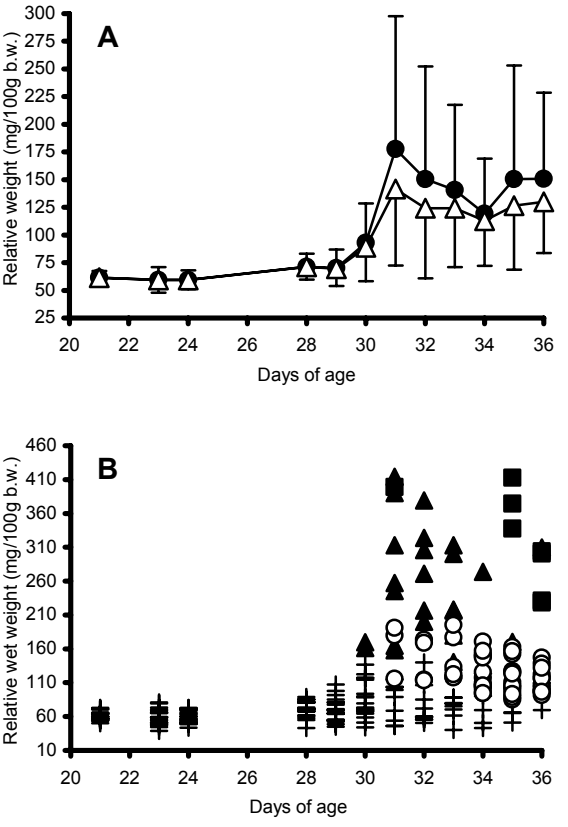

Fig. 2. Relative uterine weight in female SD IGS rats during pubertal period. (A), Uterine weight. Points are means and vertical lines represent SD (n=20). ●, wet weight; △, blotted weight. (B), Individual uterine weights with genital system findings. +, rat with neither vaginal opening nor uterine imbibition; ▲, without vaginal opening but with uterine imbibition; ○ , with vaginal opening but without uterine imbibition; ■ , with both vaginal opening and uterine imbibition.

imbibition but not vaginal opening (Fig. 2B, closed triangles.). On the other hand, some rats with uterine imbibition already showed vaginal opening (Fig. 2B, closed squares) occurring at later ages during our experimental period, i.e., 35 or 36 days of age, except in one animal.

Absolute ovarian weights gradually increased until 28 days of age and thereafter more increased (Fig. 3A). On the other hand, relative weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age followed by an increase from 30 to 34 days of age (Fig. 3B).

Three rats were dissected immediately after vaginal opening was achieved. Figure 4 presents an example of the microscopic findings of genital system when immediately vaginal opening was observed, but it could not found at the routine morning observation. The interval between the time of morning observation and the discovery of vaginal opening was approximately 3 hours. Both ovaries had multiple corpora lutea consisting of cells with scant cytoplasm containing a small number of fine vacuoles, indicating the corpora lutea to not yet be mature (Fig. 4A). A large proportion of endometrial cell was degenerative, as in the metestrus phase of the endmetrium of normal mature rats (Fig. 4B). In the vagina, cornification, matching the late estrus phase was evident (Fig. 4C).

Serum E2 and progesterone levels: The high initial E2 level at 21 days of age decreased at 28 days of age. Generally, the first peak was seen at 31 days of age, followed by a gradual decrease until 34 days of age (Fig. 5A). The serum E2 level variation after 29 days of age was very large, as was that of uterine weight. There was a significant positive relationship between the serum E2 level and uterine weight after 29 days of age (r = 0.724, p<0.001). Serum E2 levels tended to be higher in rats with uterine imbibition than in those without it (Fig. 5B). After 31 days of age, serum progesterone levels increased rapidly (Fig. 6).

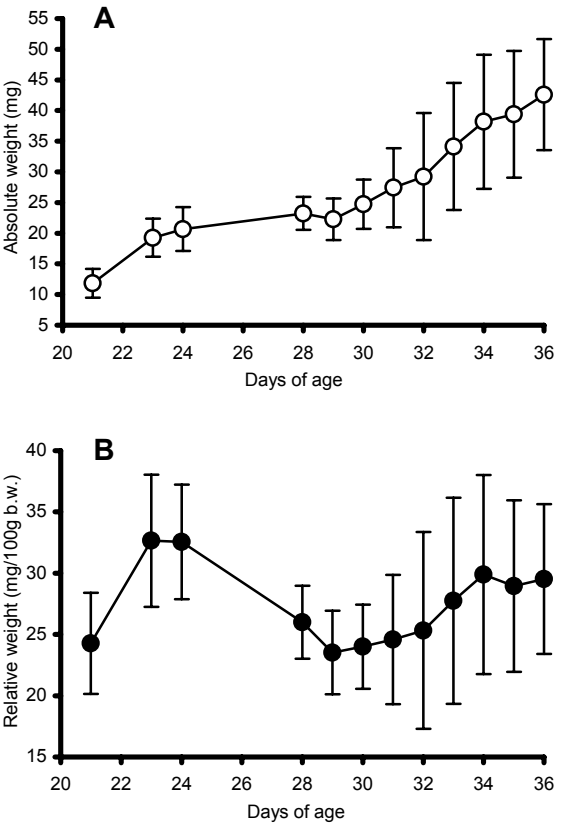

Fig. 3. Ovarian weight in female SD IGS rats during pubertal period. (A), Absolute ovarian weight. (B), Relative ovarian weight. Points are means and vertical lines represent SD (n=20).

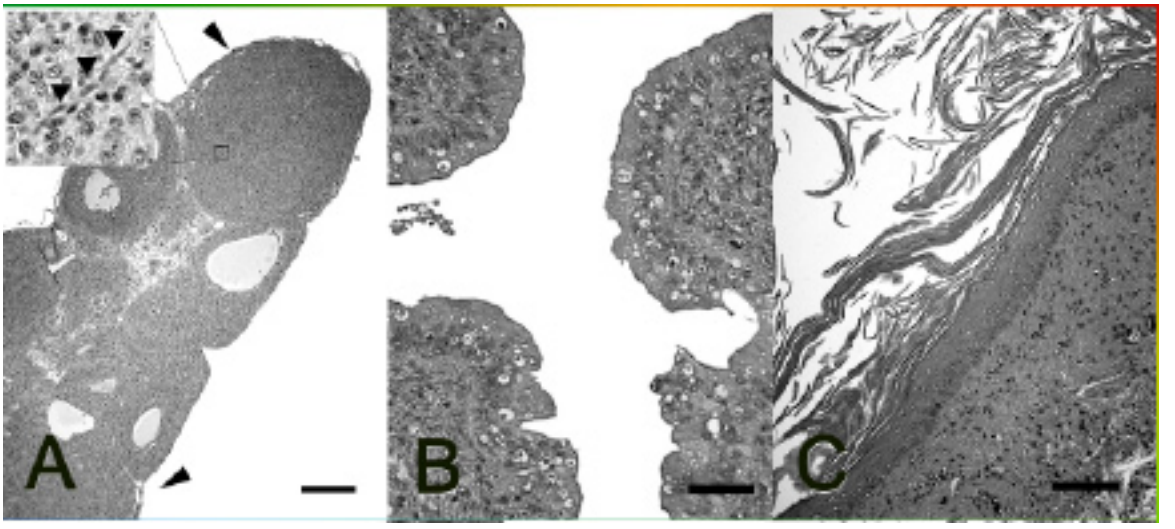

Fig. 4. Microscopic findings of the genital systems of the rat immediately after vaginal opening was noted. (A), Ovary showing a corpus luteum (upper area) and a Graafian follicle (lower area). Arrowheads point a blood vessel, which is a feature of corpora lutea. Cells with relatively rich cytoplasms and prominent nucleoli tightly contact with each other. In contrast, a Graafian follicle consists of granulosa cells with dense nuclei and occasional loose intercellular gaps. H&E staining. Bar = 50 μ m. (B), degeneration of endometrial epithelial cells. H&E staining. Bar = 50 μ m. (C), Prominent cornification in vagina. H&E staining. Bar = 100 μ m.

DISCUSSION

In this study, we measured changes in the frequency of vaginal opening, uterine weight, ovarian weight, and both serum 17 β -estradiol (E2) and progesterone levels, seen during the pubertal period in female SD IGS rats.

With the beginning of the elevation of serum E2 level from 28 days of age, uterine and ovarian weights, serum progesterone level started to show drastic change until 31 days of age. Vaginal opening and uterus imbibition was also observed in rats older than 30 days. Especially, uterine weight change correlated with the serum E2 level after 29 days of age. E2 is known to increase uterine weight and promote hypertrophy of the endometrial epithelium [2]. These changes after 28 days of age were considered to originate in pubertal onset. In general, pubertal onset in rats encompasses the period of vaginal opening and first ovulation [4]. In female SD IGS rats of this study, pubertal onset was considered to start from 28 days of age. It is noteworthy that many rats showed uterine imbibition without vaginal opening. This means that uterine imbibition, i.e., proestrus-like change, precedes vaginal opening.

The high initial E2 level at 21 days of age did not have an effect on the uterine weight. This is attributable to the high concentration of α -fetoprotein, which binds up the estrogens available around this age [1, 10]. It is generally accepted that E2 is inactive when bound to α -fetoprotein. In addition, relative ovarian weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age. Meijs-Roelofs reported that the number of follicles with a volume $\geq 100 \times 10^5 \mu m^3$ increased rapidly until 23 days of age and then more slowly until 27 days of age in contrast to the gradual body weight increase [11]. Thus, the decrease in relative ovarian weight after 24 days of age may be attributable to altered numbers of follicles at this stage.

In addition to the experiment described above, three rats were dissected immediately after vaginal opening was achieved. The histological findings of the genital systems of these rats, especially in terms of the corpora lutea formation and degenerative endometrium are indicative of the metestrus stage. These findings lead us to speculate that the first ovulation in the rat could occur before or at least at the same time as vaginal opening because it is unlikely that multiple corpora lutea formation and endometrial degeneration could occur in such a short interval. This hypothesis is supported by our observation that proestrus-like change precedes vaginal opening. It is noteworthy that our hypothesis contrasts with the literature, in which, vaginal opening is generally described as occurring with or shortly before the first ovulation [4]. However, we have not pinpointed the histology of the genital systems of these rats at the instant of vaginal opening nor have we clarified whether the histological findings of the rat genital system at the instant of vaginal opening are identical to those of mature and normally cycling rats. The further studies will be needed.

In conclusion, we obtained essential data on genital tract development of female Crj: CD® (SD) IGS rats for *in vivo* screening assays that will contribute to detect potential endocrine active chemicals. Our results will contribute to

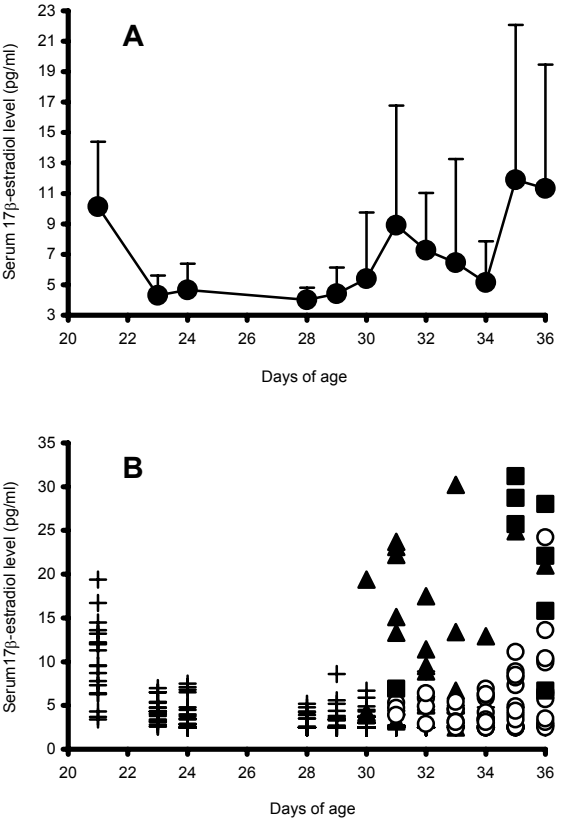

Fig. 5. Serum 17 β -estradiol (E2) levels in female SD IGS rats during pubertal period. (A), Serum E2 level. Points are means and vertical lines represent SD (n=20). Detection limit, <2.5 *p*g/m*l*, was excluded from the calculation. (B), Relationship between individual serum E2 levels and gross findings. +, rat with neither vaginal opening nor uterine imbibition; ▲ , without vaginal opening but with uterine imbibition; ○ , with vaginal opening but without uterine imbibition; ■ , with both vaginal opening and uterine imbibition.

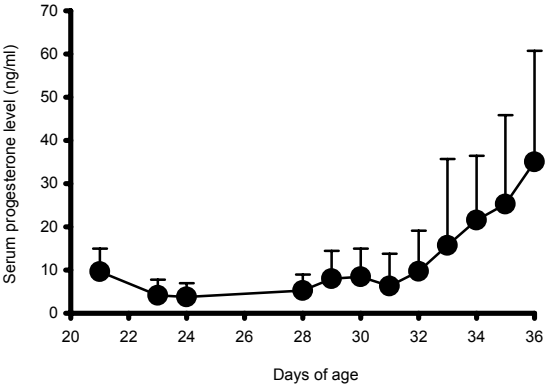

Fig. 6. Serum progesterone level in female SD IGS rats during pubertal period. Points are means and vertical lines represent SD (n=20).

analyses of studies employing the peripubertal female rats. In addition, it is assumed that the first ovulation precedes or occurs simultaneously with vaginal opening, which opposes the established theory in terms of the timing of the ovulation.

ACKNOWLEDGMENTS

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Reproductive Effects in Male and Female Rats of Neonatal Exposure to Genistein

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ABSTRACT. Sprague-Dawley rats were administered genistein orally at doses of 12.5, 25, 50, or 100 mg/kg on postnatal days 1 through 5 to examine its effects on reproductive function after puberty. In addition, preputial separation and vaginal opening as endpoints of sexual maturation, estrous cycling, sperm count, serum testosterone concentration, and histopathologic changes of reproductive organs of male and female rats were examined. Body weights of male and female rats exposed to genistein at any dose level examined were lower than those of controls.Timing of preputial separation in males and timing of vaginal opening were not affected by genistein treatment. The number of females showing estrous cycle irregularities was increased by genistein treatment. The fertility of female rats exposed neonatally to genistein at 100 mg/kg was disrupted, while neonatal exposure to genistein did not affect male fertility. Neither sperm counts nor serum testosterone concentration were changed by neonatal exposure to genistein. Female rats exposed neonatally to genistein at 100 mg/kg showed histopathologic changes in the ovaries and uterus, while male rats showed no histopathologic alterations in the gonads. The results of this study indicate that early neonatal exposure to genistein caused dysfunction of postpubertal reproductive performance as well as abonormal development of gonads in female but not in male rats. — Keywords: reproductive function, neonatal exposure, phytoestrogen, genistein, rats

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1. INTRODUCTION

Dietary estrogen consumption in humans is a matter of considerable concern [1]. Dietary phytoestrogens are naturally occurring constituents of plants that are known to elicit estrogen-like effects in animals. The richest sources among foodstuffs are legumes and grains, with soy content of the isoflavones genistein, daidzein, and their conjugates on the order of 0.5 to 3 mg/g of soy protein [2]. Human genistein exposure comes primarily from the consumption of soy products. In the typical Asian diet, 1.5 mg of genistein or other isoflavones/kg/day may be ingested, whereas the typical Western diet contains less than 0.2 mg/kg/day [3]. Soy-containing infant formulas and breast milk of mothers consuming soy foods contain isoflavones such as genistein [4,5]. Genistein competes with estradiol for binding to the estrogen receptor, with a stronger affinity for the β than the α receptor [6]. Genistein may also act through other mechanism, including inhibition of enzymes (aromatase, tyrosine kinases, and DNA topoisomerase), increased synthesis of sex hormone binding globulin, and antioxidation [7-9]. Recently, genistein has been reported to have adverse effects on animal reproductive systems [10,11], as well as several beneficial effects on human health [12].

The aim of the present study was to evaluate whether early exposure of neonates to genistein has any effect on the development of sexual organs and/or reproductive performance including sperm concentration and serum testosterone concentration, and sexual maturation was examined. In addition, the effects of genistein on the development of reproductive organs were compared with those of the synthetic estrogen ethinyl estradiol.

2. MATERIALS AND METHODS

2.1. Animals and treatment

Sprague-Dawley rats (Crj:CD, IGS) were purchased from Charles River, Atsugi, Japan, at 8 weeks of age. The animals were acclimated to the laboratory for 2 weeks prior to the start of the experiments. Animals were housed individually in metal cages in a room with controlled temperature (24 ± 1 ℃) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Rats were given access to food (CE-2, Clea Japan) and tap water ad libitum.

Estrous female rats at 10 to 11 weeks of age were cohabited overnight with a single male . The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation.Once insemination was confirmed, the females were weighed and randomly allocated to six experimental groups. The dams were allowed to deliver naturally and nurse their pups until postnatal day (PND) 21. On PND 0 (the day of birth), all pups were weighed and their sex was determined, and the litters were culled randomly to eight (four pups/sex/litter when possible). Litters of eight pups or less were not reduced. Thus, in the present study, three to five males and three to five females per litter were used. The remaining pups were discarded and the nurslings were weighed on each day of treatment (PND 1 through 5), PND 6 (the day after the final treatment) and 14, and at 3 (PND 21, the day of weaning), 5, 7, and 9 weeks of age.

All neonates were given daily gavage administration of 12.5, 25, 50, or 100 mg/kg genistein (Sigma Chemical Co., St. Louis, MO, USA), or 2 mg/kg ethinyl estradiol (abbreviated as EE; Sigma Chemical Co., St. Louis, MO, USA) from PND 1 through 5 according to our previously reported method [13,14]. The normal pattern of sexual differentiation of the genital tract as well as central nervous system are directly attributable to the presence of specific gonadal steroids during critical periods of development. Thus, neonatal rats received administration of genistein on PND 1 through 5. Control animals received an equal volume of the corn oil vehicle. The numbers of pups treated on PND 1 were 31 males and 29 females from seven

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litters in the control group, 25 and 25 from five litters in the 12.5 mg/kg group, 25 and 21 from five litters in the 25 mg/kg group, 28 and 21 from five litters in the 50 mg/kg group, 23 and 25 from six litters in the 100 mg/kg group, and 10 and 10 from five litters in the EE-treated group, respectively.

In a preliminary study, each of 10 male pups was administered genistein oreally at a dose of 0 (corn oil), 50, 100 or 200 mg/kg on PND 1 through 5. Body weight in pups given genistein at 100 and 200 mg/kg was significantly decreased as compared with that in the controls when weighed on PND 6, 14, and 21. Marked reduction of viability on PND 6 and 21 was found in pups treated with genistein at 200 mg/kg as compared with that in the controls, whereas viability in the pups treated with genistein at 50 or 100 mg/kg was comparable to that in the controls. Based in these results, genistein doses used in the present study were chosen as 100 mg/kg/day at the highest dose, 50, 25, and 12.5 mg/kg/day as the lowest dose. Dose of EE was set at 2 mg/kg/day according to the results of our previous study (Nagao, unpublished data). In the previous study, 5 male and 5 female rats per group were exposed orally to EE at 0.1, 0.5, 1, or 2 mg/kg/day from PND 1 through 5, viability and growth until weaning were monitored, and the measurement of sexual organ weight and histopathologic observation of the reproductive tracts were performed. Pups in the highest dose group showed growth retardation during the lactational period, and the development of the reproductive organs was disrupted. Pups in the other treated groups showed no evident alteration in their growth and development of gonads. From these results, we chose the dose that revealed obviously adverse effects on the development of gonads when administered during PND 1 through 5. The dosing volume was 5 ml/kg body weight in all groups, and the administration volume for each pup was individually adjusted according to the body weight on each day of dosing. The numbers of live and dead pups in each litter during the lactation period were recorded, and viability on PND 6 and 21 (weaning index) was determined. Male and female rats exposed neonatally to EE were subjected to only histopathologic inspection of reproductive organs after puberty.

2.2. Evaluation of sexual maturation

As criteria for sexual maturation, the timing of vaginal opening for female rats (beginning on PND 28) and preputial separation for male rats (beginning on PND 35) were assessed and each pup was weighed when these criteria were achieved.

2.3. Evaluation of estrous cycling

Daily vaginal lavage fluid in each female was collected from 7 weeks of age until confirmation of copulation after cohabitation for evaluation of reproductive performance. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright-Giemsa stain. Cytology was then evaluated and the stage of the estrous cycle was determined using the method of Everett [15]. The number of days in each stage of the cycle, cycle length, and the number of normally cycling females were determined. A normal cycle was defined as being from 4 to 6 days including 1 to 2 days of estrus.

2.4. Evaluation of reproductive performance

At 12 weeks of age, male and female rats in each group (number of males and females: control group, 24 and 24; 12.5 mg/kg group, 20 and 20; 25 mg/kg group, 20 and 14; 50 mg/kg group, 21 and 13; 100 mg/kg group, 18 and 20) were permitted to mate on a 1:1 basis with untreated male or female rats to evaluate their reproductive ability for up to 2 weeks. Copulation was confirmed by the presence of sperm in the vaginal smear. Genistein-treated females and untreated (intact) females that had copulated were sacrificed on day 12 of gestation, subjected to necropsy, and the number of implants and live or dead (resorption) embryos was counted. Genistein-treated males and treated females that did not show successful copulation with untreated rats were remated with new untreated rats for up to 2 weeks. Treated males that did not impregnate the copulated untreated females were also remated with new untreated females for up to 2 weeks.

2.5. Sperm concentration, measurement of reproductive organ weight, and determination of serum testosterone concentration

After the measurement of terminal body weight at least 14 days after copulation, male rats (number of males and litters tested: control group, 24 and 7; 12.5 mg/kg group, 20 and 5; 25 mg/kg group, 20 and 5; 50 mg/kg group, 21 and 5; 100 mg/kg group, 18 and 6) were anesthetized under ether, and their blood was collected via the inferior vena cava. After centrifugation (3000 *g*, 15 min), the serum was separated and stored at -80℃ until enzyme immunoassay of testosterone. The concentration of testosterone in serum was determined using a testosterone enzyme-immunoassay kit (Diagnostic System Laboratories, Inc., USA) according to the manufacture's instructions.

Subsequently, the testis, epididymis, ventral prostate, and seminal vesicles with coagulating glands of the males from the genistein-treated and control groups were weighed.

Finally, after thawing of the left cauda epididymis, 5 ml of water was added and the cauda was homogenized. The homogenates were stained with an IDENT staining kit (Hamilton Thorne prepackaged DNA-specific dye based on Hoechst 33342). The stained samples were placed onto Cell-Vu slides (Fertility Technologie, MA, USA), and the number of sperm was counted using an HTM-IVOS analyzer (Hamilitone Thorne Research, MA, USA) and the IDENT software supplied with the HTM-IVOS.

2.6. Histopathologic evaluation of reproductive organs

On PND 21, randomly selected rats in each group (five males and five females for each group except the EE-treated group) were anesthetized under ether, and subjected to necropsy. Subsequently, the testes in males were fixed in Bouin's solution, and ovaries and uterus in females were fixed in 0.1 M phosphate buffered 10% formalin solution, stained with hematoxylin and eosin, and examined histologically.

After measurement of organ weight at 18 weeks of age, the testes, epididymides, ventral prostate, and seminal vesicles with coagulating glands of all males, including those treated with EE, and the uterus and ovaries of females that did not copulate

or were not pregnant in the genisteintreated groups, and those of EE-treated females were fixed in Bouin's solution or 0.1 M phosphate buffered 10% formalin solution, stained with H&E, and examined histologically. Histopathologic observations were performed by experimenters blind to treatment conditions.

2.7. Data analyses

Statistical analysis of the data for the offspring (body weight and organ weight, organ/body weight ratios, number of implants, timing of vaginal opening, and preputial separation, sperm concentration, and serum testosterone concentration) was performed using the litter as the unit [16,17]. The body weight and organ weight, organ/body weight ratios (relative organ weight), number of implants, timing of vaginal opening, and preputial separation, sperm concentration, and serum testosterone concentration were compared by analysis of variance (one-way), Bartlett's test for homogeneity of variance, and the appropriate *t*-test (for equal and unequal variance) as described by Steel and Torrie [18] using Dunnett's [19] multiple comparison tables or the heterogeneous Student's *t*-test with Bonferroni's correction to determine the significance of differences. Nonparametric dichotomous data (frequency data such as the various indices) were initially analyzed by the Chi-square test and if significance was observed between groups then by the Fisher's exact test with the Bonferroni adjustment [20]. In addition, nonparametric data were analysed using the Kruskal-Wallis test [21], followed by the Mann-Whitney U test for pairwise comparisons [21], when appropriate to evaluate the effect of litter of origin on reproductive performance. Comparisons between the genistein-treated groups and the control group were made using $P \leqq 0.05$ and $P \leqq 0.01$ as levels of significance.

3. RESULTS

3.1. Viability

No overt signs were apparent in any pups during the postnatal period including the administration period from PND 1 through 5. There were no significant differences in the number or viability of pups on PND 6 (viability index on the day after the final treatment) or 21 (weaning index) in the genistein-treated groups as compared with those in the control group (Table 1). The numbers of pups that died during PND 1 through 6 were two males in the control group, two females in the 25 mg/kg group, and two males and two females in the 50 mg/kg group (Table 1). None of pups in the 12.5 or 100 mg/kg groups died during this period. No pups died after weaning in any group.

Table 1. Body weight changes of rats exposed neonatally to genistein

Dosage	Weeks of age						
(mg/kg/d)	0(PND 1)	1(PND 6)	2(PND 14)	3(PND 21)[a]	5	7	9
Male							
0	7.4±0.2[b](31/7)[c]	15.8±4/29[d]	33.8±0.7/29	48.4±1.3/29	157.3±2.9/24	282.2±7.1/24	388.7±7.5/24
12.5	7.3±0.2 (25/5)	15.1±0.5/25	33.6±1.2/25	47.5±1.5/25	155.1±4.2/20	280.7±7.2/20	381.1±11.1/20
25	7.3±0.2 (25/5)	15.0±0.3/25	33.1±1.2/25	47.4±1.3/25	154.2±4.0/20	273.5±6.1/20	375.4±8.8/20
50	7.2±0.2 (28/5)	14.6±0.5/26	32.4±1.4/26	46.5±1.7/26	148.6±3.5*/21	262.6±6.6*/21	370.8±5.8*/21
100	7.4±0.3 (23/6)	13.5±0.7*/23	31.2±1.5*/23	45.2±2.8*/23	146.8±2.4*/18	260.5±6.8*/18	365.7±8.4*/18
Female							
0	7.2±0.1 (29/7)	15.3±0.4/29	32.7±0.6/29	48.4±0.9/29	130.1±3.1/24	199.1±3.2/24	247.2±5.3/24
12.5	7.2±0.2 (25/5)	15.0±0.4/25	32.3±0.9/25	43.3±1.1/25	129.7±2.3/20	194.7±3.4/20	234.2±5.0*/20
25	7.2±0.2 (21/5)	14.4±0.5/19	31.5±1.5/19	44.6±1.6/19	122.7±3.2/14	188.0±4.9/14	227.0±7.2*/14
50	7.1±0.2 (20/5)	14.1±0.5/18	31.0±1.6/18	42.1±0.9/18	120.9±2.1*/13	182.7±3.9**/13	225.3±3.9**/13
100	7.2±0.2 (25/6)	13.1±0.6*/25	29.4±1.2*/25	39.7±1.1*/25	119.0±1.8**/20	178.5±4.7**/20	220.9±4.6**/20

[a] Five male or female rats were sacrificed on PND 21 and their reproductive organs were examined histologically.
[b] Values: g, Mean±S.E.
[c] Number of rats and litters weighed, [d] Number of rats weighed.
*Significantly different from the control, $P<0.05$.
**Significantly different from the control, $P<0.01$.

3.2. Growth and sexual maturation

Table 1 shows the average body weights of rats at 0, 1, 2, 3, 5, 7, and 9 weeks of age. In males, body weights in the 100 mg/kg group were significantly lower than those in the controls throughout the study after the administration period. In addition, body weights at 5, 7 and 9 weeks of age in the 50 mg/kg group were significantly lower than those in the controls. After administration, body weights were decreased in a dose-dependent manner at each measurement. However, body weights in the 12.5 and 25 mg/kg groups were not significantly different from those in the controls. In females, body weights in the 100 mg/kg group were significantly lower than those in the controls throughout the study after the administration period, and those in the 50 mg/kg group at 5, 7 weeks of age, and those in all genistein-treated groups at 9 weeks of age were significantly decreased as compared with the controls. Decreased body weight gain is common finding in rats with estrogenic exposure [22].

The times of preputial separation in males and of vaginal opening in females are shown in Table 2. The day of preputial separation in all genistein-treated groups was not significantly different from that in the controls. In females, the timing of vaginal opening in all genistein-treated groups was comparable to that in the controls.

Table 2. Preputial separation and vaginal opening of rats exposed neonatally to genistein

Dosage (mg/kg/d)	PND of	
	Preputial separation	Vaginal opening
0	44.21±0.40[a](24/7)[b]	32.56±0.39 (24/7)
12.5	44.28±0.19 (20/5)	32.44±0.43 (20/5)
25	43.73±0.41 (20/5)	32.30±0.71 (14/5)
50	43.93±0.39 (21/5)	32.88±0.56 (13/5)
100	44.35±0.41 (18/6)	32.33±0.45 (20/6)

[a] Mean±S.E.
[b] Number of rats and litters examined

3.3.Estrous cycle evaluation

Fig. 1 shows the estrous cycle of females exposed neonatally to genistein. The number of days in each stage of the cycle in the genistein-treated groups was comparable to that in the control. The estrous cycle length in the genistein-treated groups (4.2 to 5.2 days) was not significantly different from that in the control group (4.2±0.2 days). The incidences of normally cycling females were 87.5% (21 females showing a normal estrous cycle/24 females monitored) in the controls, 43.3±19.4% (8/20) in the 12.5 mg/kg group, 43.8±15.7% (6/14) in the 25 mg/kg group, 22.9±15.7% (3/13) in the 50 mg/kg group, and 33.4 ± 12.4% (8/20) in the 100 mg/kg group, respectively. There were significant differences ($P<0.01$) among the genistein-treated groups and the controls when analyzed with a χ^2 test, although the incidences did not follow a dose-response relationship.

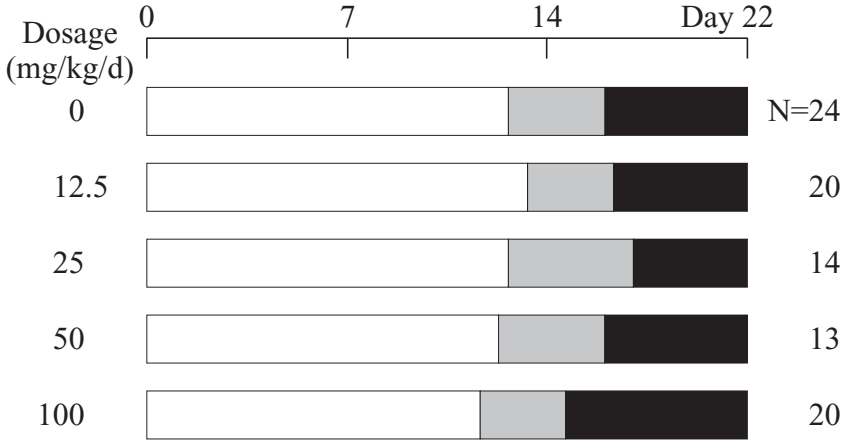

Fig. 1. Number of days of each estrous stage before cohabitation in females exposed neonatally to genistein. (open rectangle), diestrus; (shaded rectangle), proestrus; (filled rectangle), estrus. Diestrus smears contained many leukocytes, and increased numbers of basophilic epithelial cells and Schorr cells, and an increased amount of mucus. Proestrus smears contained no leukocytes, few to many clustered basophilic epithelial cells, from few nucleated epithelial cells, increased bacteria, and a varied population of cornified epithelial cells. Estrus smears contained no leukocytes, a decreased number of bacteria, and cornified epithelial cells in sheets and clumps.

3.4.Reproductive performance of male and female rats

Table 3 summarizes the data on the copulation and fertility indices of male and female rats exposed neonatally to genistein. For male reproductive performance (genistein-treated males cohabited with untreated females), copulation indices in all genistein-treated groups were 100%, and the fertility indices were 90 to 100%. The number of males that did not impregnate untreated females was 2 in the 25 mg/kg group, and one in each of the 50 and 100 mg/kg groups. These males impregnated females when remated with new untreated females in a second mating trial. Days until copulation were not different between the genistein-treated groups and the controls. The number of implants per litter in untreated females that copulated with males treated with genistein at 100 mg/kg compared to the controls (12.8 vs. 14.7) was not statistically different. The number of implants per litter in other genistein-treated groups was comparable to that in the controls. For female reproductive performance (genistein-treated females cohabited with untreated males), copulation indices in the genistein-treated groups were 91.7 to 100%, and were not significantly different among groups. The number of genistein-treated females that failed to copulate was one in each of the 12.5, 25, and 100 mg/kg groups. These females were remated with new untreated proven fertile males in a second mating trial. However, none of them copulated within 14 days. The fertility indices in all genistein-treated groups (44.4 to 77.1%) were significantly reduced as compared with the control index of 100%. Five females from four litters in the 12.5 mg/kg group, 3 from 2 litters in the 25 mg/kg group, 3 from 3 litters in the 50 mg/kg group, and 9 from 5 litters in the 100 mg/kg group did not become pregnant (Table 3).

The number of days until copulation was not significantly different between the genistein-treated groups and the control group. However, the number of implants per litter in the 100 mg/kg group was significantly decreased as compared with the controls. The numbers of dead embryos (resorptions) per litter in male and female genistein-treated groups were comparable to that in the controls (data not shown).

Table 3. Reproductive ability of rats exposed neonatally to genistein

Dosage (mg/kg/d)	No. of rats and litters examined	No. of rats and litters copulated	Copulation index [%, mean±S.E.]	No. of fertile rats and litters	Fertility index [%, mean±S.E.]	Days until copulation [mean±S.E.]	No. of implants per litter [mean±S.E.]
Male fertility[a]							
0	24/7	24/7	100	24/7	100	2.5±0.6	14.7±0.6
12.5	20/5	20/5	100	20/5	100	2.4±0.3	14.8±0.3
25	20/5	20/5	100	18/5	90.0±10.0	2.7±0.5	14.1±0.4
50	21/5	21/5	100	20/5	91.7±8.3	2.4±0.3	14.1±0.4
100	18/6	18/6	100	17/6	95.8±4.2	2.5±0.3	12.8±0.9
Female fertility[b]							
0	24/7	24/7	100	24/7	100	2.5±0.6	14.3±0.4
12.5	20/5	19/5	95.0±5.0	14/5	70.3±3.0*	2.2±0.4	14.6±0.6
25	14/5	13/5	91.7±8.3	10/4	72.9±10.4*	2.6±0.5	14.7±1.3
50	13/5	13/5	100	10/5	77.1±7.9*	1.8±0.3	13.0±0.7
100	20/6	19/6	95.8±4.2	10/6	44.4±17.2**	2.1±0.5	11.8±1.1*

[a] Genistein-treated males were cohabited with intact (untreated) females.
[b] Genistein-treated females were cohabited with intact (untreated) males.
Copulation index (%)=(No. copulated/No.cohabited)×100
Fertility index (%)=(No. pregnant/No.copulated)×100
*Significantly different from the control, $P<0.05$.
**Significantly different from the control, $P<0.01$.

3.5.Sperm counts, serum testosterone level and reproductive organ weight

Fig. 2 shows the sperm concentration and serum testosterone levels in males exposed neonatally to genistein. Epididymal sperm counts in all genistein-treated groups were comparable to that in the controls. Serum testosterone concentrations in the genistein-treated groups were comparable to that in the controls.

At necropsy after evaluation of reproductive ability, no pathologic changes in the reproductive organs were observed in any male of female rat in the control group. In one male of the 100 mg/kg group, severe atrophy of both testes and epididymides was found. In other males and females in the genistein-treated groups, no macroscopic alterations were noted.

The weights of the paired testes and epididymides, seminal vesicles, and ventral prostate, and the organ weight/terminal body weight ratio (relative organ weight), as well as the body weight of males at 18 weeks of age (terminal body weight) are shown in Table 4. Terminal body weights and paired epididymides weights in all genistein-treated groups were significantly lower than those in the controls. Weights of paired testes, seminal vesicles with coagulating glands, and ventral prostate in all genistein-treated groups were comparable to those in the controls. The relative organ weights of the testes in the 25 mg/kg and more groups, and those of the ventral prostate in all genistein-treated group were significantly increased as compared with those in the controls, due to the decreased terminal body weights.

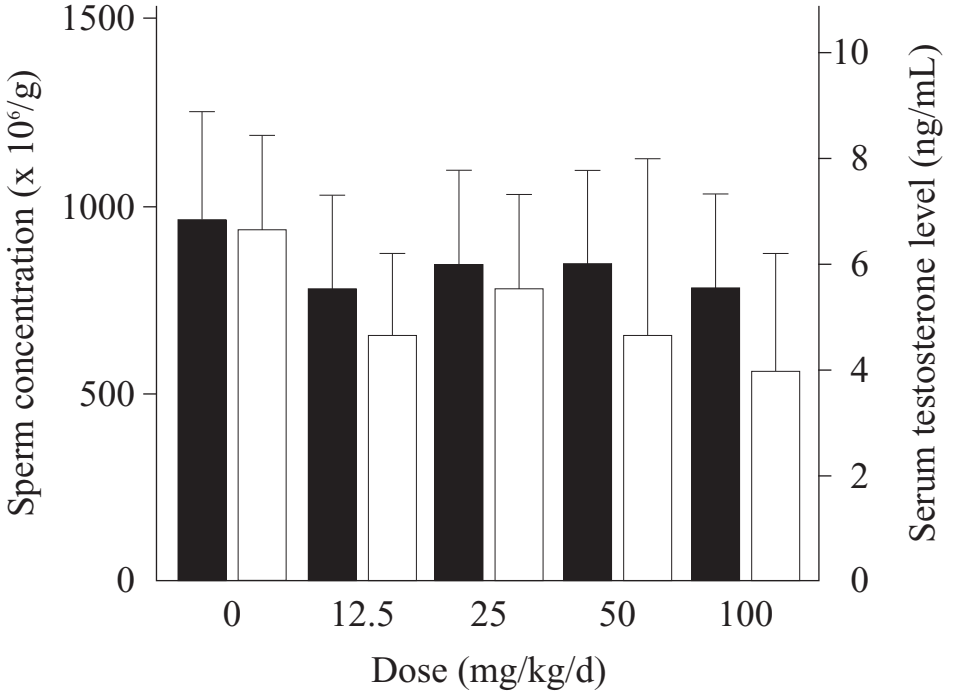

Fig 2. Epididymal sperm concentration (filled rectangle) and serum testosterone level (open rectangle) of males exposed neonatally to genistein.

Table 4. Terminal body weight and reproductive organ weight (g) of male rats exposed neonatally to genistein

Dosage (mg/kg/d)	No. of males and litters	Terminal body weight [A]	Testes [B]	Epididymides [C]	Seminal vesicle [D]	Ventral prostrate [E]	[B/A]	[C/A]	[D/A]	[E/A]
0	24/7	491.0±16.2[a] (100%)[b]	3.27±0.06 (100%)	0.98±0.03 (100%)	1.75±0.08 (100%)	0.39±0.03 (100%)	0.669±0.015 (100%)	0.201±0.005 (100%)	0.360±0.021 (100%)	0.079±0.006 (100%)
12.5	20/5	459.4*±18.8 (94%)	3.21±0.06 (98%)	0.90**±0.02 (92%)	1.61±0.08 (97%)	0.41±0.02 (105%)	0.699±0.014 (104%)	0.202±0.006 (100%)	0.368±0.016 (102%)	0.092**±0.004 (116%)
25	20/5	451.7*±14.8 (92%)	3.43±0.07 (105%)	0.92*±0.03 (94%)	1.78±0.10 (102%)	0.41±0.03 (105%)	0.760**±0.016 (114%)	0.201±0.005 (100%)	0.394±0.018 (109%)	0.093**±0.005 (118%)
50	21/5	430.8**±12.2 (88%)	3.41±0.09 (104%)	0.92*±0.03 (94%)	1.73±0.07 (99%)	0.40±0.03 (103%)	0.788**±0.020 (118%)	0.208±0.007 (103%)	0.396±0.017 (110%)	0.093**±0.008 (118%)
100	18/6	425.1**±11.0 (87%)	3.40±0.05 (104%)	0.91*±0.03 (93%)	1.68±0.07 (96%)	0.41±0.03 (105%)	0.793**±0.019 (119%)	0.211±0.005 (105%)	0.395±0.015 (110%)	0.095**±0.005 (120%)

[a] Mean±S.E.

[b] Number in parentheses represent percentages of control.

*Significantly different from the control, $P<0.05$.

**Significantly different from the control, $P<0.01$.

3.6.Histopathology of gonads in males and females

On histopathologic observation of male rats at 21 days old (weanlings), the most differentiated cells in the seminiferous tubules were at the diplotene spermatocyte stage, and there were no obvious differences between the controls and the genistein-treated groups. In female weanlings, polyovular follicles containing more than two oocytes in a single follicle were detected in rats of each genistein-treated group (Fig. 3). However, control rats had no polyovular follicles in their ovaries. No obvious differences were observed in the uteri between the genistein-treated and the control groups.

On histopathologic observation of 18-week-old male rats that were evaluated for reproductive performance, no obvious abnormalities were observed in the testes, epididymides, seminal vesicles, or prostate in any genistein-treated group. On histopathologic examination of the genistein-treated female rats that failed to copulate or did not become pregnant after mating with untreated males (Table 5), one of five rats in the 50 mg/kg group, and five of 10 rats in the 100 mg/kg group had atrophic ovaries with many atretic follicles (Fig 4A). Corpora lutea

were absent in these ovaries. The uterus of these rats showed hypertrophy of uterine epithelial cells and myometrium, and the numbers of uterine glands were decreased (Fig. 5A and B). Hypertrophy of uterine epithelial cells was also observed in two of 6 infertile rats in the 12.5 mg/kg group. Diffuse hyperplasia of uterine epithelial cells with an increased number of epithelial folds was noted in other rats (Fig. 5C). This change was detected in each genistein-treated group. Many of these rats in the 50 mg/kg or lower dose groups showed hypertrophy of the corpora lutea in their ovaries, and luteal cells showed acidophilic changes (Fig. 4B). The myometrium of some of these rats was also hypertrophic.

In 18-week-old male rats treated neonatally with EE, an increase in the multinucleated giant cells and decrease in number of germ cells in the seminiferous tubules of the testes were observed, while no histopathologic abnormalities were noted in the epididymides, ventral prostate or seminal vesicles. Ovaries of 18-week-old female rats exposed neonatally to EE showed increased atresia of follicles and contained few corpora lutea (Fig.6A). Uterine luminal epithelial cells in the EE-treated group were hypertrophic and the height of the epithelium was increased relative to the controls (Fig. 6B). The number of uterine glands was decreased in the EE-treated females (Fig. 6C). Squamous metaplasia of glandular epithelium (Fig. 6D) was noted and the myometrium was hypertrophic in the EE-treated females.

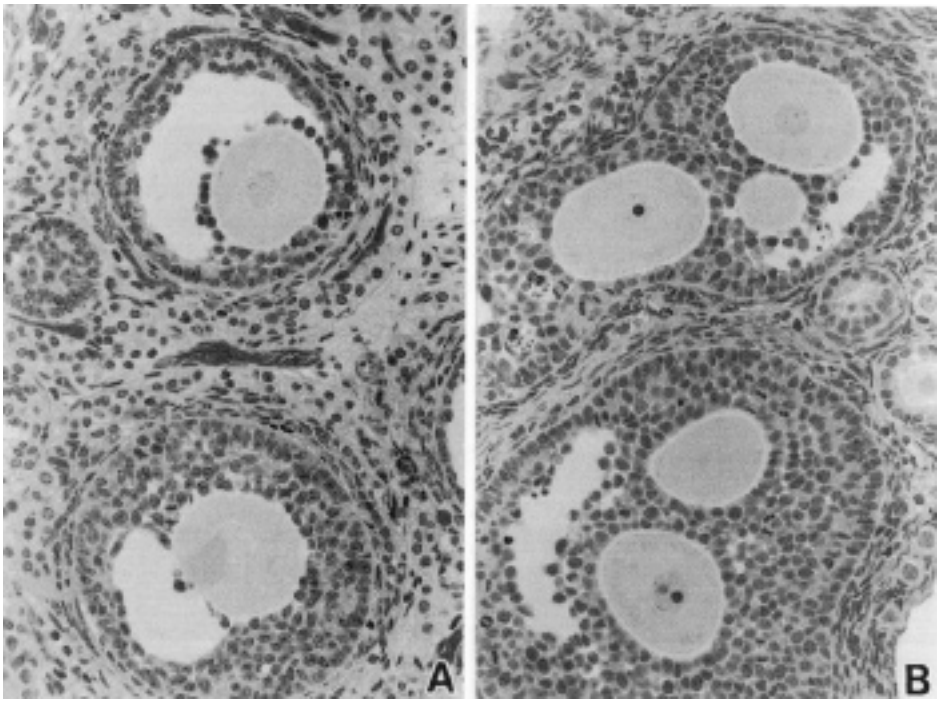

Fig. 3. Histology in female weanlings exposed neonatally to genistein. A:Ovary from a weanling control rat showing no abnormalities. H&E, × 350. B:Ovary from a weanling rat orally administered 50 mg/kg of genistein from PND 1 through 5, and sacrificed on PND 21. Note polyovular follicles with two or three oocytes. H&E, × 350.

Table 5. Histopathologic findings of 18-week-old female rats exposed neonatally to genistein or ethinyl estradiol.

Dosage(mg/kg/d)	12.5						25				50				100									EE									
Animal No.	1-1	2-2	3-1	4-1	4-4	5-3	1-1	2-2	2-4	4-3	1-3	2-1	3-4	1-2	1-3	2-1	2-2	4-1	4-2	5-1	5-3	5-4	6-2	1	2	3	4	5	6	7	8	9	10
Course	NP	NC	NP	NP	NP	NP	NP	NP	NP	NC	NP	NP	NP	NC	NP	NP	NP	NP	NP	NP	NP	NP	NP										
Ovary																																	
Increase in atretic follicles	-	-	-	-	-	-	-	+	+	-	2+	-	-	3+	3+	3+	3+	+	3+	+	3+	3+	+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Decrease in corpus luteum	-	-	-	-	-	-	-	±	-	-	3+	-	-	3+	3+	2+	3+	±	3+	±	3+	3+	+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Hemorrhage in cyst	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypertrophy of corpus luteum	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uterus																																	
Diffuse hyperplasia of luminal epithelial cells	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Hypertrophy of luminal epithelial cell	+	-	-	2+	-	-	-	-	-	-	+	-	-	-	2+	2+	2+	+	2+	+	2+	-	+	+	+	+	2+	+	+	+	+	+	+
Hypertrophy of glandular epithelial cells	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	-	+	+	-	±	-	-	+	-	+	+	±	+	+
Squamous metaplasia of glandular epithelium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Decrease in number of glands	-	-	-	-	-	-	-	-	-	-	+	-	-	2+	2+	+	2+	-	+	-	+	2+	-	2+	2+	2+	+	2+	2+	+	+	+	+
Hypertrophy of myometrium	-	+	±	+	-	-	-	-	±	-	2+	+	+	+	2+	2+	2+	+	+	±	+	+	+	2+	+	+	+	+	+	±	+	+	+
Cellular infiltration of eosinophils	2+	-	-	2+	+	-	2+	±	-	+	2+	+	+	+	2+	2+	2+	±	+	±	+	2+	+	2+	+	+	+	+	2+	+	2+	2+	2+

-, Negative; ±, Very slight; +, Slight; 2+, Moderate; 3+, Severe NP, Nonpregnant female; NC, Noncopulated female

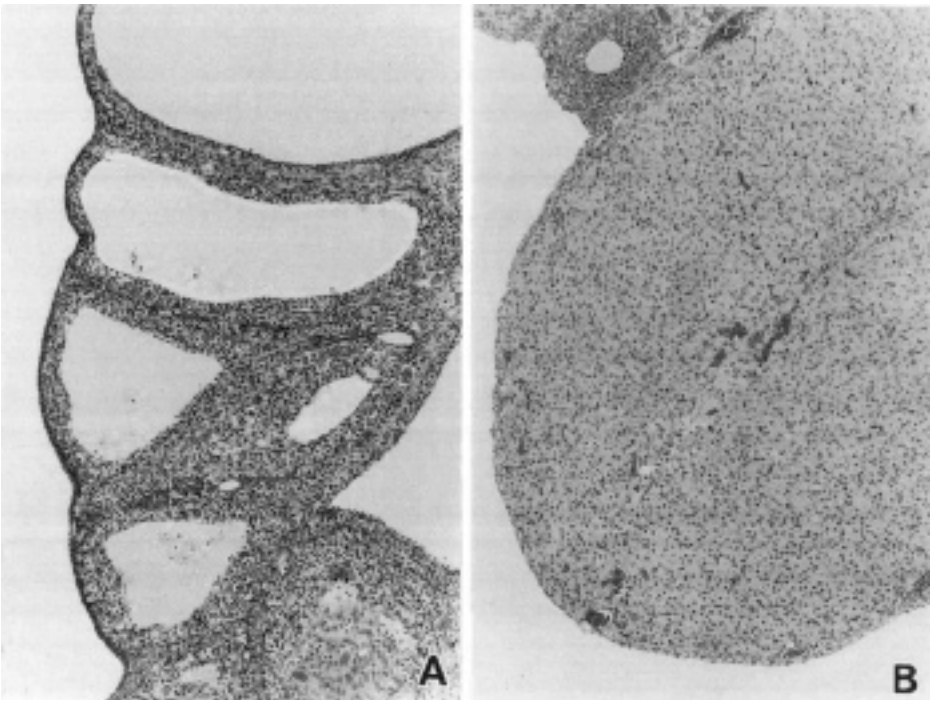

Fig. 4. Histology in postpubertal female rats exposed neonatally to genistein. A:Ovary from a rat orally administered 100 mg/kg of genistein from PND 1 through 5, and sacrificed at 18 weeks of age. Note atrophy or the ovary with increased atretic follicles. H&E, × 88. B:Ovary from a rat orally administered 50 mg/kg of genistein from PND 1 through 5, and sacrificed at 18 weeks of age. Note hypertrophy of the corpus luterum. H&E, × 88.

4. DISCUSSION

The present syudy, evaluating exposure of rats to genistein by oral gavage from postnatal days 1 through 5, clearly showed female reproductive toxicity after puberty, evidenced by the increased number of females showing irregular estrous cycling, markedly decreased fertility, and histopathologic changes of reproductive organs. To our knowledge, this is the first demonstration of the reproductive and developmental effects of early neonatal exposure (by oral gavage) to the phytoestrogen genistein on hormone-sensitive characteristics. The incidence of irregularly cycling females was increased by early neonatal exposure to genistein, whereas the number of days in each stage of the cycle and the estrous cycle length were not affected. We reported previously that female rats treated with 17β-estra-diol on postnatal days 1 through 5 showed an irregular estrous cycle afrer 7 weeks of age [14]. Levy et al. [10] demonstrated that prenatal exposure to genistein in rats did not significantly disrupt vaginal smear cyclicity. Persistent vaginal estrus or cornification, anovulation, and infertility during adulthood were shown to be characteristic responses to androgen and estrogen exposure in rodents during critical periods of neuroendocrine differentiation (less than 5 to 10 days of age) [23]. Thus, it is reasonable to suggest that early neonatal exposure to genistein disrupts estrous cyclicity in rats.

Coumestrol is the most potent phytoestrogen known, as determined by the uterotrophic assay using young adult mice [24]. It has been reported that neonatal coumestrol exposure is capable of inducing complete vaginal opening between day 5 and 12, before that seen in untreated controls, but not as early as that induced by dose of diethylstilbestrol employed [25]. In the present study, however, early neonatal exposure to genistein had no unique influence on puberty onset in females as evaluated by the day of vaginal opening. Vaginal opening is a maturational sign in mammals. Specifically, vaginal opening in mice and rats is characteristic of puberty and is followed by a shot estrous period [26]. Presumably, vaginal opening is the result of rising titers of estrogen produced by the ovary under the influence of a functional hypothalamo-hypophysial axis [26]. Estrogen (5μg 17β-estradiol) given for the first 5 days of life induced complete vaginal opening between day 5 and day 7; increased doses of estradiol resulted in earlier complete vaginal openig [26]. It has been demonstrated that there were no treatment-related effects on anogenital distance, timing of testes descent, vaginal opening, or estrous cycle for male or female offspring of dams exposed to genistein in the diet at a concentration of 250 mg genistein/kg AIN-76 diet [27] from conception to day 21 postpartum. In this previous study, total genistein concentration in stomach milk and serum of 7-day-old animals were determined to be 4439 and 726 pmol/ml, respectively. Postpubertal females exposed perinatally to genistein had similar numbers of corpora lutea compared to controls. In addition, histopathologic examination of 100-day-old females exposed perinatally to genistein did not reveal significant alterations in the vaginal, uterine, or ovarian tissues. Thus, route of administration (dietary versus direct dosing) may play a significant role in bioavailability and potential for toxicity

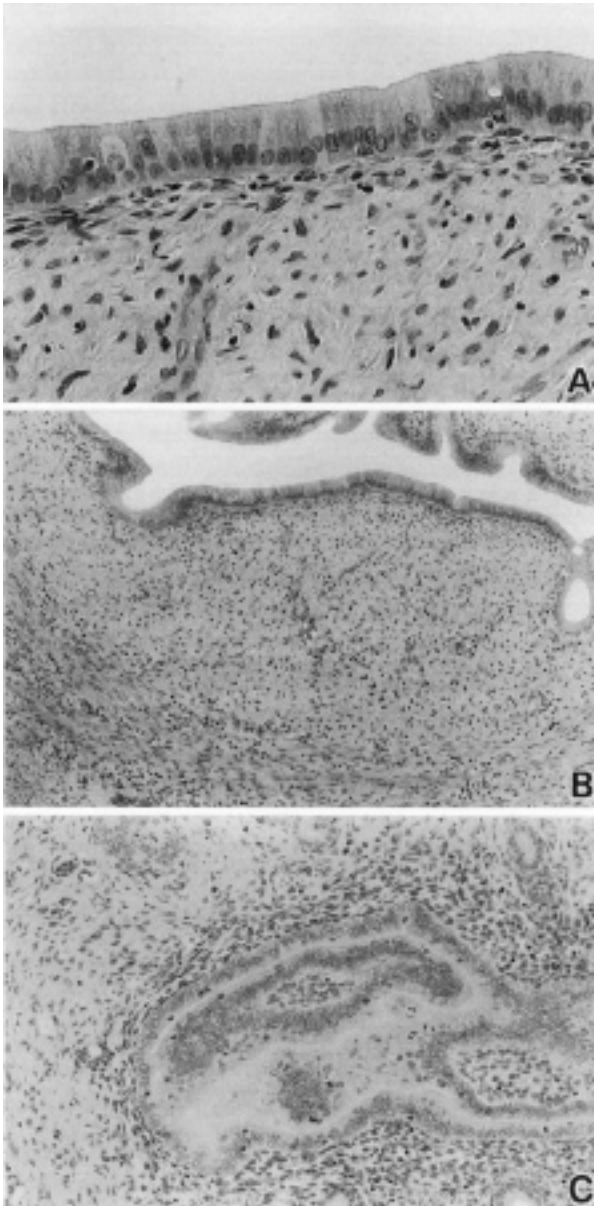

Fig. 5. Histology in postpubertal female rats exposed neonatally to genistein. A:Uterus from a rat orally administered 100 mg/kg of genistein from PND 1 through 5, and sacrificed at 18 weeks of age. Note hypertrophy of luminal epithelial cells. H&E, × 350. B:Uterus from a rat orally administered 100 mg/kg of genistein from PND 1 through 5, and sacrificed at 18 weeks of age. Note decrease in the number of uterine glands. H&E, × 88. C:Uterus from a rat orally administered 50 mg/kg of genistein from PND 1 through 5, and sacrificed at 18 weeks of age. Note diffuse hyperplasia of luminal epithelial cell and folds of the uterine epithelium. H&E, × 175.

[27].

The major finding in rats in the present study was that the number of sterile females was significantly increased even by exposure to genistein at 12.5 mg/kg/day, while copulation ability of the genistein-treated femakes was not disrupted. Conversely, male rats exposed neonatally to genistein even at the fainly high dose of 100 mg/kg/day showed normal fertility (fertility index: 95.8%) as well as normal copulation ability (copulation index: 100%) as evaluated by cohabitation with untreated females (see Table 3). Body weight reduction observed in the highest dose group may have been responsible for the reproductive disruption in the female rats. It has been demonstrated that serum levels of gonadotropins (FSH, LH) in female rats after intrauterine and neonatal exposure to genistein were comparable to those in the controls whereas body weight reduction and decreased serum levels of 17β -estradiol and progesterone were found [28]. Thus, the mechanism of disruption of genistein-treated female reproduction, and the relation between reduced body weight, stress, and gonadotropin secretion remain unclear in the present study. Further studies on the effects of neonatal exposure to genistein on female reproduction are needed.

Recently, the estrogenic activity of genistein has raised the possibility that this agent may affect the estrogen-sen-sitive sexual differentiation of the central nervous system, including the neural basis of sexually dimorphic behavior. Developmental treatment with genistein at 1250 ppm (approximately 100 mg/kg/day) from rat embryonic day 7 through postnatal day 77 caused subtle alterations in some sexually dimorphic behaviors: open field activity, play behavior, residential running wheel activity, and consumption of saccharin-and sodium chloride-flavored solutions [29]. It has been demonstrated that neonatal exposure of rat pups through milk to coumestrol at 100 mg/kg/day during the critical period of the first 10 postnatal days produced marked effects in males, resulting reductions in mounting and ejaculation frequency and prolongation of the latencies to mount and ejaculate, whereas testicular weights and plasma testosterone levels were not altered [30]. These deficits resembled the demasculinizing effects of acute perinatal estrogen treatment [31,32]. However, in the present study, copulation ability in male and female rats exposed to genistein at fairly high doses during the critical period was not affected.

With respect to the decreased fertility ability in females exposed neonatally to genistein, histopathologic investigation of reproductive organs revealed atrophic ovaries with many atretic follicles, hypertrophy of uterine epithelial cells and myometrium, hypertrophy of corpus luteum in the ovaries, acidophilic changes of luteal cells and hypertrophic myometrium. Neonatal coumestrol treatment of mice has also been shown to cause a number of histopathologic alterations in the female reproductive tract [33], which were similar to the morphologic changes observed in the present study.

Two types of histopathologic changes were observed in the ovaries or uteri of female rats exposed to genistein in the present study. The first type was characterized as the induction of atrophy of the ovary and hypertrophy of uterine luminal epithelial cells, and hypertrophy of the myometrium, which were found in females exposed to estrogens including ethinyl estradiol. The other type was hypertrophy of corpora lutea, increases in the number of luminal epithelial cells and in folds of the epithelial layer, although hypertrophy of luminal epithelial cells was not apparent. The latter type was considered

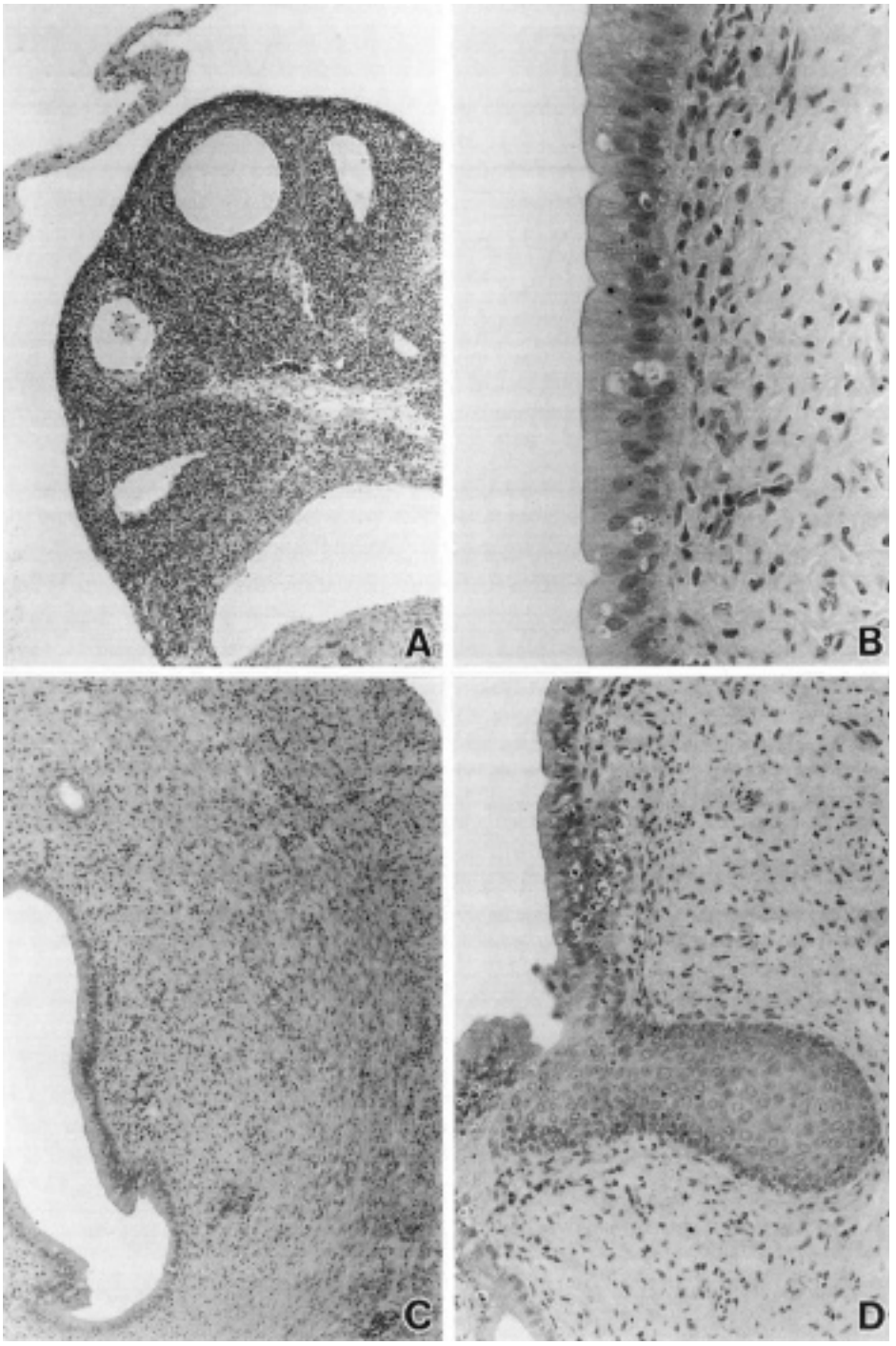

Fig. 6. Histology in postpubertal female rats exposed neonatally to ethinyl estradiol. A:Ovary from a rat orally administered 2 mg/kg of ethinyl estradiol from PND 1 through 5, and sacrificed at 18 weeks of age. Note severe increase in the follicular atresia. H&E, × 88. B:Uterus from a rat orally administered 2 mg/kg of ethinyl estradiol from PND 1 through 5, and sacrificed at 18 weeks of age. Note hypertrophy of luminal epithelial cells. H&E, × 350. C:Uterus from a rat orally administered 2 mg/kg of ethinyl estradiol from PND 1 through 5, and sacrificed at 18 weeks of age. Note decrease in uterine gland. H&E, × 88. D:Uterus from a rat orally administered 2 mg/kg of ethinyl estradiol from PND 1 through 5, and sacrificed at 18 weeks of age. Note squamous metaplasia of glandular epithelium. H&E, × 175

to be a pseudopregnant-like change, accompanied by increases in the prolactin level [34]. It has been demonstrated that the plasma prolactin level is significantly greater in ovariectomized rats fed genistein (750 μg/g) compared with comparable rats not receiving genistein [11]. However, histopathologic alterations seen frequently in pseudopregnant rats were not observed in the ovaries or uteri of females exposed neonatally to ethinyl estradiol. In addition, the latter type of changes was not observed in postubertal females exposed to diethylstilbestrol, 17β-estradiol, or estriol on postnatal day 1 through 5 [14,35]. Of particular interest in the histopathologic evaluation in the present study was that polyovular follicles with two or three oocytes were observed in 21-day-old females treated neonatally with genistein. These changes were also seen in immature mice or rats exposed neonatally to diethylstilbestrol [36,37] or estradiol benzoate [13]. Polyovular follicles seen in 21-day-old females in this study were not observed in 18-week-old females exposed neonatally to genistein, and the females after puberty showed marked atrophy of the ovaries (follicles).

One of the variables not controlled in the present study was the amount of phytoestrogens contained in the diet, water, and nest meterials. The concentrations of phytoestrogens (genistein and daidzein) were determined as follows: diet, genistein, ND-2.1 mg/100 *g*; daidzein, ND-1.9 mg/100 *g*; tap water and nest materials, ND. Relative amount of diet consumed by each lactating dam and the pup were not recorded. Thus, it is unclear dam and the pup were not recorded. Thus, it is unclear how much impact this baseline exposure to phytoestrogens had on the resulting measures. Casanova et al. [38] demonstrated that it is not necessary to replace diet containing phytoestrogens with phytoestrogen-free diet in most developmental toxicology studies. However, phytoestrogen-free diets are recommended for endocrine toxicology studies at low doses to determine the effects of chemicals on reproduction. In the present study, phytoestrogen-free diets were not used because fairly high doses of genistein were applied to newborn animals.

The results of the present study indicated that direct neonatal dosing of genistein at the fairly high dose of 100 mg/kg/day caused dysfunction of postpubertal reproductive performance as well as abnormal development of gonads in female rats. Human milk concentrations of the isoflavones genistein, daidzein, and their glycosides are very low (5 to 15 ng/ml) and although they can increase up to 10-fold when the lactating mother consumes soy foods [39], the daily intake by the infant of phytoestrogens from human milk is only 0.005 to 0.01 mg [40]. Because of the weak estrogenic activity of isoflavones (10^{-2} to 10^{-3} that of 17 β -estradiol) [41], the dietary intake of phytoestrogens from human milk is unlikely to exert biologic effects. It would be difficult for human beings to consume sufficient amounts of isoflavones from natural soy-foods to reach the toxicologic levels that induce the pathologic effects seen in animals. However, with the recent trend toward extracting isoflavones from soy for commercialized over-the-counter soy isoflavone supplements, and because such products and not closely regulated, the potential dangerous effects from self-induced megadosing are a concern [40].

Before recommendations are made regarding consumption of specific phytoestrogens in humans, it will be important to further characterize their pharmacologic effects and margins of safety [42]. Further research is needed to evaluate effects at physiologic and pharmacologic levels; to determine the effective doses for beneficial as well as harmful effects; and to evaluate the interactions of phytoestrogens with each other and with other dietary components.

ACKNOWLEDGMENTS

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CHAPTER 4

Carcinogenicity Related To

Effects of Dietary Restriction on General Signs, Body Weight, Food Consumption, Blood Parameters, and Pathological Findings in Male Crj: CD (SD)IGS Strain Rats Aged 24 Months

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ABSTRACT. Male rats were given CR-LPF or CRF-1 diet *ad libitum* or restrictedly for 24 months to examine how dietary restriction and low protein diet affect general signs, body weight, food consumption, blood parameters, and pathological findings.

Low body weight was noted in the CRF-1 and CR-LPF restricted feeding groups compared with the CRF-1 and CR-LPF *ad libitum* feeding groups. Body weight gain was suppressed in the restricted feeding groups. It can definitely be said that this finding was caused by the dietary restriction. Body weight gain in the CR-LPF restricted feeding group was markedly more suppressed than that in the CRF-1 restricted feeding group.

No significant biological differences were seen between the CR-LPF and CRF-1 *ad libitum* and restricted feeding groups in general signs, hematological examination, blood parameters, macroscopic pathology, or organ weights. In conclusion, there were no toxicologically significant differences in the biological parameters examined in this long-term toxicity study between Crj: CD (SD) IGS rats which had been given low protein diet and those which had been given normal protein diet. — Key words: long-term toxicity studies, CD(SD)IGS rat, low protein diet

CD(SD)IGS-2002/2003 : 191-207

INTRODUCTION

Male rats were given CR-LPF diet or CRF-1 diet *ad libitum* or restrictedly for 24 months to examine how dietary restriction and low-protein diet affect general signs, body weight, food consumption, blood parameters, and pathological findings.

MATERIALS AND METHODS

Animals

For the present study, 170 male Crj: CD (SD)IGS strain rats aged 4 weeks were obtained from Charles River Japan Inc. (Hino Breeding Center, Shiga, Japan). The animals were quarantined and acclimated for about 1 week. Animals showing healthy, favorable growth during this period were selected and assigned to 4 test groups by stratified randomization so as to distribute the mean body weights evenly among the groups. Each test group consisted of 5 - 30 animals. Animals remaining after group assignment are to be necropsied at the age of 24 months. The test animals were individually identified by ear-punching.

Housing Conditions

The animals were kept in an animal room located in an SPF animal facility with a 12-hour light and dark cycle (lighting: 06:00-18:00) and fresh air changes of 12 times per our. Temperature and relative humidity in the animal room were set at 20 - 26℃ and 40 - 70%, respectively. The animals were housed individually in suspended stainless steel cages (W: 240 × D: 380 × H: 200 mm). The animal room was cleaned daily, and cages were replaced with sterilized ones at least once every 2 weeks. All the animals were allowed free access to tap water. Regarding diet supply, CR-LPF (protein content; 18.2%, Oriental Yeast Co., Ltd.) was given to a group of animals *ad libitum* (CR-LPF *ad libitum* feeding group), and to another group of animals restrictedly (CR-LPF restricted feeding group). Similarly, CRF-1 (protein content; 22.6%, Oriental Yeast Co., Ltd.) was given to a group of animals *ad libitum* (CRF-1 *ad libitum* feeding group), and to another group of animals restrictedly (CRF-1 restricted feeding group). Each animal of the restricted feeding groups was given about 18 g (3.2 - 3.3 g/pellet) of the diet per day.

Observation for General Signs

The animals were observed for mortality once a day and for general signs once a week.

Body Weight Measurement

The animals were weighed once a week until the 13th week of test feeding and once every 4 weeks thereafter.

Food Consumption Measurement

Food consumption was measured once a week until the 13th week of test feeding and once every 4 weeks thereafter.

Hematological Examination

The animals were sacrificed at the age of 24 months. Blood was collected via the abdominal aorta under anesthesia with sodium pentobarbital. In addition, the animals were not deprived of food before blood collection. Red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined or calculated with an automated hematology analyzer (Sysmex K-4500, Sysmex Co., Ltd.) using EDTA-2K as an anticoagulant. Differential white blood cell count (by May-Giemsa staining) and reticulocyte count (RET; by Brecher's method) were obtained under a microscope

using EDTA-2K as an anticoagulant. Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration (FIB) were measured with an automated coagulation analyzer (Coagmaster Ⅱ, Sankyo Co., Ltd.) using citric acid sodium as an anticoagulant.

Blood Chemical Analysis

Blood was collected via the abdominal aorta at the time of blood collection for the hematological examination. Serum obtained from the blood by centrifugation (at about 4℃ and 3000 r.p.m. for 15 minutes) were divided into that for measurement and that for storage at -80℃ until analysis. Methods for determination of serum biochemical parameters are shown in Table 1.

Organ Weight Measurement

The animals, whose blood had been collected, were sacrificed by bleeding via the abdominal aorta, and the following organs were weighed (pair organs were weighed together): the pituitary, heart, lungs, liver, spleen, kidneys, and adrenals. The relative organ weight, i.e., the ratio of each organ weight to the body weight measured before necropsy, was calculated.

Histopathological Examination

The following organs were fixed in 20% neutral buffered formalin: the pituitary, heart, lungs, liver, spleen, kidneys, and adrenals. Then the organs were embedded in paraffin according to the usual methods to stain them with H.E., and the H.E. stained specimens were examined histopathologically.

Statistical Methods

Data were statistically analyzed as described below. Significance tests were performed for the CR-LPF and CRF-1 groups between the *ad libitum* feeding group and the restricted feeding groups, for the *ad libitum* feeding groups between the CR-LPF group and the CRF-1 group, and for the restricted feeding groups between the CR-LPF group and the CRF-1 group. Probabilities less than 5% were considered statistically significant and shown as $p < 0.05$ (less than 5%) or $p < 0.01$ (less than 1%).

Significant Tests

Group mean values with standard deviations in the CR-LPF *ad libitum* and restricted feeding groups and the CRF-1 *ad libitum* and restricted feeding groups were calculated for body weight, food consumption, hematological parameters, serum chemical parameters, and organ weights (relative weights included). A significance test assuming equal variance in 2 groups was performed by an *F* test. When each group variance was equal, Student's *t* test was performed. When the group variances differed significantly, differences in mean value were statistically tested by Aspin-Welch's test.

RESULTS

Mortality

Results of the observation for mortality during the study period are shown in Table 2. The survival rate tended to be higher in the restricted feeding groups than the *ad libitum* feeding groups both for the animals which had been given CR-LPF and for those which had been given CRF-1. The survival rate also tended to be higher in the CR-LPF *ad libitum* feeding group than the CRF-1 *ad libitum* feeding group. No significant differences were seen in the survival rate between the CR-LPF restricted feeding group and the CRF-1 restricted feeding group.

General Signs

The types and incidences of general signs observed during the experimental period are summarized in Table 3. No particular tendencies were seen for the symptoms noted either in the CR-LPF or CRF-1 restricted feeding group or in the CR-LPF or CRF-1 *ad libitum* feeding group.

Body Weight

Body weight changes are shown in Fig. 1 and Tables 4 and 5. Regarding the groups which had been given CRF-1, body weight in the restricted feeding group was significantly lower than that in the *ad libitum* feeding group on the 8th day of test feeding and thereafter. Similar body weight changes were noted in the groups which had been given CR-LPF. When the restricted feeding groups were compared with each other, body weight in the CR-LPF restricted feeding group was significantly lower than that in the CRF-1 restricted feeding group on the 8th day of test feeding and thereafter. When the *ad libitum* feeding groups were compared with each other, body weight in the CR-LPF *ad libitum* feeding group was significantly lower than that in the CRF-1 *ad libitum* feeding group on the 8th day of test feeding and thereafter, as was seen in the CR-LPF restricted feeding group.

Food Consumption

Changes in food consumption are shown in Fig. 2 and Tables 6 and 7. Food consumption in the CR-LPF *ad libitum* feeding group tended to be slightly higher than that in the CRF-1 *ad libitum* feeding group. There were no abnormal changes in food consumption either in the CRF-1 or CR-LPF *ad libitum* feeding group or in the CRF-1 or CR-LPF restricted feeding group.

Hematology

Group mean values of hematological parameters are shown in Tables 8 and 9. Regarding the groups which had been given CR-LPF, more prolonged PT, lower lymphocytic ratio, and higher neutrophilic ratio were noted in the restricted feeding group compared with the *ad libitum* feeding group and the CRF-1 restricted feeding group. No differences were seen in the hematological parameters either between the CR-LPF restricted feeding group and the CRF-1 restricted feeding group or between the CR-LPF *ad libitum* feeding group and the CRF-1 *ad libitum* feeding group.

Blood Chemistry

Group mean values of blood chemistry parameters are shown in Tables 10 and 11. Regarding the groups which had been given CR-LPF and CRF-1, lower TG was noted in the restricted feeding group compared with the *ad libitum* feeding group.

Regarding the groups which had been given CRF-1, lower ALP was noted in the restricted feeding group compared with the *ad libitum* feeding group. Regarding the groups which had been given CR-LPF, lower UN was noted in the restricted feeding group compared with the CRF-1 restricted feeding group. No differences were seen in the blood chemistry parameters between the CR-LPF *ad libitum* feeding group and the CRF-1 *ad libitum* feeding group.

Necropsy

Macroscopic findings are shown in Table 12-1 and 12-2. Regarding the groups which had been given CRF-1, decubitus was noted in hind-limbs of 5 animals of the *ad libitum* feeding group and 7 animals of the restricted feeding group. Regarding the groups which had been given CR-LPF, decubitus was noted in hind-limbs of 5 animals of the *ad libitum* feeding group and 6 animals of the restricted feeding group. No particular tendencies were seen for the findings noted either in the CR-LPF or CRF-1 restricted feeding group or in the CR-LPF or CRF-1 *ad libitum* feeding group.

Organ Weight

Group mean values of the absolute and relative organ weights are shown in Table 13 and 14. The absolute and relative weights of all the organs measured were significantly lower or tended to be lower in the restricted feeding groups than the *ad libitum* feeding groups both for the animals which had been given CR-LPF and for those which had been given CRF-1. The liver and kidney weights were significantly lower in the CR-LPF restricted feeding group than the CRF-1 restricted feeding group.

Histopathological Findings

Histopathological findings are summarized in Table 15-1 - 15-3. The histopathological findings in some of the organs examined were noted sporadically in the CR-LPF and CRF-1 *ad libitum* and restricted feeding groups. All of these findings were incidental changes. Findings which deserve special mention, though the degrees were slight or mild, were confined to the chronic nephropathy in the CR-LPF and CRF-1 restricted feeding groups. The cardiac changes noted in the CR-LPF restricted feeding group were of smaller magnitude than those in the CR-LPF *ad libitum* feeding group. The incidence of adrenal tumors tended to be lower in the restricted feeding group than the *ad libitum* feeding group both for the animals which had been given CR-LPF and for those which had been given CRF-1.

DISCUSSION

Male rats were given CR-LPF diet or CRF-1 diet *ad libitum* or restrictedly for 24 months to examine how dietary restriction and low-protein diet affect general signs, body weight, food consumption, blood parameters, and pathological findings.

The survival rate in males was 80% in the CR-LPF and CRF-1 restricted feeding groups, suggesting no differences from that in the CR-LPF and CRF-1 *ad libitum* feeding groups.

Body weight was low in the CR-LPF *ad libitum* and restricted feeding groups, and food consumption was high in the CR-LPF *ad libitum* feeding group during the experimental period. It has been reported that food consumption of animals which had been given low protein diet was higher than that which had been given normal protein diet, which may be due to the higher fiber content of lower protein diet [3, 5, 6]. It has also been reported that food consumption decreased with increase in the level of digestible energy in diet; animals regulated their food consumption in response to the energy density of diet to meet their energy demand [2]. These findings support the results of the present study that consumption of CR-LPF was higher than that of CRF-1 when the animals were given the diets *ad libitum*. In addition, the digestible energy of CR-LPF is 299 kcal/100g, and that of CRF-1 is 332 kcal/100 g [1]. It has been suggested that lowering dietary protein level reduces the incidence of spontaneous lesions and prolongs rats life span [4, 7]. These suggestions tended to be actualized in the CR-LPF and CRF-1 restricted feeding groups and the CR-LPF *ad libitum* feeding group in the present study, although no significant differences were seen in the life-prolong effect of these diets. No significant biological differences were seen in general signs, hematological examination, blood parameters, macroscopic pathology, or organ weights among the CR-LPF and CRF-1 *ad libitum* and restricted feeding groups. It has been reported that a low protein diet decreases the severity of cardiomyopathy, prevents nephrocalcinosis, and decreases the incidence and severity of nephropathy in F344 rats [6]. In the present study, cardiomyopathy and nephropathy were noted less frequently in the CR-LPF restricted feeding group than the CR-LPF and CRF-1 *ad libitum* feeding groups and the CRF-1 restricted feeding group. The restricted feeding of the low protein diet for 24 months is considered to tend to prevent cardiomyopathy and nephropathy in Crj: CD (SD) IGS rats.

It can be concluded, although from the data obtained in a small number of animals, that, as pointed by Nagayabu et al. [5], there were no toxicologically significant differences in the biological parameters examined in this long-term toxicity study between Crj: CD (SD) IGS rats which had been given low protein diet and those which had been given normal protein diet.

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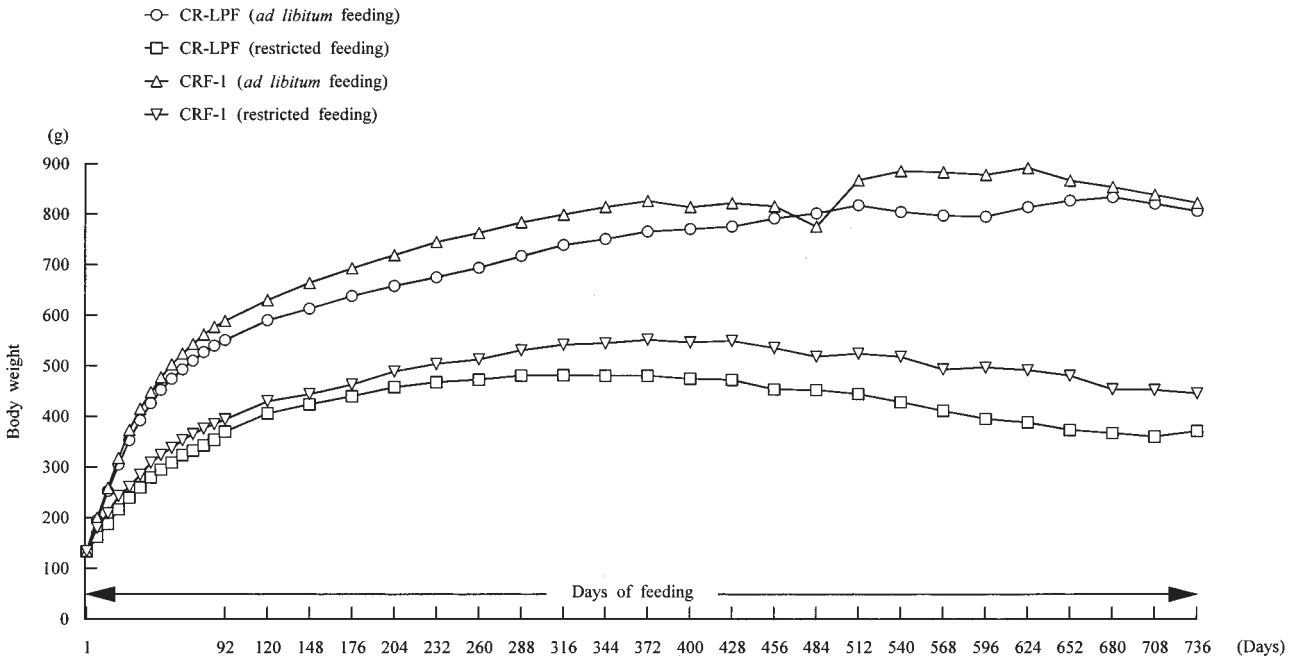

Figure 1. Body weights of male rats.

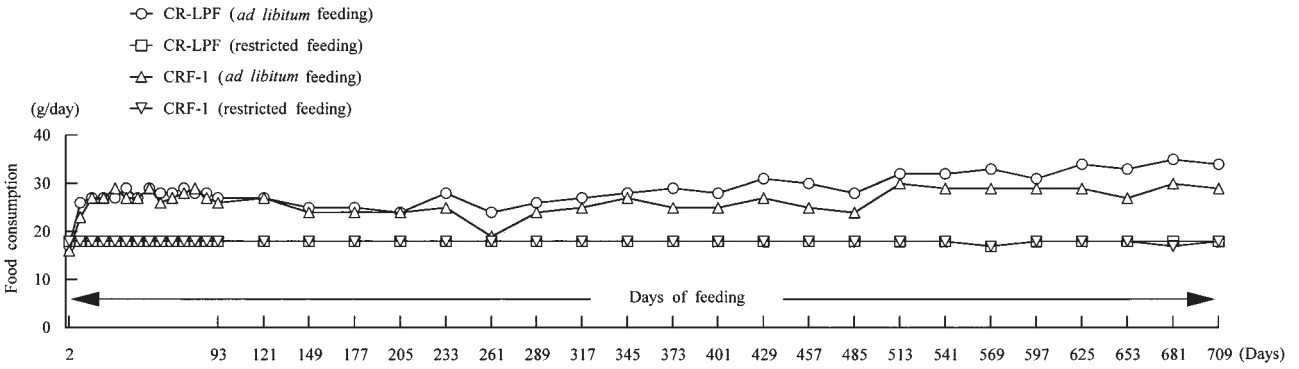

Figure 2. Food consumption of male rats.

Table 1. Blood chemistry parameters and methods used for the blood chemical analysis

Parameter	Method
AST	JSCC standard method
ALT	JSCC standard method
ALP	JSCC standard method
T-Cho	Cholesterol oxidase・peroxidase
TG	Glycerol-3-phosphate oxidase・peroxidase
TP	Biuret
Alb	Calculation ; TP×Alb ratio
Protein fraction	Electrophoresis
UN	Urease・glutamate dehydrogenase
CRE	Creatininase
T-Bil	Azobilirubin
Glu	Hexokinase・G-6-PDH
IP	Purine nucleotide phosphorylase・XDH
Ca	o-Cresolphthalein complexone
Na	Ion selective electrode
K	Ion selective electrode
Cl	Ion selective electrode

JSCC: Japan Socitey of Clinical Chemistry, XDH: Xanthine dehydrogenase
G-6-PDH: Glucose-6-phosphate dehygrogenase.

Table 2. The number of dead animals and survival rate for the entire study period

Group	CRF-1(*ad libitum* feeding)	CR-LPF(*ad libitum* feeding)	CRF-1(restricted feeding)	CR-LPF(restricted feeding)
Animals examined	30* (20)	30* (20)	30* (20)	30* (20)
Found dead	12	8	6	6
Killed in extremis	0	0	0	0
Total	12	8	6	6
Survival rate (%)	60.0 (40.0)	73.3 (60.0)	80.0 (70.0)	80.0 (70.0)

*: Figures in initiation of feeding.
Figures in parentheses indicate number of males in the entire study period.

Table 3. Summary incidence of general signs

Group	CRF-1(*ad libitum* feeding)	CR-LPF(*ad libitum* feeding)	CRF-1(restricted feeding)	CR-LPF(restricted feeding)
Number of males	30	30	30	30
General signs				
Prone position	1	0	0	0
Decreased locomotor activity	1	1	0	0
Staggering gait	0	0	0	1
Bradypnea	1	0	0	0
Hypothermia	1	0	0	0
Soiled hair	1	0	1	0
Soiled hair, around nose	2	0	0	0
Soiled hair, around mouth	2	0	0	0
Cornea, opacity	2	2	3	0
Ptosis	1	0	0	0
Decubitus, hindlimb	11	10	11	14
Swelling, hindlimb	2	7	4	2
Loss, tail	0	0	0	6
Palpable masses	4	8	4	3

Table 4. Body weights of male rats

Group		CR-LPF (*ad libitum* feeding)		CR-LPF (restricted feeding)		CRF-1 (*ad libitum* feeding)		CRF-1 (restricted feeding)	
Number of males		30		30		30		30	
Days of feeding	1	133±6		133±6		134±4		134±5	
	8	195±10		162±6**		200±8		180±6##	
	15	253±14		188±9**		259±11		209±9##	
	22	305±14		217±13**		318±15		243±12##	
	29	353±17		240±13**		373±20		261±17##	
	36	392±22		260±15**		415±24		285±18##	
	43	426±25		280±17**		448±27		309±17##	
	50	452±27		295±18**		477±28		324±16##	
	57	474±28		309±18**		503±30		338±17##	
	64	493±30		324±18**		524±32		353±17##	
	71	510±32		333±19**		543±33		365±18##	
	78	527±32		343±18**		562±36		376±17##	
	85	540±34		354±17**		577±37		385±16##	
	92	551±35		370±17**		589±38		394±16##	
	120	590±43		406±18**		630±47		430±17##	
	148	613±49		424±18**		664±52		444±19##	
	176	638±54		440±20**		693±61		463±18##	
	204	658±52	(25)	458±19**	(25)	719±71	(25)	489±14##	(25)
	232	675±56	(25)	468±23**	(25)	745±76	(25)	504±17##	(25)
	260	694±62	(25)	473±24**	(25)	763±80	(25)	513±17##	(25)
	288	717±66	(25)	481±23**	(25)	784±88	(25)	531±16##	(25)
	316	739±71	(25)	482±21**	(25)	799±96	(25)	542±17##	(25)
	344	751±72	(25)	481±22**	(25)	814±101	(25)	545±17##	(25)
	372	766±76	(25)	481±23**	(25)	826±111	(25)	552±19##	(25)
	400	771±80	(20)	475±24**	(20)	814±111	(20)	547±27##	(20)
	428	776±80	(19)	473±27**	(20)	822±119	(20)	550±29##	(19)
	456	792±86	(19)	454±36**	(20)	816±138	(20)	536±30##	(19)
	484	802±92	(19)	453±39**	(20)	776±183	(20)	519±37##	(19)
	512	818±102	(19)	445±46**	(20)	868±122	(17)	525±43##	(19)
	540	805±90	(17)	429±54**	(20)	886±129	(16)	519±48##	(19)
	568	798±94	(16)	412±55**	(18)	884±133	(16)	494±62##	(19)
	596	796±112	(15)	396±58**	(18)	879±166	(16)	498±64##	(18)
	624	815±99	(14)	389±59**	(18)	893±133	(15)	493±72##	(18)
	652	828±104	(13)	374±59**	(18)	868±112	(13)	482±83##	(17)
	680	835±106	(12)	368±59**	(17)	855±114	(12)	455±70##	(16)
	708	822±95	(12)	361±58**	(16)	840±129	(9)	454±59##	(14)
	736	808±102	(12)	372±54**	(14)	824±155	(8)	447±62##	(14)

Each value shows mean (g)±S.D.
**: P<0.01, Significantrly different from CR-LPF(*ad libitum* feeding) group.
##: P<0.01, Significantrly different from CRF-1(*ad libitum* feeding) group.
Figures in parentheses indicate number of males.

Table 5. Body weights of male rats

Group	CRF-1 (*ad libitum* feeding)		CR-LPF (*ad libitum* feeding)		CRF-1 (restricted feeding)		CR-LPF (restricted feeding)	
Number of males	30		30		30		30	
Days of feeding								
1	134±4		133±6		134±5		133±6	
8	200±8		195±10*		180±6		162±6##	
15	259±11		253±14*		209±9		188±9##	
22	318±15		305±14**		243±12		217±13##	
29	373±20		353±17**		261±17		240±13##	
36	415±24		392±22**		285±18		260±15##	
43	448±27		426±25**		309±17		280±17##	
50	477±28		452±27**		324±16		295±18##	
57	503±30		474±28**		338±17		309±18##	
64	524±32		493±30**		353±17		324±18##	
71	543±33		510±32**		365±18		333±19##	
78	562±36		527±32**		376±17		343±18##	
85	577±37		540±34**		385±16		354±17##	
92	589±38		551±35**		394±16		370±17##	
120	630±47		590±43**		430±17		406±18##	
148	664±52		613±49**		444±19		424±18##	
176	693±61		638±54**		463±18		440±20##	
204	719±71	(25)	658±52**	(25)	489±14	(25)	458±19##	(25)
232	745±76	(25)	675±56**	(25)	504±17	(25)	468±23##	(25)
260	763±80	(25)	694±62**	(25)	513±17	(25)	473±24##	(25)
288	784±88	(25)	717±66**	(25)	531±16	(25)	481±23##	(25)
316	799±96	(25)	739±71*	(25)	542±17	(25)	482±21##	(25)
344	814±101	(25)	751±72*	(25)	545±17	(25)	481±22##	(25)
372	826±111	(25)	766±76*	(25)	552±19	(25)	481±23##	(25)
400	814±111	(20)	771±80	(20)	547±27	(20)	475±24##	(20)
428	822±119	(20)	776±80	(19)	550±29	(19)	473±27##	(20)
456	816±138	(20)	792±86	(19)	536±30	(19)	454±36##	(20)
484	776±183	(20)	802±92	(19)	519±37	(19)	453±39##	(20)
512	868±122	(17)	818±102	(19)	525±43	(19)	445±46##	(20)
540	886±129	(16)	805±90*	(17)	519±48	(19)	429±54##	(20)
568	884±133	(16)	798±94*	(16)	494±62	(19)	412±55##	(18)
596	879±166	(16)	796±112	(15)	498±64	(18)	396±58##	(18)
624	893±133	(15)	815±99	(14)	493±72	(18)	389±59##	(18)
652	868±112	(13)	828±104	(13)	482±83	(17)	374±59##	(18)
680	855±114	(12)	835±106	(12)	455±70	(16)	368±59##	(17)
708	840±129	(9)	822±95	(12)	454±59	(14)	361±58##	(16)
736	824±155	(8)	808±102	(12)	447±62	(14)	372±54##	(14)

Each value shows mean (g)±S.D.

*: P<0.05, **: P<0.01, Significantrly different from CRF-1(*ad libitum* feeding) group.

##: P<0.01, Significantrly different from CRF-1(restricted feeding) group.

Figures in parentheses indicate number of males.

Table 6. Food consumption of male rats

Group	CR-LPF (*ad libitum* feeding)		CR-LPF (restricted feeding)		CRF-1 (*ad libitum* feeding)		CRF-1 (restricted feeding)	
Number of males	30		30		30		30	
Days of feeding								
2	17±1		18±0		16±1		18±0	
9	26±2		18±0		23±3		18±0	
16	27±4		18±2		27±3		18±0	
23	27±4		18±0		27±5		18±0	
30	27±12		18±0		29±9		18±0	
37	29±7		18±0		27±6		18±0	
44	27±4		18±0		27±2		18±0	
51	29±3		18±0		29±3		18±0	
58	28±3		18±0		26±2		18±0	
65	28±3		18±0		27±2		18±0	
72	29±3		18±0		28±3		18±0	
79	28±3		18±0		29±3		18±0	
86	28±3		18±0		27±2		18±0	
93	27±3		18±0		26±2		18±0	
121	27±3		18±0		27±3		18±0	
149	25±3		18±0		24±2		18±0	
177	25±4	(25)	18±1	(25)	24±2	(25)	18±0	(25)
205	24±4	(25)	18±0	(25)	24±3	(25)	18±0	(25)
233	28±4	(25)	18±0	(25)	25±3	(25)	18±0	(25)
261	24±7	(25)	18±1	(25)	19±10	(25)	18±1	(25)
289	26±5	(25)	18±0	(25)	24±3	(25)	18±0	(25)
317	27±4	(25)	18±0	(25)	25±2	(25)	18±0	(25)
345	28±4	(25)	18±1	(25)	27±8	(25)	18±0	(25)
373	29±4	(20)	18±0	(20)	25±4	(20)	18±0	(20)
401	28±4	(19)	18±0	(20)	25±6	(20)	18±0	(20)
429	31±6	(19)	18±0	(20)	27±4	(20)	18±0	(19)
457	30±4	(19)	18±0	(20)	25±7	(20)	18±0	(19)
485	28±5	(19)	18±2	(20)	24±9	(20)	18±0	(19)
513	32±5	(19)	18±0	(20)	30±5	(17)	18±0	(19)
541	32±5	(17)	18±0	(20)	29±3	(16)	18±0	(19)
569	33±5	(16)	17±3	(18)	29±5	(16)	17±4	(19)
597	31±7	(15)	18±0	(18)	29±3	(15)	18±0	(18)
625	34±4	(14)	18±0	(18)	29±3	(15)	18±0	(18)
653	33±4	(13)	18±1	(18)	27±4	(13)	18±0	(17)
681	35±5	(12)	18±1	(17)	30±4	(12)	17±4	(16)
709	34±4	(12)	18±1	(16)	29±6	(9)	18±0	(14)

Each value shows mean (g/day)±S.D.
Figures in parentheses indicate number of males.

Table 7. Food consumption of male rats

Group	CRF-1 (*ad libitum* feeding)		CR-LPF (*ad libitum* feeding)		CRF-1 (restricted feeding)		CR-LPF (restricted feeding)	
Number of males	30		30		30		30	
Days of feeding								
2	16±1		17±1*		18±0		18±0	
9	23±3		26±2**		18±0		18±0	
16	27±3		27±4		18±0		18±2	
23	27±5		27±4		18±0		18±0	
30	29±9		27±12		18±0		18±0	
37	27±6		29±7		18±0		18±0	
44	27±2		27±4		18±0		18±0	
51	29±3		29±3		18±0		18±0	
58	26±2		28±3		18±0		18±0	
65	27±2		28±3*		18±0		18±0	
72	28±3		29±3		18±0		18±0	
79	29±3		28±3*		18±0		18±0	
86	27±2		28±3*		18±0		18±0	
93	26±2		27±3		18±0		18±0	
121	27±3		27±3		18±0		18±0	
149	24±2		25±3*		18±0		18±0	
177	24±2	(25)	25±4	(25)	18±0	(25)	18±1	(25)
205	24±3	(25)	24±4	(25)	18±0	(25)	18±0	(25)
233	25±3	(25)	28±4*	(25)	18±0	(25)	18±0	(25)
261	19±10	(25)	24±7*	(25)	18±1	(25)	18±1	(25)
289	24±3	(25)	26±5	(25)	18±0	(25)	18±0	(25)
317	25±2	(25)	27±4**	(25)	18±0	(25)	18±0	(25)
345	27±8	(25)	28±4	(25)	18±0	(25)	18±1	(25)
373	25±4	(20)	29±4**	(20)	18±0	(20)	18±0	(20)
401	25±6	(20)	28±4	(19)	18±0	(20)	18±0	(20)
429	27±4	(20)	31±6*	(19)	18±0	(19)	18±0	(20)
457	25±7	(20)	30±4*	(19)	18±0	(19)	18±0	(20)
485	24±9	(20)	28±5	(19)	18±0	(19)	18±2	(20)
513	30±5	(17)	32±5	(19)	18±0	(19)	18±0	(20)
541	29±3	(16)	32±5	(17)	18±0	(19)	18±0	(20)
569	29±5	(16)	33±5*	(16)	17±4	(19)	17±3	(18)
597	29±3	(15)	31±7	(15)	18±0	(18)	18±0	(18)
625	29±3	(15)	34±4**	(14)	18±0	(18)	18±0	(18)
653	27±4	(13)	33±4**	(13)	18±0	(17)	18±1	(18)
681	30±4	(12)	35±5*	(12)	17±4	(16)	18±1	(17)
709	29±6	(9)	34±4	(12)	18±0	(14)	18±1	(16)

Each value shows mean (g/day)±S.D.
*: P<0.05, **: P<0.01, Significantrly different from CRF-1(*ad libitum* feeding) group.
Figures in parentheses indicate number of males.

Table 8. Hematological finding of male rats

Group		CR-LPF (*ad libitum* feeding)	CR-LPF (restricted feeding)	CRF-1 (*ad libitum* feeding)	CRF-1 (restricted feeding)
Number of males		5	5	5	5
RBC	($10^4/\mu$ L)	635±157	502±143	550±176	626±122
HGB	(g/dL)	11.3±3.4	8.3±2.5	9.3±3.4	11.4±2.3
HCT	(%)	35.4±8.4	28.1±6.0	30.0±9.4	35.5±6.5
MCV	(fL)	55.9±2.6	57.2±6.5	55.1±7.7	56.9±4.0
MCH	(pg)	17.7±1.2	16.5±2.2	16.8±3.0	18.1±0.7
MCHC	(g/dL)	31.6±2.1	28.9±2.9	30.4±2.1	31.9±1.8
PLT	($10^4/\mu$ L)	132.8±37.6	136.4±38.9	107.9±26.1 (4)	127.7±38.9
RET	(‰)	91±73	206±163	126±84	107±68
PT	(sec.)	16.6±0.3	18.0±0.9*	16.0±0.6	16.6±1.2
APTT	(sec.)	22.6±2.8	20.1±2.1	21.0±2.8	23.5±3.0
FIB	(mg/dL)	357±57	331±50	393±157	310±62
WBC	($10^2/\mu$ L)	134±87	156±152	121±61	50±12
Differential leukocyte	(%)				
Lymphocyte		72.8±9.0	45.8±6.9**	66.4±17.8	72.6±7.9
Neutrophil		25.4±9.0	52.8±7.7**	30.0±18.1	26.4±8.0
Eosinophil		0.4±0.5	0.6±0.5	0.8±1.1	0.2±0.4
Basophil		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Monocyte		1.4±0.5	0.8±0.4	2.8±2.5	0.8±0.4

Each value shows mean±S.D.
*: P<0.05, **: P<0.01, Significantrly different from CR-LPF(*ad libitum* feeding) group.
Figures in parentheses indicate number of males.

Table 9. Hematological finding of male rats

Group		CRF-1 (*ad libitum* feeding)	CR-LPF (*ad libitum* feeding)	CRF-1 (restricted feeding)	CR-LPF (restricted feeding)
Number of males		5	5	5	5
RBC	($10^4/\mu$ L)	550±176	635±157	626±122	502±143
HGB	(g/dL)	9.3±3.4	11.3±3.4	11.4±2.3	8.3±2.5
HCT	(%)	30.0±9.4	35.4±8.4	35.5±6.5	28.1±6.0
MCV	(fL)	55.1±7.7	55.9±2.6	56.9±4.0	57.2±6.5
MCH	(pg)	16.8±3.0	17.7±1.2	18.1±0.7	16.5±2.2
MCHC	(g/dL)	30.4±2.1	31.6±2.1	31.9±1.8	28.9±2.9
PLT	($10^4/\mu$ L)	107.9±26.1 (4)	132.8±37.6	127.7±38.9	136.4±38.9
RET	(‰)	126±84	91±73	107±68	206±163
PT	(sec.)	16.0±0.6	16.6±0.3	16.6±1.2	18.0±0.9
APTT	(sec.)	21.0±2.8	22.6±2.8	23.5±3.0	20.1±2.1
FIB	(mg/dL)	393±157	357±57	310±62	331±50
WBC	($10^2/\mu$ L)	121±61	134±87	50±12	156±152
Differential leukocyte	(%)				
Lymphocyte		66.4±17.8	72.8±9.0	72.6±7.9	45.8±6.9##
Neutrophil		30.0±18.1	25.4±9.0	26.4±8.0	52.8±7.7##
Eosinophil		0.8±1.1	0.4±0.5	0.2±0.4	0.6±0.5
Basophil		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Monocyte		2.8±2.5	1.4±0.5	0.8±0.4	0.8±0.4

Each value shows mean±S.D.
##: P<0.01, Significantrly different from CRF-1(restricted feeding) group.
Figures in parentheses indicate number of males.

Table 10. Blood chemical analysis of male rats

Group		CR-LPF (*ad libitum* feeding)	CR-LPF (restricted feeding)	CRF-1 (*ad libitum* feeding)	CRF-1 (restricted feeding)
Number of males		5	5	5	5
AST	(IU/L)	227.9±93.9	216.2±46.5	212.0±63.6	174.7±74.1
ALT	(IU/L)	59.5±25.3	53.9±25.1	66.1±33.6	82.9±78.1
ALP	(IU/L)	448.2±199.2	330.9±80.6	460.0±163.8	254.7±64.5#
TP	(g/dL)	5.6±0.5	5.5±0.2	5.7±0.6	5.5±0.4
Alb	(g/dL)	1.74±0.71	1.91±0.36	1.61±0.35	2.08±0.55
Protein fraction (%)					
alb		30.5±9.7	34.4±5.8	28.8±7.4	37.3±8.0
α 1-glb		26.6±5.3	24.5±4.5	25.7±3.6	25.3±5.4
α 2-glb		5.9±1.7	4.4±2.4	6.3±2.9	5.5±1.9
β -glb		25.2±3.8	25.6±3.6	25.7±1.7	22.2±3.7
γ -glb		11.8±4.6	11.1±7.1	13.6±4.2	9.6±2.4
A/G		0.46±0.21	0.53±0.15	0.42±0.16	0.62±0.21
T-Bil	(mg/dL)	0.14±0.05	0.13±0.02	0.08±0.01	0.13±0.05
UN	(mg/dL)	15.1±2.6	12.5±1.3	25.8±18.6	17.1±2.6
CRE	(mg/dL)	0.30±0.05	0.32±0.02	0.49±0.43	0.37±0.07
Glu	(mg/dL)	102.7±31.0	103.3±33.5	103.9±25.8	128.8±31.1
T-Cho	(mg/dL)	97.4±33.4	72.0±11.7	100.7±41.9	78.7±15.4
TG	(mg/dL)	89.3±29.1	31.3±14.1**	100.3±51.4	34.6±24.8#
Na	(mEq/L)	144.5±1.2	143.4±3.0	144.1±2.0	144.7±3.0
K	(mEq/L)	4.50±0.51	4.60±0.43	4.78±0.71	4.58±0.69
Cl	(mEq/L)	105.2±1.3	107.0±3.2	105.0±4.1	104.6±2.1
Ca	(mg/dL)	8.8±0.6	8.5±0.5	8.9±0.8	8.8±0.3
IP	(mg/dL)	5.0±1.0	5.5±1.3	5.5±1.1	5.9±1.1

Each value shows mean±S.D.

**: P<0.01, Significantrly different from CR-LPF(*ad libitum* feeding) group.

#: P<0.05, Significantrly different from CRF-1(*ad libitum* feeding) group.

Table 11. Blood chemical analysis of male rats

Group		CRF-1 (*ad libitum* feeding)	CR-LPF (*ad libitum* feeding)	CRF-1 (restricted feeding)	CR-LPF (restricted feeding)
Number of males		5	5	5	5
AST	(IU/L)	212.0±63.6	227.9±93.9	174.7±74.1	216.2±46.5
ALT	(IU/L)	66.1±33.6	59.5±25.3	82.9±78.1	53.9±25.1
ALP	(IU/L)	460.0±163.8	448.2±199.2	254.7±64.5	330.9±80.6
TP	(g/dL)	5.7±0.6	5.6±0.5	5.5±0.4	5.5±0.2
Alb	(g/dL)	1.61±0.35	1.74±0.71	2.08±0.55	1.91±0.36
Protein fraction (%)					
alb		28.8±7.4	30.5±9.7	37.3±8.0	34.4±5.8
α 1-glb		25.7±3.6	26.6±5.3	25.3±5.4	24.5±4.5
α 2-glb		6.3±2.9	5.9±1.7	5.5±1.9	4.4±2.4
β -glb		25.7±1.7	25.2±3.8	22.2±3.7	25.6±3.6
γ -glb		13.6±4.2	11.8±4.6	9.6±2.4	11.1±7.1
A/G		0.42±0.16	0.46±0.21	0.62±0.21	0.53±0.15
T-Bil	(mg/dL)	0.08±0.01	0.14±0.05	0.13±0.05	0.13±0.02
UN	(mg/dL)	25.8±18.6	15.1±2.6	17.1±2.6	12.5±1.3##
CRE	(mg/dL)	0.49±0.43	0.30±0.05	0.37±0.07	0.32±0.02
Glu	(mg/dL)	103.9±25.8	102.7±31.0	128.8±31.1	103.3±33.5
T-Cho	(mg/dL)	100.7±41.9	97.4±33.4	78.7±15.4	72.0±11.7
TG	(mg/dL)	100.3±51.4	89.3±29.1	34.6±24.8	31.3±14.1
Na	(mEq/L)	144.1±2.0	144.5±1.2	144.7±3.0	143.4±3.0
K	(mEq/L)	4.78±0.71	4.50±0.51	4.58±0.69	4.60±0.43
Cl	(mEq/L)	105.0±4.1	105.2±1.3	104.6±2.1	107.0±3.2
Ca	(mg/dL)	8.9±0.8	8.8±0.6	8.8±0.3	8.5±0.5
IP	(mg/dL)	5.5±1.1	5.0±1.0	5.9±1.1	5.5±1.3

Each value shows mean±S.D.
##: P<0.01, Significantrly different from CRF-1(restricted feeding) group.

Table 12-1. Macroscopic findings in male rats

Group	CRF-1(*ad libitum* feeding)	CR-LPF(*ad libitum* feeding)	CRF-1(restricted feeding)	CR-LPF(restricted feeding)
Number of males	5	7	9	9
Findings				
Lung				
Nodule	0	0	0	1
Hepatization	1	0	0	0
Stomach				
Red spot	1	0	0	0
Brown in color	1	0	0	0
Red in color, glandular mucosa	0	0	1	0
Liver				
Nodule	0	0	1	0
Tumor	1	0	0	0
Dark red spot	0	1	1	0
Kidney				
Swelling	1	0	0	0
Granular surface	1	0	0	0
Cyst	0	0	2	0
Testis				
Small in size	0	0	1	0
Softness	1	1	1	0
White in color	1	0	0	0
Thymus				
Not found	4	6	8	9
Spleen				
Large in size	2	3	0	0
Adhesion to omentum	1	0	0	1
Lymph node				
Large in size	1	0	1	0

Table 12-2. Macroscopic findings in male rats

Group	CRF-1(*ad libitum* feeding)	CR-LPF(*ad libitum* feeding)	CRF-1(restricted feeding)	CR-LPF(restricted feeding)
Number of males	5	7	9	9
Findings				
Pituitary				
Tumor	1	2	0	1
Dark red spot	0	2	2	0
Cyst	1	0	0	0
Adrenal				
Large in size	2	1	0	0
Thoracic cavity				
Pleural effusion, transparent	1	0	0	0
Eyeball				
Opacity	1	1	0	0
Purulent discharge	1	0	0	0
Skin/subcutis				
Tumor	2	1	1	1
Papilloma	0	1	0	0
Hindlimb				
Decubitus	5	5	7	6
Swelling/induration, ankle	0	1	0	0
Tail				
Mass	2	5	1	3
Defect	0	0	0	1
Ulcer	0	1	0	0

Table 13. Organ weights of male rats

Group		CR-LPF (*ad libitum* feeding)	CR-LPF (restricted feeding)	CRF-1 (*ad libitum* feeding)	CRF-1 (restricted feeding)
Number of males		5	5	5	5
Body weight	(g)	856±130	382±50**	825±175	481±86##
Pituitary	(mg)	21.3±4.2	12.4±1.3**	22.9±5.6	15.0±3.4#
	(mg%)	2.5±0.5	3.3±0.4*	2.9±1.0	3.1±0.5
Lungs	(g)	2.11±0.35	1.56±0.14*	2.28±0.50	1.66±0.10
	(g%)	0.25±0.05	0.42±0.08**	0.29±0.11	0.35±0.07
Heart	(g)	2.28±0.25	1.41±0.13**	2.51±0.27	1.60±0.14##
	(g%)	0.27±0.05	0.37±0.05*	0.32±0.08	0.34±0.06
Liver	(g)	21.84±2.56	9.31±1.10**	24.79±6.71	12.46±1.07#
	(g%)	2.57±0.27	2.44±0.09	3.15±1.26	2.66±0.54
Spleen	(g)	2.01±0.98	0.99±0.22	4.26±5.70	1.06±0.22
	(g%)	0.24±0.14	0.26±0.06	0.60±0.87	0.23±0.11
Kidneys	(g)	4.11±0.31	2.74±0.10**	5.77±2.22	3.31±0.44
	(g%)	0.49±0.06	0.72±0.08**	0.74±0.39	0.70±0.14
Adrenals	(mg)	86.7±10.1	50.9±7.5**	105.9±59.8	60.1±13.8
	(mg%)	10.2±1.4	13.6±3.2	14.1±10.0	13.2±5.6

Each value shows mean±S.D.
*: P<0.05, **: P<0.01, Significantrly different from CR-LPF(*ad libitum* feeding) group.
#: P<0.05, ##: P<0.01, Significantrly different from CRF-1(*ad libitum* feeding) group.

Table 14. Organ weights of male rats

Group		CRF-1 (*ad libitum* feeding)	CR-LPF (*ad libitum* feeding)	CRF-1 (restricted feeding)	CR-LPF (restricted feeding)
Number of males		5	5	5	5
Body weight	(g)	825±175	856±130	481±86	382±50
Pituitary	(mg)	22.9±5.6	21.3±4.2	15.0±3.4	12.4±1.3
	(mg%)	2.9±1.0	2.5±0.5	3.1±0.5	3.3±0.4
Lungs	(g)	2.28±0.50	2.11±0.35	1.66±0.10	1.56±0.14
	(g%)	0.29±0.11	0.25±0.05	0.35±0.07	0.42±0.08
Heart	(g)	2.51±0.27	2.28±0.25	1.60±0.14	1.41±0.13
	(g%)	0.32±0.08	0.27±0.05	0.34±0.06	0.37±0.05
Liver	(g)	24.79±6.71	21.84±2.56	12.46±1.07	9.31±1.10##
	(g%)	3.15±1.26	2.57±0.27	2.66±0.54	2.44±0.09
Spleen	(g)	4.26±5.70	2.01±0.98	1.06±0.22	0.99±0.22
	(g%)	0.60±0.87	0.24±0.14	0.23±0.11	0.26±0.06
Kidneys	(g)	5.77±2.22	4.11±0.31	3.31±0.44	2.74±0.10#
	(g%)	0.74±0.39	0.49±0.06	0.70±0.14	0.72±0.08
Adrenals	(mg)	105.9±59.8	86.7±10.1	60.1±13.8	50.9±7.5
	(mg%)	14.1±10.0	10.2±1.4	13.2±5.6	13.6±3.2

Each value shows mean±S.D. Figures in parentheses indicate number of males.
#: P<0.05, ##: P<0.01, Significantrly different from CRF-1(restricted feeding) group.

Table 15-1. Histopathological findings in male rats

Group	CRF-1(*ad libitum* feeding)					CR-LPF(*ad libitum* feeding)					CRF-1(restricted feeding)					CR-LPF(restricted feeding)				
Number of males	5					5					5					5				
Grade	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+
Non-neoplastic lesions																				
Pituitary																				
Hyperplasia, anterior lobe	3	1	1	0	0	3	1	1	0	0	3	2	0	0	0	5	0	0	0	0
Cyst, intermediate lobe	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0	4	1	0	0	0
Cyst	4	0	1	0	0	4	0	1	0	0	3	2	0	0	0	4	1	0	0	0
Angiectasis, intermediate lobe	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0
Heart																				
Cellular infiltration, histiocyte	0	1	4	0	0	0	4	1	0	0	1	1	3	0	0	4	1	0	0	0
Fibrosis	0	0	5	0	0	0	1	4	0	0	0	1	4	0	0	0	5	0	0	0
Lung																				
Accumulation, foam cell	0	5	0	0	0	1	3	1	0	0	1	2	2	0	0	1	2	1	1	0
Granulation tissue	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Liver																				
Proliferation, bile duct	0	1	2	2	0	0	3	1	0	1	0	3	2	0	0	1	3	1	0	0
Altered hepatocellular focus	0	2	2	1	0	1	3	1	0	0	2	2	1	0	0	5	0	0	0	0
Microgranuloma	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0	3	2	0	0	0
Hematopoiesis, extramedullary	4	1	0	0	0	4	1	0	0	0	4	1	0	0	0	2	3	0	0	0
Angiectasis	5	0	0	0	0	4	0	1	0	0	4	0	1	0	0	5	0	0	0	0
Cellular infiltration, lymphoid cell, focal	4	1	0	0	0	3	2	0	0	0	4	1	0	0	0	3	2	0	0	0
Spleen																				
Hematopoiesis, extramedullary	1	1	2	0	1	1	0	3	0	1	0	3	2	0	0	2	0	1	1	1
Cellular infiltration, inflammatory cell, capsule	4	0	1	0	0	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Kidney																				
Pyelitis	5	0	0	0	0	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0
Nephropathy, chronic	2	1	1	0	1	3	0	2	0	0	4	0	1	0	0	4	1	0	0	0
Cellular infiltaration, lymphoid cell, focal	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Hyaline droplet, tubular epithelium	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0
Pyelonephritis	5	0	0	0	0	3	1	1	0	0	3	0	2	0	0	4	1	0	0	0
Cyst	5	0	0	0	0	5	0	0	0	0	4	0	1	0	0	5	0	0	0	0

Grade of histopathological findings: −: no abnormality detected, ±: slight, +: mild, 2+: moderate, 3+: marked.

Table 15-2. Histopathological findings in male rats

Group	CRF-1(*ad libitum* feeding)					CR-LPF(*ad libitum* feeding)					CRF-1(restricted feeding)					CR-LPF(restricted feeding)				
Number of males	5					5					5					5				
Grade	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+
Non-neoplastic lesions																				
Adrenal																				
Hyperplasia, cortex, focal	0	3	2	0	0	3	2	0	0	0	3	2	0	0	0	2	3	0	0	0
Hyperplasia, medulla, focal	1	4	0	0	0	5	0	0	0	0	3	2	0	0	0	5	0	0	0	0
Hematopoiesis, extramedullary	3	2	0	0	0	5	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Head			(1)*					NE					NE					NE		
Abscess, skin	0	0	0	1	0															
Eye ball			(2)*					(1)*					NE					NE		
Cataract	1	0	1	0	0	0	0	0	1	0										
Iritis	1	0	1	0	0	1	0	0	0	0										
Abscess	1	0	1	0	0	1	0	0	0	0										
Stomach			(1)*					NE					(1)*					NE		
Hemorrhage, lamina propria mucosa, glandular stomach	0	0	0	1	0						1	0	0	0	0					
Congestion, mucosa, glandular stomach	0	1	0	0	0						1	0	0	0	0					
Congestion, muscle layer, glandular stomach	1	0	0	0	0						0	1	0	0	0					
Lymph node, mandibular			(1)*					NE					NE					NE		
Dilatation, sinus	0	0	1	0	0															
Lymph node, iliac			(1)*					NE					NE					NE		
Dilatation, sinus	0	0	1	0	0															
Testis			(1)*					NE					(1)*					NE		
Atrophy, seminiferous tubule	0	0	0	0	1						0	0	1	0	0					
Tail			NE					(1)*					NE					NE		
Ulcer						0	0	1	0	0										
Hyperkeratosis						0	0	1	0	0										
Hindlimb			NE					(1)*					(1)*					NE		
Decubitus						0	0	0	1	0	1	0	0	0	0					
Abscess						1	0	0	0	0	0	0	1	0	0					
Skin/subcutis			(1)*					NE					NE					NE		
Crystal deposition, cholesterin	0	0	1	0	0															
Foreign body giant cell	0	0	1	0	0															

Grade of histopathological findings: −: no abnormality detected, ±: slight, +: mild, 2+: moderate, 3+: marked.
*Examined only one or two animals.
NE: not examined.

Table 15-3. Histopathological findings in male rats

Group	CRF-1(*ad libitum* feeding)	CR-LPF(*ad libitum* feeding)	CRF-1(restricted feeding)	CR-LPF(restricted feeding)
Number of males	5	5	5	5
Neoplastic lesions				
Pituitary				
Adenoma, anterior lobe	1	1	1	0
Heart				
Large granular lymphocyte leukemia	1	0	0	0
Lung				
Large granular lymphocyte leukemia	1	0	0	0
Liver				
Hepatocellular carcinoma	1	0	0	0
Large granular lymphocyte leukemia	1	0	0	0
Spleen				
Large granular lymphocyte leukemia	1	0	0	0
Adrenal				
Pheochromocytoma	2	3	0	1
Ganglioneuroma	0	1	0	1
Adenoma, cortex	0	1	0	0
Large granular lymphocyte leukemia	1	0	0	0
Testis	(1)*	NE	NE	NE
Leydig cell tumor	1			
Skin/subcutis	NE	(1)*	NE	(1)*
Fibroma		1		0
Trichoepithelioma		0		1

*Examined only one animal.
NE: not examined.

The Effects of Moderate Dietary Restriction on Biological Characteristics in Crj:CD(SD)IGS Rats

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ABSTRACT. A 2-year study was conducted in CD(SD)IGS rats (100/sex/group) under moderate dietary restriction. For the moderate dietary restriction, the animals were fed a commercial diet (CRF-1) daily of approximately 21g in males or 14g in females during the experimental period. To simulate conditions of a 2-year carcinogenicity study, 10 mL/kg of a 0.5% methylcellulose solution was administered orally, once a day by gavage, to each rat. After 104 weeks the survival rate was 68% in males and 73% in females, and was significantly higher when compared to the published literature values in previous studies at different facilities under *ad libitum* feeding condition of CRF-1 diet. In this study, moderate dietary restriction (ca. 20-25% restriction of daily food intake) improved longevity of CD(SD)IGS rats by controlling body weight changes and lowering the incidences of some spontaneous tumors and life-threatening non-neoplasitc lesions. In conclusion, a dietary restriction rate of approximately 20-25% appears to be appropriate for CD(SD)IGS rats in chronic studies to improve survivability and could possibly reduce variability in carcinogenicity studies in CD(SD)IGS rats. — Key words: carcinogenicity, dietary restriction, IGS rats.

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INTRODUCTION

For the past decade, CD(SD)IGS rats have been widely used for pre-clinical toxicology studies, including carcinogenicity bioassays, for pharmaceuticals. The background data for CD(SD)IGS rats, such as mortality, body weight change, clinical pathology parameters, and macro- and micro-pathology have been reported by many different testing facilities [1-7]. As with other strains of rats, one of the biggest concerns for carcinogenicity bioassays with CD(SD)IGS rats is high mortality. The survival rates of CD(SD)IGS rats under life-time ad-lib feeding condition are gradually declining year by year, and the average survival rate is documented to be less than 50% for both males and females. Although there were no significant effects of a low protein diet on the survival rate and biological parameters in rats [1-4], it has been demonstrated that one way to improve longevity in rats is calorie restriction [1-4, 8-12]. In this study, to establish background data on biological characteristics in CD(SD)IGS rats, we examined the effects of dietary optimization by controlling daily dietary intake in CD(SD)IGS rats.

MATERIALS AND METHODS

1. Animals: One hundred male and 100 female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc., (Hino, Shiga, Japan). The animals were 6 weeks old at time of receipt and 8 weeks old at study initiation. Following the 2 week acclimation period, all animals were individually housed in stainless steel wire-meshed cages under the following conditions: target temperature of 20 - 26℃ and target humidity of 35 - 75% in a clean air room with 12-hour light cycles. The animals were provided a commercial diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) of approximately 21 g/day for males and approximately 14 g/day for females, and water was given *ad libitum*. The mean diet composition of the CRF-1 diet was as follows; moisture: <9.9%, crude protein: >21.0%, crude fat: >4.0%, crude ash: <8.0%, crude fiber: 2.0-5.0%, nitrogen-free extract: 52-55%, calorie: 3.7 kcal/g. The total calorie intake was approximately 78 kcal/day in males and 52 kcal/day in females. To simulate conditions of a 2-year carcinogenicity study, all animals were also given 0.5% methylcellulose solution (10 mL/kg) by gavage daily for 104 weeks.

2. Observations and Examinations: The first day and the first week of administration were defined as Day 1 and Week 1, respectively.

1) General Observations: All animals were observed daily for clinical signs and mortality and detailed physical examinations were conducted weekly during the experimental period.

2) Body Weights: All animals were weighed once a week.

3) Food Consumption: Food consumption was recorded weekly.

3. Hematology: On the day prior to necropsy, fasted blood samples were collected from all animals. Blood samples were collected from the tail vein using EDTA-2K or sodium citrate as anti-coagulant for hematology and blood coagulation, respectively. The following parameters were examined: erythrocyte (RBC) count, hematocrit, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count, white blood cell (WBC) count, differential WBC count (neutrophils, lymophocytes, monocytes, eosinophils, basophils, and large unstained cells) (Total Hematology Management System, Technicon H･1E, Bayer Corporation, USA), reticulocyte count (Automatic Reticulocyte analyzer R-2000, Sysmex Co., Japan), and prothrombin time (PT) and activated partial thromboplastin time (APTT) (Automated coagulation analyzer KC10A, Amelung GmbH, Germany).

4. Blood Chemistry: On the day prior to necropsy, fasted blood samples were collected from all animals. Blood

samples were also collected from the tail vein without anticoagulant. The following parameters were examined: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, total cholesterol, glucose, urea nitrogen, creatinine, total bilirubin, albumin, globulin, A/G ratio, chloride (Cl), sodium (Na), potassium (K), and calcium (Ca) (Automated analyzer, 7170, Hitachi, Ltd., Japan).

5. **Necropsy:** All animals were sacrificed by CO_2 asphyxiation after 104 weeks of the experiment. A complete necropsy was performed after exsanguination from the vena cava. All animals found dead and moribund-sacrificed animals were also examined grossly at necropsy.

6. **Histopathological Examination:** Samples of the following organs were collected from all animals: liver, kidneys, brain, spinal cord, sciatic nerve, trachea, lung (including bronchi), heart, aorta, spleen, thymus, adrenals, thyroids, parathyroids, pituitary, testes, epididymides, ovaries, uterus, vagina, sublingual glands, submaxillary glands, Harderian glands, eyes, optic nerve, tongue, submaxillary lymph node, mesenteric lymph node, pancreas, urinary bladder, prostate, seminal vesicles, coagulating glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, bone marrow (sternum and femur), sternum, femur, thigh muscle, mammary gland, skin, and gross lesions. The eyes, Harderian glands, and optic nerve were preserved in glutaraldehyde-formalin solution and the testes and epididymides were fixed in formalin-sucrose-acetic acid (FSA) solution. Other organs and tissues were preserved in neutral buffered 10 % formalin. All organs and tissues from all animals were processed for microscopic examination of routine-paraffin embedded sections, stained with hematoxylin and eosin, and examined histopathologically.

7. **Data Evaluation:** The data from this study were evaluated and compared with the published values in previous studies at different facilities under *ad libitum* feeding condition of CRF-1 diet [1, 4, 6].

RESULTS

1. Mortality:

The numbers of animals found dead or sacrificed moribund and survival rates are shown in Table 1, and survival curves are presented in Fig. 1. In this study, the survival rates in male and female CD(SD)IGS rats after 104 weeks of experiment (112weeks-old at study termination) were 68% and 73%, respectively. The first death due to non-neoplastic lesion was observed in Week 41 in males (urogenital inflammation) and Week 87 in females (pan-arteritis). The first death attributed to tumor formation occurred in week 67 in males (liposarcoma in abdominal cavity), and Week 50 in females (mammary adenocarcinoma).

2. Body Weights:

Changes in mean body weights are shown in Fig. 2. Mean body weights after 104 weeks of experiment were 545g in males and 290g in females, and mean body weight gains from the beginning to the end of the experiment were 274g in males and 109g in females. When compared to the published literature values for mean body weights of CD(SD)IGS rats fed CRF-1 diet *ad libitum* (mean body weight: 814g in males, 529g in females), the terminal mean body weights of the rats in this study were significantly lower (67% in males and 55% in females lower than published reference values).

3. Food Consumption:

Approximately 21g (males) or 14g (females) CRF-1 diet was given to each animal daily, and residual food was seldom observed during the 2-year experimental period (data not shown). In this study, the daily food intake was estimated to be restricted by approximately 20-25% when compared to the literature reference data of rats fed CRF-1 diet *ad libitum* (literature reference mean food consumption: ca. 26 g/day in males, ca. 18g/day in females).

4. Hematology:

Group means of hematological parameters at terminal necropsy (at 104 weeks of experiment) are summarized in Table 2.

5. Blood Chemistry:

Group means of blood chemistry parameters at terminal necropsy are summarized in Table 3.

6. Histopathological Findings:

1) Cause of Death:

The causes of death are shown in Table 4. In males, the pituitary tumor (5/32, 16%), malignant lymphoma/leukemia (5/32, 16%), and urogenital inflammation (6/32, 19%) were the most common lesions associated with cause of death. In females, the pituitary tumor (13/27, 48%) and mammary tumors (6/27, 22%) were the most common lesions associated with cause of death.

2) Neoplastic Findings:

Neoplastic lesions are listed in Table 5. In males, significant incidences of anterior pituitary tumors (adenoma: 33%, adenocarcinoma: 3%, combined: 36%) and adrenal pheochromocytoma (benign: 14%, malignant: 4%, combined: 18%) were observed. In females, significant incidences of anterior pituitary tumors (adenoma: 59%, adenocarcinoma: 10%, combined: 69%) and mammary tumors (adenoma: 10%, adenocarcinoma, 22%, combined: 32%, fibroadenoma: 19%) were observed. The incidences of anterior pituitary tumors in males, fibroadenoma in mammary glands in females, and adrenocortical tumors in both sexes were lower in the diet-restricted animals in this study when compared to those in the published literature for animals under *ad libitum* feeding condition of CRF-1 diet (see below table). There were no substantial differences in the incidence of other neoplasitc lesions between diet-restricted and ad-lib feeding condition.

Neoplastic lesions comparison

Neoplastic lesions	Incidence (%)			
Feeding condition	Diet restricted		Ad-lib fed*	
Sex	Male	Female	Male	Female
Pituitary: Adenoma+adenocaricnoma	36	69	67	78
Mammary: Fibroadenoma	1	19	0	46
Adrenal cortex: Adenoma+carcinoma	0	2	6	36

*from T. Nagayabu et al. (2000)

3) Non-neoplastic Findings:

Non-neoplastic lesions are listed in Table 6. The incidences of the following non-neoplastic lesions in dietary restricted animals were significantly decreased when compared with those in the published literature for *ad libitum* fed animals; chronic nephropathy in the kidneys, myocardial fibrosis/cardiomyopathy in the heart, adrenal cortical hyperplasia, C-cell hyperplasia in the thyroids, bile duct hyperplasia in the liver, and inflammation in the prostate. In this study, the incidence and severity of chronic nephropathy, which is considered to be one of the most common life-threatening spontaneous lesions in chronic rat studies, was dramatically decreased under chronic diet optimization (incidence rates of 6% in males and 2% in females) when compared with the published literature data from *ad libitum* feeding studies (incidence rates of 94% in males and, 60% in females) (See below table). Although the biological significance is unclear, the incidence of adrenal medullary hyperplasia was increased in the diet-restricted animals when compared to that in ad-lib fed animals.

There were no substantial differences in the incidence of other non-neoplasitc lesions between diet-restricted and ad-lib feeding condition.

Non-neoplastic lesions comparison

Non-neoplastic lesions	Incidence (%)			
Feeding condition	Diet restricted		Ad-lib fed*	
Sex	Male	Female	Male	Female
Kidney: Chronic nephropathy	6	2	94	60
Heart: Myocardial fibrosis/cardiomyopathy	63	9	92	34
Liver: Bile duct hyperplasia	33	25	78	62
Prostate: Inflammation	14	-	76	-
Adrenal: Cortical hyperplasia	4	0	18	12
Thyroid: C-cell hyperplasia	10	17	34	52

*from T. Nagayabu et al. (2000)

DISCUSSION

It has been widely reported that moderate diet restriction (15 to 25% lower than ad-lib intake) results in increased longevity, reduced variability in body weights, delayed onset and reduced variability in the incidences of spontaneous tumors, and delayed onset and reduced severity of degenerative lesions in some different strains of rats, including Sprague- Dawley and F344 rats [8-15]. In this study, it was convincing that in the CD(SD)IGS rats, life-span diet optimization, restricting by daily food intake, can improve survival rate by controlling body weight changes, and by lowering the incidence of some spontaneous tumors and life-threatening non-neoplasitc lesions. Although, it has been reported that the incidences of spontaneous tumors, especially endocrine tumors, are significantly decreased by life-time food restriction [16], there are speculations that diet restriction may result in significant alterations in a test chemical's pharmacokinetics, metabolism, and toxicity [17]. Extensive comparative carcinogenicity and repeat-dose studies in rats under both ad-lib and restricted feeding conditions have concluded that moderate dietary restriction does not change the sensitivity, toxicity, or detection of carcinogenic potential of a test chemical [12-15]. This is further substantiated in the FDA's points-to-consider documents addressing the need for dietary control in chronic toxicity and carcinogenicity studies [18].

In conclusion, a dietary restriction rate of approximately 20-25% appears to be appropriate for CD(SD)IGS rats in chronic studies to improve survivability and would possibly be the best way to reduce variability in carcinogenicity studies in rats.

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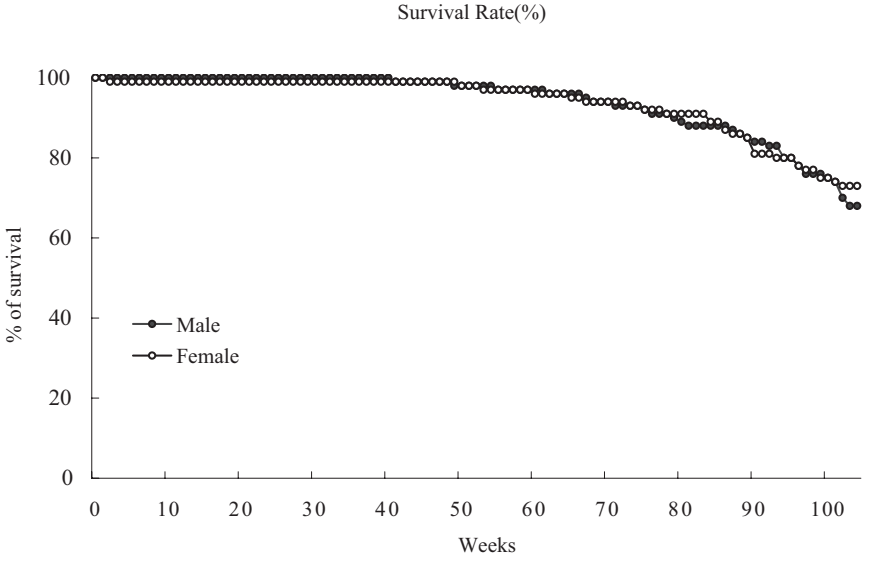

Fig. 1. Survival rates in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

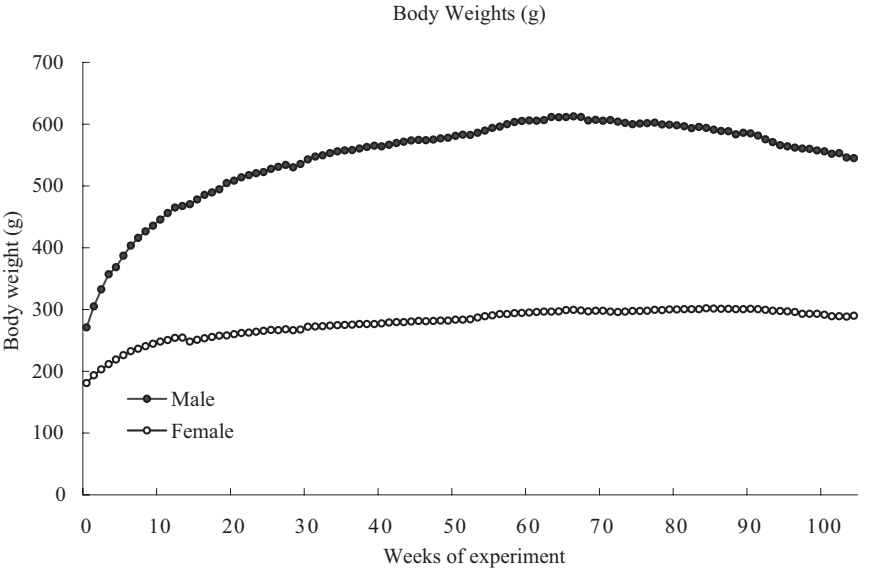

Fig. 2. Body weights in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

Table 1. Survival rates in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

Sex	Male	Female
No. of animals	100	100
Found dead	14	7
Moribund-killed	18	20
Total	32	27
Survival rate	68	73

Table 2. Mean values of hematological parameters in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

Sex	Male	Female
Number of animals	66	73
WBC ($\times 10^3/\mu$ L)	8.647±3.919	5.030±1.887
RBC ($\times 10^3/\mu$ L)	6.514±1.367	6.099±1.426
Hemoglobin (g/dL)	11.83±2.82	12.27±2.55
Hematocrit (%)	37.23±6.91	37.01±6.17
MCV (fL)	57.58±4.38	62.12±7.12
MCH (pg)	18.09±1.65	20.30±1.41
MCHC (%)	31.45±2.25	32.87±2.05
Platelet count ($\times 10^3/\mu$ L)	1348.1±311.2	1059.0±261.0
Reticulocyte count ($\times 10^4/\mu$ L)	34.596±16.179	23.158±15.684

Values were expressed as mean ± S.D.

Table 3. Mean values of blood chemistry parameters in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

Sex	Male	Female
Number of animals	66	73
Alp (mU/mL)	183.9±78.3	103.4±59.6
ALT (mU/mL)	45.3±24.2	44.0±36.1
AST (mU/mL)	121.0±48.0	109.4±78.5
γ -GT (mU/mL)	1.6±0.9	1.1±0.8
Total protein (g/dL)	5.94±0.59	6.57±0.63
Albumin (g/dL)	2.28±0.35	2.88±0.48
Globulin (g/dL)	3.66±0.44	3.68±0.48
A/G ratio	0.630±0.114	0.798±0.182
Urea nitrogen (mg/dL)	14.80±2.54	17.20±4.99
Creatinine (mg/dL)	0.316±0.048	0.348±0.075
Glucose (mg/dL)	101.6±22.1	97.5±18.9
Total cholesterol (mg/dL)	78.0±23.1	91.5±21.3
Phospholipid (mg/dL)	123.5±34.3	160.2±32.5
Triglyceride (g/dL)	59.4±35.3	41.9±23.5
Total bilirubin (mg/dL)	0.132±0.045	0.150±0.046
Na (mEq/L)	145.2±1.4	144.5±1.3
K (mEq/L)	4.22±0.42	3.65±0.56
Cl (mEq/L)	103.7±2.1	101.7±2.9
Inorganic Phosphorus (mg/dL)	5.05±0.64	5.01±0.87
Ca (mg/dL)	9.13±0.42	9.75±0.45

Values were expressed as mean ± S.D.

Table 4. Cause of death in Crj:CD(SD)IGS rats under chronic moderate dietary restriction

	Sex	Male	Female
Cause of death	No. of deaths	32	27
Neoplastic Lesion			
Pituitary tumor		5 (15.6)	13 (48.1)
Mammary gland tumor		1 (3.1)	6 (22.2)
Squamous cell carcinoma		1 (3.1)	0 (0.0)
Intestinal adenocarcinoma		1 (3.1)	0 (0.0)
Subcutanious fibroma		1 (3.1)	0 (0.0)
Histiocytic sarcoma		1 (3.1)	2 (7.4)
Malignant lymphoma/Leukemia		5 (15.6)	1 (3.7)
Malignant mesothelioma		0 (0.0)	1 (3.7)
Malignant schwannoma		1 (3.1)	0 (0.0)
Hemangiosarcoma		0 (0.0)	1 (3.7)
Liposarcoma		2 (6.3)	0 (0.0)
Rhabdomyosarcoma		1 (3.1)	0 (0.0)
Total		19 (59.3)	24 (88.9)
Non-Neoplastic Lesion			
Abscess		1 (3.1)	0 (0.0)
Esophagoectasis		1 (3.1)	0 (0.0)
Gastrointestinal failure		1 (3.1)	0 (0.0)
Liver necrosis		1 (3.1)	0 (0.0)
Nephropathy		0 (0.0)	1 (3.7)
Panarteritis nodosa		0 (0.0)	1 (3.7)
Thrombosis		1 (3.1)	0 (0.0)
Urogenital inflammation		6 (18.8)	0 (0.0)
Total		11 (34.3)	2 (7.4)
Undetermined		2 (6.3)	1 (3.7)

Number in parenthesis indicates the percentage

Table 5-1. Incidence of neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Integumentary System			
Mammary gland		(98)	(99)
Adenoma		0	10
Adenocarcinoma		0	22
Fibroadenoma		1	19
Skin		(100)	(99)
Basal cell adenoma		1	2
Squamous cell carcinoma		2	0
Subcutis		(5)	(3)
Fibroma		3	0
Fibrosarcoma		1	0
Hemangiosarcoma		0	1
Histiocytic sarcoma		0	1
Hematopoietic and Lymphatic System			
Spleen		(100)	(99)
Histiocytoma		1	0
Malignant lymphoma		2	0
Thymus		(100)	(99)
Histiocytic sarcoma		1	0
Malignant lymphoma		1	0
Mesenteric lymph node		(100)	(99)
Lymphangioma		2	2
Malignant lymphoma		2	0
Other lymph node		(0)	(3)
Histiocytic sarcoma		-	1
Bone marrow		(100)	(99)
Granulocytic leukemia		0	1
Musculoskeletal System			
Muscle		(1)	(0)
Rhabdomyosarcoma		1	0
Respiratory Tract			
Nasal cavity		(2)	(0)
Squamous cell carcinoma		1	-
Cardiovascular System			
Heart		(100)	(99)
Schwannoma		1	1
Digestive System			
Jejunum		(100)	(99)
Leiomyoma		0	1
Colon		(100)	(99)
Adenocarcinoma		1	0

( ) : Number of organs/tissues examined

Table 5-2. Incidence of neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Digestive System (continued)			
Liver		(100)	(99)
Hepatocellular adenoma		3	0
Pancreas		(100)	(99)
Islet-cell adenoma		8	1
Malignant schwannoma		1	0
Urinary System			
Kidneys		(100)	(99)
Adenoma		1	0
Lipoma		1	0
Liposarcoma		1	0
Ureter		(0)	(1)
Fibrosarcoma		-	1
Male Genital Tract			
Testes		(100)	(-)
Interstitial cell tumor		1	-
Prostate		(100)	(-)
Adenoma		1	-
Female Genital Tract			
Ovaries		(-)	(99)
Granulosa cell tumor		-	1
Uterus		(-)	(99)
Endometrial stromal polyp		-	8
Endometrial stromal sarcoma		-	1
Vagina		(-)	(99)
Vaginal polyp		-	2
Stromal sarcoma		-	2
Endocrine System			
Pituitary		(100)	(99)
Adenoma, pars distalis		33	59
Adenoma, pars intermedia		2	1
Adenocarcinoma, pars distalis		3	10
Craniopharyngioma		1	0
Adrenals		(100)	(99)
Cortical adenoma		0	2
Pheochromocytoma		14	2
Malignant pheochromocytoma		4	0
Complex pheochromocytoma		0	1

( ) : Number of organs/tissues examined

Table 5-3. Incidence of neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Endocrine System (continued)			
Thyroids		(100)	(99)
C-cell adenoma		3	3
C-cell carcinoma		1	0
Follicular adenoma		1	0
Follicular carcinoma		1	0
Parathyroids		(98)	(99)
Adenoma		4	0
Nervous System and Special Sense Organs			
Eyes		(100)	(99)
Malignant schwannoma		0	1
Harderian glands		(100)	(99)
Adenoma		0	1
Other Organs			
Thoracic wall		(3)	(0)
Lipoma		1	-
Abdominal cavity		(4)	(1)
Mesothelioma		1	0
Malignant mesothelioma		1	1
Liposarcoma		1	0

( ) : Number of organs/tissues examined

Table 6-1. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Integumentary System			
Mammary gland		(98)	(99)
Cyst		6	15
Ductal ectasia		2	2
Lobular hyperplasia		2	28
Arteritis		0	1
Atypical hyperplasia		0	3
Skin		(100)	(99)
Epidermal cyst		1	2
Inflammatory cell infiltration, cutis		1	1
Subcutis		(5)	(3)
Cyst		1	0
Abscess		0	1
Hematopoietic and Lymphatic System			
Spleen		(100)	(99)
Accessory spleen		0	1
Arteritis		0	1
Extramedullary hematopoiesis		34	32
Focal necrosis		0	1
Hyperplasia, myelopoietic cell		0	1
Lymphoid atrophy		2	1
Pigmentation		7	58
Stromal hyperplasia		1	0
Thymus		(100)	(97)
Atrophy		92	87
Hyperplasia, epithelial cell		0	8
Mesenteric lymph node		(100)	(99)
Arteritis		0	1
Cyst		1	0
Lymphadenitis		1	0
Lymphangiectasis		0	1
Lymphoid atrophy		2	0
Submaxillary lymph node		(100)	(98)
Cyst		1	0
Lymphoid atrophy		2	0
Lymphangiectasis		0	1
Plasma cell hyperplasia		1	0
Other lymph node		(20)	(3)
Abscess		1	0
Lymphangiectasis		3	0
Plasma cell hyperplasia		17	2
Femoral marrow		(100)	(99)
Fibrosis		1	0
Hyperplasia		37	25
Hyperplasia, granulocytic cell		0	2
Hyperplasia, myelopoietic cell		0	1
Hypoplasia, myelopoietic cell		0	1
Hypocellularity		1	0

( ) : Number of organs/tissues examined

Table 6-2. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Hematopoietic and Lymphatic System (continued)			
Sternal marrow		(99)	(99)
Fibrosis		1	4
Hyperplasia		39	25
Hyperplasia, granulocytic cell		0	2
Hyperplasia, myelopoietic cell		1	1
Hypocellularity		1	0
Musculoskeletal System			
Thigh muscle		(100)	(99)
Abscess		1	0
Arteritis		0	1
Inflammatory cell infiltration		0	1
Femur		(100)	(99)
Hyperostosis		1	0
Osteosis		1	0
Condromucinous degeneration		0	1
Sternum		(99)	(99)
Chondromucinous degeneration		5	8
Respiratory Tract			
Trachea		(100)	(99)
Inflammatory cell infiltration, submucosa		2	2
Lung		(100)	(99)
Alveolar hyperplasia, focal		2	0
Edema, alveolar		3	2
Foamy cell accumulation, alveolar, focal		19	31
Granuloma		0	1
Inflammatory cell infiltration, interstitial		1	0
Cardiovascular System			
Aorta		(99)	(99)
Inflammatory cell infiltration		1	0
Mineralization		3	0
Heart		(100)	(99)
Endocardial proliferation		0	1
Inflammatory cell infiltration, focal		1	0
Myocardial fibrosis		63	9
Myocardial necrosis, focal		2	1
Thickened, epicardium		1	0
Thrombosis		2	0
Digestive System(1)			
Tongue		(100)	(99)
Arteritis		0	1
Atrophy		1	0
Inflammatory cell infiltration, submucosa		1	0
Esophagus		(100)	(99)
No non-neoplastic lesions		-	-

( ) : Number of organs/tissues examined

Table 6-3. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Digestive System(1) (continued)			
Stomach		(100)	(99)
Arteritis		0	2
Cell debris, glandular stomach		1	0
Edema		2	0
Epithelial hyperplasia		1	0
Erosion, forestomach		1	0
Erosion, glandular stomach		7	5
Inflammatory cell infiltration, forestomach		1	0
Inflammatory cell infiltration, glandular stomach		0	1
Mineralization, mucosa		1	0
Ulcer, forestomach		5	0
Duodenum		(100)	(99)
Arteritis		0	1
Jejunum		(100)	(99)
Inflammatory cell infiltration, mucosa		0	1
Ileum		(100)	(99)
No non-neoplastic lesions		-	-
Colon		(100)	(99)
Arteritis		0	1
Hemorrhage, serosa		1	0
Inflammatory cell infiltration, serosa		1	0
Rectum		(100)	(99)
Abscess		1	0
Arteritis		0	1
Cecum		(100)	(99)
Arteritis		0	1
Erosion		1	0
Inflammatory cell infiltration, mucosa		1	0
Inflammatory cell infiltration, submucosa		1	1
Digestive System(2)			
Liver		(100)	(99)
Altered cell foci		39	30
Anisonucleosis, hepatocytes		0	1
Biliary cyst		0	1
Bile duct hyperplasia		33	25
Cirrhosis		1	0
Cyst		0	1
Cystic degeneration		0	1
Extramedullary hematopoiesis		7	15
Fibrosis, focal		1	0
Focal necrosis, hepatocytes		3	0
Necrosis, hepatocytes, centrilobular		2	1
Peliosis hepatis		9	15
Single cell necrosis, hepatocytes		0	1
Thickened, capsule		1	1
Vacuolation, hepatocytes, periportal		5	1

( ) : Number of organs/tissues examined

Table 6-4. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Digestive System(2) (continued)			
Pancreas		(100)	(99)
Acinar cell atrophy, diffuse		1	1
Acinar cell atrophy, focal		12	4
Arteritis		0	1
Basophilic foci		2	0
Focal fibrosis		1	0
Islet-cell hyperplasia		5	3
Lipomatosis		1	0
Vacuolation, acinar cells		0	1
Periarteritis		1	0
Submaxillary glands		(100)	(99)
Acinar atrophy, bilateral		2	0
Sublingual glands		(100)	(99)
No non-neoplastic lesions		-	-
Urinary System			
Kidneys		(100)	(99)
Abscess		1	0
Arteritis		0	1
Atypical hyperplasia		3	2
Chronic nephropathy		6	2
Cyst, cortex		0	3
Dilatation, pelvis		1	0
Focal fibrosis		0	2
Glomerular sclerosis		2	0
Hemorrhage		1	0
Hyaline droplet		1	1
Hydronephrosis		0	1
Infarct		2	1
Inflammatory cell infiltration, focal		1	0
Inflammatory cell infiltration, pelvis		3	1
Mineralization		6	48
Mononuclear cell infiltration, focal		1	0
Necrosis, papilla		3	0
Pigmentation		1	0
Pyelitis		4	0
Simple hyperplasia		9	21
Tubular basophilia		1	0
Tubular dilatation		2	1
Urinary bladder		(100)	(99)
Cystitis		4	0
Hemorrhage		1	0
Hemorrhage, submucosa		1	0
Inflammatory cell infiltration, submucosa		3	0
Mineralization		1	0
Ureter		(2)	(0)
Ulcer		1	-
Inflammatory cell infiltration		1	-

( ) : Number of organs/tissues examined

Table 6-5. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Male Genital Tract			
Prostate		(100)	(-)
Abscess		1	-
Acinar atrophy		8	-
Acinar hyperplasia		3	-
Hemorrhage		2	-
Inflammation		14	-
Seminal vesicles		(100)	(-)
Atrophy		9	-
Hemorrhage		1	-
Inflammation		3	-
Coagulating glands		(100)	(-)
Atrophy		8	-
Hemorrhage		1	-
Inflammation		4	-
Testes		(100)	(-)
Atrophy, seminiferous tubules		13	-
Degeneration, seminiferous epithelium		3	-
Edema, unilateral		2	-
Hypocellularity, seminiferous epithelium		2	-
Epididymides		(100)	(-)
Arteritis		1	-
Decreased spermatozoa		10	-
Desquamated seminiferous epithelial cells		8	-
Granuloma		1	-
Mononuclear cell infiltration, interstitium, focal		1	-
Spermatic granuloma		1	-
Vacuolation, epithelium		3	-
Female Genital Tract			
Ovaries		(-)	(99)
Arteritis		-	1
Cyst		-	15
Uterus		(-)	(99)
Arteritis		-	1
Cyst		-	4
Vagina		(-)	(99)
Arteritis		-	1
Granuloma		-	1
Endocrine System			
Pituitary		(100)	(99)
Angiectasis, pars intermedia		1	0
Cyst, pars distalis		7	2
Cyst, pars intermedia		8	0
Hyperplasia, focal, pars distalis		12	14
Telangiectasis, pars intermedia		0	1

( ) : Number of organs/tissues examined

Table 6-6. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic dietary restriction

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Endocrine System (continued)			
Adrenals		(100)	(99)
Arteritis		0	1
Cortical hyperplasia		4	0
Cortical hypertrophy		26	4
Cyst, cortex		1	0
Cystic degeneration		34	89
Fibrosis		1	0
Focal necrosis		1	0
Hemorrhage		1	0
Medullary hyperplasia		51	25
Vacuolation, cortex		1	0
Thyroids		(100)	(99)
C-cell hyperplasia		10	17
Cyst		1	0
Follicular atrophy		1	0
Follicular hyperplasia		2	0
Parathyroids		(98)	(94)
Cyst		1	0
Hyperplasia, diffuse		5	1
Hyperplasia, focal		6	4
Nervous System and Special Sense Organs			
Brain		(100)	(99)
Gliosis		1	0
Hemorrhage		0	1
Vacuolation, focal		1	0
Spinal cord		(100)	(99)
No non-neoplastic lesions		-	-
Sciatic nerve		(99)	(98)
Inflammatory cell infiltration, focal		1	1
Mononuclear cell infiltration, perineurium		0	1
Eyes		(100)	(99)
Cataract		2	2
Granuloma		1	0
Inflammatory cell infiltration, focal		1	0
Uveitis		1	0
Optic nerves		(99)	(99)
No non-neoplastic lesions		-	-
Harderian glands		(100)	(99)
Abscess		1	0
Acinar atrophy, bilateral		1	0
Hyperplasia, focal, unilateral		1	0
Inflammatory cell infiltration, unilateral		1	0

( ) : Number of organs/tissues examined

Table 6-7. Incidence of non-neoplastic lesions in Crj:CD(SD)IGS rats under chronic diet optimization

Organs	Sex	Male	Female
Findings	No.of animals	100	100
Other Organs			
Limb		(81)	(32)
Abscess		1	0
Edema		1	0
Hyperkeratosis		81	32
Ulcer		81	32
Peritoneum		(1)	(0)
Inflammation		1	-
Thoracic wall		(3)	(0)
Abscess		1	-
Nasal cavity		(2)	(0)
No non-neoplastic lesions		-	-
Tail		(42)	(21)
Abscess		25	12
Edema		1	0
Epidermal cyst		13	7
Fibrosis		5	0
Inflammatory cell infiltration		10	4
Preputial/Clitoral glands		(2)	(0)
Abscess		2	-

( ) : Number of organs/tissues examined

Survival Rate, Body Weight and Tumor Data in Crj:CD(SD)IGS Rats Fed Low Protein Commercial Diet CR-LPF

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ABSTRACT. Survival rate, body weight and tumor data of a recently terminated carcinogenicity study with two identical control groups were compared to those in our previous report. The survival rate in the present study was markedly decreased in males and slightly decreased in females as compared to that in the previous report. The body weight in both sexes in the present study was slightly higher than in the previous report. In the tumor data, increased incidences in anterior adenoma in the pituitary of both sexes, islet cell adenoma in the pancreas of males and fibroadenoma in the mammary gland of females were observed. However, the incidences of other tumors did not have great difference as compared to those in the previous report.
－Key words: Crj:CD(SD)IGS rats, low protein diet, tumor data, 104-week observation period

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INTRODUCTION

In the previous papers [1, 2], the mortality, body weight and histopathological data of Crj:CD(SD)IGS rats, those reared for 104 weeks fed low protein commercial diet (CR-LPF), were reported. Recently, we conducted a carcinogenicity study with two identical control groups in Crj:CD(SD)IGS rats fed CR-LPF. In the present paper, the mortality, body weight and tumor data are in the carcinogenicity study are compared with those in the previous report. In addition, tumor data of Crj:CD(SD) IGS rats, those reared for 104 weeks fed relatively high protein commercial diet (CRF-1) are also described.

MATERIALS AND METHODS

Animals and Husbandry: In the previous report (experiment-I), 50 male and female Crj:CD(SD)IGS rats, at 4 weeks of age, were obtained twice on different dates (Lot 1: October 2, 1996, Lot 2: October 9, 1996) from Charles River Japan Inc. (Hino Breeding Center, Japan). In total, 100 males and 100 females were used in the above study. For each lot, the animals were acclimatized for 2 weeks and healthy animals were used at 6 weeks of age. The animals were housed individually in hanging stainless-steel wire mesh cages in an animal room under the following conditions: temperature at 23 ± 3 ℃, relative humidity at 50 ± 20%, air ventilation at 10 to 15 times per hour and 12-hour illumination (07:00 to 19:00). The animals were supplied commercial low protein feed (approximately 18% crude protein), CR-LPF (Oriental Yeast Co., Ltd., Japan), and tap water *ad libitum*. In a recent carcinogenicity study (experiment-II), about 440 of each sex of Crj:CD(SD)IGS rats, at 4 weeks of age, were obtained (November 15, 1999) from Charles River Japan Inc. (Atsugi Breeding Center, Japan) and two identical control groups (60 animals/sex/group) were provided and used at 6 weeks of age. These animals received 0.5 w/v% methylcellulose solution (5 mL/kg body weight) by gavage using a stomach tube for 104 weeks. Other conditions were the same as those in the previous report. In other previous study (experiment-III), 75 male and female Crj:CD(SD) IGS rats, at 4 weeks of age, were obtained (July 27, 1998) from Charles River Japan Inc. (Hino Breeding Center, Japan). The animals were housed in the same manner in the above studies, excepting that they were supplied ordinary commercial diet CRF-1 (approximately 23% crude protein).
Body weight: The body weight was recorded once weekly.
Histopathology: The animals were sacrificed at the termination of a 104-week observation period. The animals found dead or sacrificed moribund were necropsied soon after discovery. After necropsy, all organs and tissues of all animals were dissected and fixed in phosphate buffered 10 vol% formalin. The eyeballs, Harderian glands and optic nerves were fixed with phosphate buffered fixative containing 3 w/v% glutaraldehyde and 2.5 vol% formalin, and then preserved in phosphate buffered 10 vol% formalin. All organs/tissues (approximately 50 per animal) were embedded in paraffin, stained with hematoxylin and eosin (H.E.) by a routine procedure and examined histopathologically.

Survival rate: The survival rates in IGS rats fed CR-LPF are summarized in the following Table 1.

The terminal survival rate in male IGS rats in the previous report (experiment-I) was 54.0% in males and 49.0% in females. On the other hand, those in the recent carcinogenicity study (experiment-II) was 30.0% in males in both the control-I and control-II and 35.0% in females in the control-I and 40.0% in females in the control-II; therefore, markedly decreased survival rate in males and slightly decreased survival rate in females were noted as compared to those in the previous report.
Body weight: The changes in body weight in IGS rats fed CR-LPF are summarized in the following Table 2.

The mean terminal body weight in the previous report and the control-I and control-II in the present carcinogenicity study was 716, 735 and 796 g for males and 446, 481 and 502 for females, respectively, and the body weight in the previous study was the slightly lower than other two group for both sexes.
Cause of death: The cause of demise in IGS rats fed CR-LPF are summarized in the following Table 3. The cause of death was assigned based on the clinical signs and gross/histopathology. A high mortality in the experiment-II (70% in the control-I and 70% in the control-II for males and 65% in the control-I

Table 1. Summary of the survival rates of Crj:CD(SD)IGS rats fed CR-LPF.

Sex	Male			Female		
Experiment No.	I	II		I	II	
		Control-I	Control-II		Control-I	Control-II
No. of animals used	100	60	60	100	60	60
Week of experiment						
0(initial)	100[a)]	100	100	100	100	100
26	100	100	98.3	100	98.3	98.3
52	99.0	96.7	91.7	98.0	95.0	96.7
78	87.0	83.3	71.7	85.0	73.3	83.3
91	81.0	63.3	53.3	69.0	51.7	61.7
104	54.0	30.0	30.0	49.0	35.0	40.0

a): Survival rate (%)

Table 2. Summary of body weight changes in Crj:CD(SD)IGS rats fed CR-LPF.

Sex	Male			Female		
Experiment No.	I	II		I	II	
		Control-I	Control-II		Control-I	Control-II
No. of animals used	100	60	60	100	60	60
Week of experiment						
0 (initial)	183±7[a)]	216±14	215±12	141±7	172±10	172±11
26	585±57	655±80	686±77	306±33	336±36	340±42
52	669±79	762±121	798±115	361±47	422±58	435±75
78	730±99	825±141	836±136	419±65	482±95	489±113
91	737±103	800±162	807±170	444±80	500±93	521±135
104	716±110	735±138	796±138	446±88	481±91	502±136
Gain (104 weeks)	533±109	517±134	583±181	304±86	309±87	333±133

a): Mean±S.D. (unit: g)

and 60% in the control-II for females) as compared to that in the previous report (53% for males and 49% for females) was considered to be attributable to increases in death of pituitary tumor in both sexes, deaths of subcutaneous tumor and chronic nephropathy and unclear death in males and death of mammary gland tumor in females. Above increased cause of death except unclear death may be related to increased body weight.

Tumors: The tumors observed in 3 experiments are shown in Table 2. The incidence of almost all tumors did not have great difference among 3 experiments.

However, the incidences of some tumors in the experiment-II was higher than those in the experiment-I as shown in the following.

Males

islet cell adenoma in the pancreas (38 - 40% in experiment-II vs. 10 - 16% in experiment-I)

anterior adenoma in the pituitary (68 - 73% in experiment-II vs. 47 - 53% in experiment-I)

Females

fibroadenoma in the mammary gland (40 - 53% in experiment-II vs. 37 - 43% in experiment-I)

anterior adenoma in the pituitary (80 - 81% in experiment-II vs. 60 - 69% in experiment-I)

In addition, the incidence of anterior adenoma in the pituitary in the experiment-III was higher than that in the experiment-I as shown in the following.

Females

anterior adenoma in the pituitary (76% in experiment-III vs. 60 - 68% in experiment-I)

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Table 3. Cause of death in IGS rats fed CR-LPF

.	Experiment No	I	II	
			Control-I	Control-II
Cause of demise	No. of animals	100	60	60
Male				
No. of deaths		47[a)]	42	42
Tumor				
ituitary tumor		15(31.9)[b)]	14(33.3)	18(42.9)
Leukemia		4(8.5)	1(2.4)	0(0.0)
Malignant lymphoma		0(0.0)	0(0.0)	1(2.4)
Histiocytic sarcoma		2(4.2)	1(2.4)	1(2.4)
Liposarcoma		2(4.2)	0(0.0)	0(0.0)
Zymbal gland tumor		2(4.2)	0(0.0)	0(0.0)
Malignant mesothelioma		2(4.2)	0(0.0)	0(0.0)
Islet cell adenocarcinoma		2(4.2)	0(0.0)	0(0.0)
Pheochromocytoma		1(2.1)	1(2.4)	0(0.0)
Keratoacanthoma, oral cavity		1(2.1)	0(0.0)	0(0.0)
Hemangiosarcoma		1(2.1)	0(0.0)	0(0.0)
Heart tumor		0(0.0)	0(0.0)	1(2.4)
Fibro sarcoma		1(2.1)	0(0.0)	0(0.0)
Prostate tumor		0(0.0)	1(2.4)	0(0.0)
Renal tumor		0(0.0)	0(0.0)	2(4.8)
Subcutaneous tumor		0(0.0)	3(7.1)	3(7.1)
Squamous cell carcinoma, oral cavity		0(0.0)	0(0.0)	1(2.4)
Total deaths of tumor		33(70.2)	21(50.0)	27(64.3)
Non-tumor				
Hemorrhage, trauma		2(4.2)	0(0.0)	0(0.0)
Urination disturbance		2(4.2)	1(2.4)	1(2.4)
Subcutaneous hematoma		1(2.1)	0(0.0)	0(0.0)
Chronic nephropathy		1(2.1)	4(9.5)	3(7.1)
Cyst, abdominal cavity		1(2.1)	0(0.0)	0(0.0)
Circulatory disturbance		3(6.3)	1(2.4)	0(0.0)
Brain lesion		0(0.0)	1(2.4)	0(0.0)
Foot lesion		0(0.0)	2(4.8)	0(0.0)
Liver lesion		0(0.0)	1(2.4)	0(0.0)
Lung lesion		0(0.0)	0(0.0)	1(2.4)
Total deaths of non-tumor lesions		10(21.3)	10(23.8)	5(11.9)
Gavage error		-(-)	0(0.0)	1(2.4)
Unclear		4(8.5)	11(26.2)	9(21.4)
Female				
No. of deaths		51	40	36
Tumor				
Pituitary tumor		43(84.3)	29(72.5)	27(75.0)
Leukemia		1(1.9)	0(0.0)	0(0.0)
Malignant lymphoma		0(0.0)	2(5.0)	1(2.8)
Histiocytic sarcoma		2(3.9)	0(0.0)	0(0.0)
Mammary gland tumor		2(2.0)	3(7.5)	6(16.7)
Adrenocortical tumor		0(0.0)	1(2.5)	0(0.0)
Thymic lymphoma		1(1.9)	0(0.0)	0(0.0)
Urerine tumor		0(0.0)	1(2.5)	0(0.0)
Vaginal tumor		0(0.0)	1(2.5)	0(0.0)
Subcutaneous tumor		0(0.0)	2(5.0)	0(0.0)
Squamous cell carcinoma, stomach		1(1.9)	0(0.0)	0(0.0)
Total deaths of tumor		50(98.0)	39(97.5)	34(94.4)
Non-tumor				
Chronic nephropathy		0(0.0)	1(2.5)	0(0.0)
Cyst, abdominal cavity		0(0.0)	0(0.0)	0(0.0)
Circulatory disturbance		1(1.9)	0(0.0)	0(0.0)
Tooth abnormality		0(0.0)	0(0.0)	1(2.8)
Total deaths of non-tumor lesions		1(1.9)	1(2.5)	1(2.8)
Gavage error		-(-)	0(0.0)	0(0.0)
Unclear		0(0.0)	0(0.0)	1(2.8)

a) : One male that died during the necropsy period was excluded from calculation of the survival rate.
b) : Number in parenthesis indicates the percentage (%).

Table 4. Tumors in IGS rats fed CR-LPF or CRF-1.

	Diet	CR-LPF										CRF-1	
	Experiment No.	I		II		I		II				III	
		Lot 1	Lot 2	Control-I	Comtrol-II	Lot 1	Lot 2	Control-I	Comtrol-II	TOTAL			
Tissue	Sex:	Male	Male	Males	Males	Females	Females	Female	Female	Male	Female	Male	Female
Observation	Number:	50	50	60	60	50	50	60	60	220	220	75	75
Abdominal cavity													
Number examined		50	50	60	60	50	50	60	60	220	220	75	75
LIPOSARCOMA		0	1	0	0	0	0	0	0	1	0	0	0
MESOTHELIOMA, MALIGNANT		3	0	1	0	0	0	0	0	4	0	0	0
Adrenal													
Number examined		50	49	60	59	49	50	60	60	218	219	75	75
ADENOMA, CORTICAL CELL		0	2	2	0	1	1	0	4	4	6	0	1
PHEOCHROMOCYTOMA		5	9	7	4	0	1	4	4	25	9	10	5
CARCINOMA, CORTICAL CELL		1	0	0	0	0	0	1	0	1	1	0	0
Brain													
Number examined		50	50	60	60	50	50	60	60	220	220	72	74
GRANULAR CELL TUMOR		1	0	0	0	0	0	0	0	1	0	2	0
MENINGIOMA		0	0	1	0	0	0	0	0	1	0	0	0
OLIGODENDROGLIOMA		1	0	0	0	0	0	0	0	1	0	0	0
ASTROCYTOMA		0	0	2	1	0	0	0	0	3	0	3	0
Ear													
Number examined		50	50	60	60	50	50	60	60	220	220	75	75
NEURAL CREST TUMOR		0	1	0	0	0	0	0	0	1	0	1	0
Hemolymphoreticular													
Number examined		50	50	60	60	50	50	60	60	220	220	75	75
LYMPHOMA, MALIGNANT		0	0	1	1	0	0	2	1	2	3	0	0
SARCOMA, HISTIOCYTIC		2	1	2	1	1	1	0	1	6	3	0	0
LEUKEMIA, GRANULOCYTIC		1	1	1	0	0	0	0	0	3	0	0	0
LEUKEMIA, LYMPHOCYTIC		1	1	0	0	1	0	0	0	2	1	0	0
Small intestine, jejunum													
Number examined		48	48	46	52	47	46	53	57	194	203	67	67
LEIOMYOMA		1	0	0	0	0	1	0	0	1	1	0	0
ADENOCARCINOMA		1	0	0	0	0	0	0	0	1	0	0	0
Heart													
Number examined		50	50	60	60	50	48	60	60	220	218	75	75
MESOTHELIOMA, MALIGNANT, ATRIOCAVAL		0	0	0	1	0	0	0	0	1	0	0	0
Kidney													
Number examined		48	48	60	60	47	48	60	60	216	215	74	74
LIPOMA		1	0	1	1	0	0	0	0	3	0	0	0
LIPOSARCOMA		1	0	0	0	0	0	0	0	1	0	0	0
SCHWANNOMA, MALIGNANT		0	0	0	1	0	0	0	0	1	0	0	0
Liver													
Number examined		50	50	60	60	50	49	60	60	220	219	75	75
ADENOMA, HEPATOCELLULAR		1	1	0	3	0	0	1	1	5	2	3	3
CYSTADENOMA		0	0	0	0	3	3	0	0	0	6	0	1
ADENOMA, HEPATOCELLULAR		0	0	0	0	0	0	0	0	0	0	1	0
Lymph node, mesenteric													
Number examined		50	48	60	60	46	49	60	60	218	215	72	73
HEMANGIOMA		0	0	2	1	0	0	0	0	3	0	0	0
LYMPHANGIOMA		0	0	0	1	0	0	0	0	1	0	0	0
HEMANGIOSARCOMA		0	0	0	0	0	0	0	0	0	0	1	0
Mammary gland													
Number examined		50	50	60	60	49	49	60	60	220	218	66	75
ADENOMA, ACINAR CELL		0	0	1	0	3	0	1	1	1	5	0	1
FIBROADENOMA		0	0	1	0	21	18	32	24	1	95	0	25
FIBROMA		0	0	0	0	0	0	0	0	0	0	2	0
CARCINOMA, ACINAR CELL		0	0	0	0	13	16	13	16	0	58	0	23
HEMANGIOSARCOMA		0	0	0	0	0	0	0	1	0	1	0	0
Nasal cavity													
Number examined		50	50	0	0	50	50	0	0	100	100	0	0
SCHWANNOMA, MALIGNANT		0	1	0	0	0	0	0	0	1	0	0	0
Oral cavity													
Number examined		1	0	0	1	1	0	0	1	2	2	0	0
KERATOACANTHOMA		1	0	0	0	0	0	0	0	1	0	0	0
CARCINOMA, SQUAMOUS CELL		0	0	0	1	1	0	0	0	1	1	0	0
Ovary													
Number examined		-	-	-	-	50	49	60	60	-	219	-	75
GRANULOSA-THECA CELL TUMOR, BENIGN		-	-	-	-	1	0	0	0	-	1	-	0
CYSTADENOCARCINOMA		-	-	-	-	0	1	0	0	-	1	-	0

Table 4. Tumors in IGS rats fed CR-LPF or CRF-1.

	Diet	CR-LPF										CRF-1	
	Experiment No.	I		II		I		II				III	
		Lot 1	Lot 2	Control-I	Comtrol-II	Lot 1	Lot 2	Control-I	Comtrol-II	TOTAL			
Tissue	Sex:	Male	Male	Males	Males	Females	Females	Female	Female	Male	Female	Male	Female
Observation	Number:	50	50	60	60	50	50	60	60	220	220	75	75
Pancreas													
Number examined		49	49	60	60	48	50	60	60	218	218	75	75
ADENOMA, ACINAR CELL		1	3	2	4	0	0	1	1	10	2	2	0
ADENOMA, ISLET CELL		8	5	23	24	1	3	6	4	60	14	6	3
CARCINOMA, ACINAR CELL		0	1	0	0	0	0	0	0	1	0	0	0
CARCINOMA, ISLET CELL		4	7	1	2	0	2	0	1	14	3	6	2
HEMANGIOSARCOMA		0	0	0	0	0	0	0	0	0	0	0	1
Pituitary													
Number examined		47	49	59	60	50	49	60	60	215	219	75	75
ADENOMA, ANTERIOR		25	23	40	44	30	34	49	48	132	161	38	57
CARCINOMA, ANTERIOR		1	2	0	0	11	8	2	3	3	24	12	6
Prostate													
Number examined		50	49	60	60	-	-	-	-	219	-	75	-
ADENOMA, ACINAR CELL		0	1	0	0	-	-	-	-	1	-	1	-
CARCINOMA, ACINAR CELL		0	0	1	0	-	-	-	-	1	-	0	-
Skin/subcutis													
Number examined		50	50	60	60	49	49	60	60	220	218	75	74
ADENOMA, SEBACEOUS		1	1	1	0	0	0	0	1	3	1	0	0
FIBROMA		1	0	6	1	1	1	0	0	8	2	1	1
KERATOACANTHOMA		1	3	1	0	0	0	0	1	5	1	0	0
LIPOMA		1	0	2	0	1	0	0	2	3	3	1	2
FIBROSARCOMA		0	0	2	2	0	0	1	0	4	1	0	0
HEMANGIOSARCOMA		1	0	0	0	0	0	0	0	1	0	0	0
HEMANGIOPERICYOMA, MALIGNANT		0	0	0	1	0	0	0	0	1	0	0	0
LIPOSARCOMA		0	1	0	0	0	0	0	0	1	0	2	0
PAPILLOMA, SQWAMOUS CELL		0	0	0	2	0	0	0	0	2	0	0	0
CARCINOMA, SQWAMOUS CELL		0	0	0	1	0	0	0	0	1	0	0	0
OSTEOSARCOMA		0	0	0	1	0	0	0	0	1	0	0	0
SARCOMA, NOS		0	0	0	0	0	0	2	0	0	2	0	0
Spinal cord													
Number examined		50	49	60	60	50	50	60	60	219	220	74	74
ASTROCYTOMA		0	0	0	0	0	0	0	0	0	0	1	0
ASTROCYTOMA, MALIGNANT		0	0	0	1	0	0	0	0	1	0	0	0
Stomach													
Number examined		50	49	60	60	49	50	60	60	219	219	75	74
CARCINOMA, SQUAMOUS CELL		1	0	1	0	0	0	0	0	2	0	0	0
TERATOMA, MALIGNANT		0	0	0	0	1	0	0	0	0	1	0	0
Testis													
Number examined		50	50	60	60	-	-	-	-	220	-	74	-
LEYDIG CELL TUMOR		3	0	3	0	-	-	-	-	6	-	1	-
Thymus													
Number examined		41	45	60	60	47	48	59	58	206	212	67	73
THYMIC LYMPHOMA		0	0	0	0	0	1	0	0	0	1	0	0
Tongue													
Number examined		50	50	60	60	50	49	60	60	220	219	75	75
GRANULAR CELL TUMOR		0	0	0	0	0	1	0	0	0	1	0	0
Thyroid													
Number examined		48	50	41	46	50	50	53	57	185	210	75	75
ADENOMA, C CELL		1	3	6	2	3	2	2	3	12	10	1	1
ADENOMA, FOLLICULAR CELL		1	1	0	1	1	0	0	0	3	1	2	2
CARCINOMA, C CELL		0	0	0	1	0	0	0	3	1	3	0	0
CARCINOMA, FOLLICULAR CELL		0	0	0	0	1	0	0	0	0	1	1	0
Uterus													
Number examined		-	-	-	-	50	50	60	60	-	220	-	74
GRANULAR CELL TUMOR		-	-	-	-	0	0	1	2	-	3	-	0
POLYP, ADENOMATOUS		-	-	-	-	0	0	1	1	-	2	-	0
POLYP, ENDOMETRIAL STROMAL		-	-	-	-	1	9	5	3	-	18	-	6
ADENOCARCINOMA		-	-	-	-	0	1	0	0	-	1	-	1
SARCOMA, NOS		-	-	-	-	1	0	0	0	-	1	-	0
Zymbal gland													
Number examined		50	50	0	0	50	50	0	0	100	100	0	0
ADENOMA		1	0	0	0	0	0	0	0	1	0	0	0
CARCINOMA		2	1	0	0	0	0	0	0	3	0	0	0

CHAPTER 5

Information

Comparative Genetic Monitoring of Crl:CD®(SD)IGS BR Rat Colonies Worldwide

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ABSTRACT. The frequencies of satellite DNA markers in 140 Crl:CD®(SD)IGS BR outbred rats representing 7 separate Charles River colonies from around the world were analyzed to assess heterozygosity and genetic divergence among colonies. Microsatellite allele frequencies were remarkably consistent in the IGS foundation and production colonies. This supports the conclusion that genetic divergence among CD rat colonies, by this analysis, is minimal. The microsatellite panel used in this evaluation consisted of just 6 loci on 4 chromosomes. Additional loci represented by a much greater number of microsatellites could be used to further refine the analysis of allele frequencies and is currently being pursued as a method for regular monitoring of genetic drift. Analyses such as these coupled with phenotypic data and sharing of control data through mechanisms such as the IGS study group further enable the ongoing assessment of the CD IGS production colonies. DNA fingerprints of minisatellite markers showed a high degree of band sharing. Therefore, monitoring of minisatellite markers is not an especially sensitive method for detecting genetic drift among CD rat colonies. —Key words: Crl:CD®(SD)IGS BR, rat, genetic monitoring, worldwide

CD(SD)IGS-2002 /2003: 231-238

INTRODUCTION

While inbred strains of rats and mice, as well as certain transgenic lines, have been utilized in the drug discovery process and in other biomedical research studies, outbred rodent stocks still represent approximately 75% rodents used in research and are especially popular to toxicology. Outbred rodent stocks are valuable to the biomedical research community, because like people (and in contrast to inbred strains) they are characterized by genotypic and phenotypic variation. That is, no two outbred rats are expected to be genetically the same. Outbreds are more vigorous than inbreds because heterozygosity masks detrimental recessive mutations.

The Crl:CD®(SD)IGS BR is the principal outbred rat stock used in toxicology worldwide. The stock was started with rats acquired from Sprague Dawley Inc., in 1950. These SD rats were Cesarean-derived in 1955, after which CD was added to the stock nomenclature. BR refers to barrier-reared and has begun to fall out of favor as part of the nomenclature. In all likelihood, this portion of the designation will be dropped in the near future. Charles River Laboratories developed the international genetic management system (IGS) for the CD in 1992 to address the concerns of researchers that the lifespan of the CD and other rat stocks was decreasing, possibly because of genetic drift. The letters IGS were added to the nomenclature during the implementation of this genetic management system as a means of distinguishing CD stock being managed in this fashion from those not managed under this system. By 1997, the IGS was in place in all Charles River CD production rooms worldwide. Since then other outbred stocks of rats and mice have been brought under a similar management system [1].

Simply put, the purpose of the IGS system for outbred rodent stocks is to preserve heterozygosity and limit genetic divergence among colonies and within a colony over time.

Loss of heterozygosity, or inbreeding, and genetic divergence inevitably occur because of mutation, selection (whether intentional or not) and most importantly, random genetic drift due to sampling error. Random genetic drift occurs because not every individual in a generation contributes equally to the next. From an essentially infinite population of eggs and sperm, only a few will by chance combine to create the next generation of animals. Moreover, as gametes are haploid, the likelihood of inheriting a parental allele is just 50% [6].

Heterozygosity is preserved by:

- Avoiding genetic bottlenecks.
- pt Employing rotational breeding systems [3].
- Migrating animals among colonies [6].

Inbreeding and genetic divergence are worsened when a colony is founded and maintained with a small number of breeding pairs. To prevent genetic bottlenecks (i.e., severe temporary reduction in the number of animals), we have chosen to start new outbred rodent colonies with at least 200 breeders, selected from 200 separate matings (Figure 1).

Rotational breeding schemes are employed to further prevent inbreeding (Figure 2). In CD rat colonies, a circular population breeding scheme that is composed of three blocks (A, B and C) is utilized such that female breeders are selected in one block are rotated to the next to set up new mating pairs. Male breeders are not rotated; they remain in the block where they were born and are used to set up new breeding pairs. Animals not used for breeding from each of the lines are combined as stock for sale and use in biomedical research. To eliminate the possibility that a male would mate with his mother, male replacement breeders are selected from the 3rd to the 5th litter only. When choosing future breeders, only one animal per litter is selected to avoid any inadvertent brother x sister matings.

Random genetic drift and other factors can be expected to cause at least moderate genetic divergence among outbred colonies unless steps are taken to migrate animals. Colony divergence can be controlled through migration, which refers to the movement of individuals among colonies. Migration acts as a "glue" that sets a limit on the amount of genetic divergence that can occur, but has rarely been practiced by others because of the potential for transferring microbial contamination along with the migrating animals. This has been overcome by utilizing a highly secure, isolator-maintained foundation colony

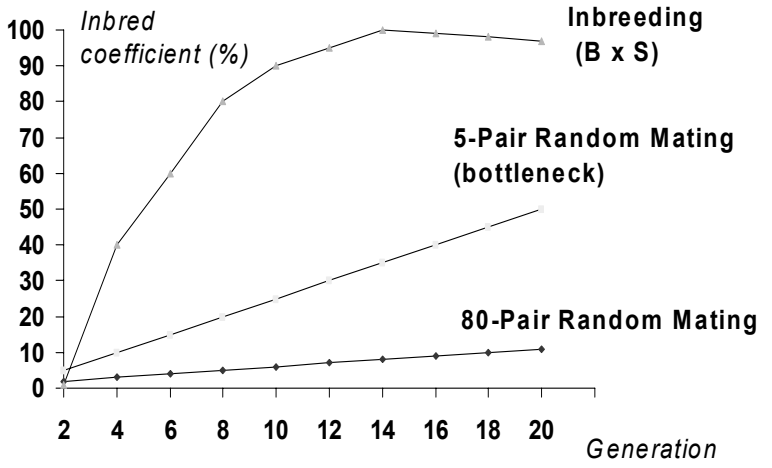

Fig.1 Coefficient of Inbreeding with different Colony Size and Mating Sytems

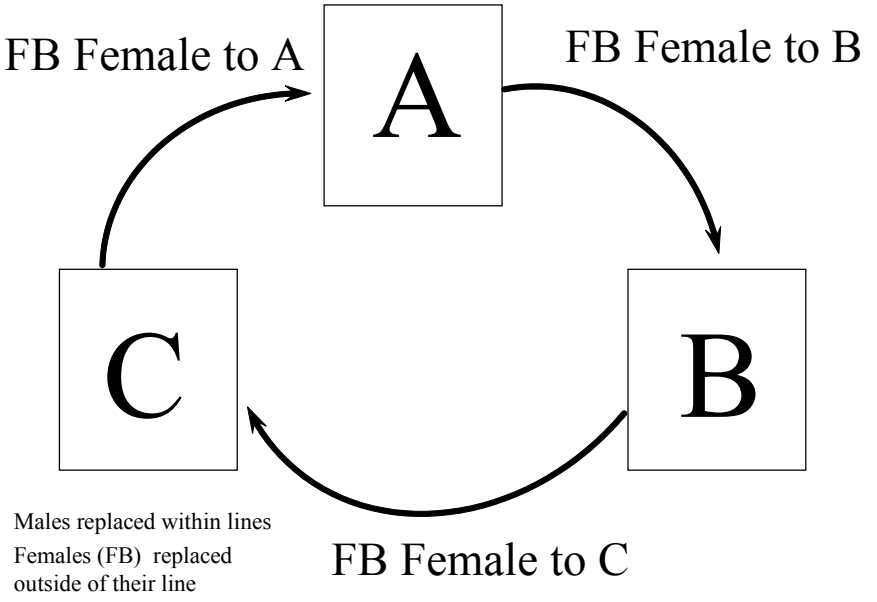

Fig 2. Rotational Breeding Scheme For IGS Rats

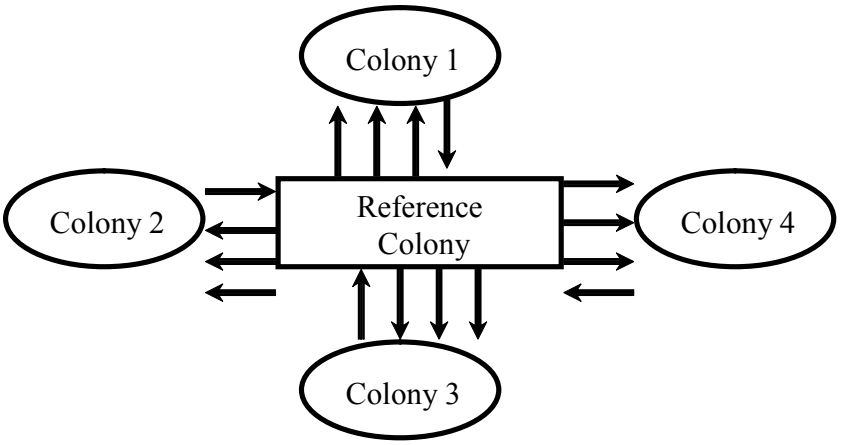

Fig.3 Regular Forward and Backward Migration Using a Reference Colony System

system whose health status is assured. In the case of the CD rat, the foundation colony was derived from

Charles River colonies all over the world and consists of 250 breeding pairs, which are maintained using a circular-pair mating system. This system has been described previously [1].

Every three years, 25% of the male breeders in a production colony are replaced with CD rats from the foundation. This is referred to as forward (or outward) migration. Once per year, 1% of the breeding pairs in the foundation colony are replaced with cesarean-derived rats from healthy production colonies. This is termed backward (or inward) migration (Figure 3).

The IGS is a proactive approach to minimize colony divergence and genetic drift, and should minimize inter-colony variation. In order to test the success of the program, we have undertaken the present study to evaluate the similarity between the colonies in allele frequency.

Approach

The same assays used to establish genetic authenticity in inbred and F1 hybrid rodents are used to evaluate outbred stocks but the results are analyzed differently. The goal of genetic monitoring for inbred strains is to determine whether genetic contamination (i.e., an unintentional outcross) has occurred. Each inbred strain has a constant and often unique genetic profile characterized by homozygosity. Only one allele is present at each gene locus that is segregating in the species. Deviation from the expected strain profile typically indicates that genetic contamination has occurred and is rarely due to mutation [2,7,8].

By contrast, individuals in an outbred rodent stock are expected to be genetically different from one another, or allogenic; multiple alleles may segregate at a polymorphic gene locus. The allele frequency is a proportion of all alleles at a genetic locus that are of a prescribed type.

Calculation of Allele Frequencies

	Genotype			Total
	F/F	F/S	S/S	
# of Individuals	8	6	2	16
# of F Alleles	16	6	0	22
# of S Alleles	0	6	4	10

Allele Frequency F = 22/32 = 69%, S = 10/32 = %31

Allele frequencies for polymorphic gene loci can be used to assess the level of heterozygosity in a population and evaluate the degree to which genetic divergence has occurred among subpopulations (i.e., colonies) [9]. In colonies that have not drifted apart, one would expect that the alleles represented and their frequencies will be similar. In theory, if the frequency of migration of stock and the number of migrating breeders were great enough, the allele frequencies in the colonies involved in the migration would approach identity. Further complicating this ideal analysis is the sample size required to completely characterize the population. Simply selecting a small number of animals and extrapolating the percentage of any given allele assayed back to the population can lead to significant error. On the other hand, taking large numbers of samples and assaying for large numbers of alleles can prove to be impractical. Given the nature of the use of outbred animals, rather than absolute identity, only some level of similarity between colonies is necessary. There are no fixed rules for judging acceptability. However, if there is too much variation in allele frequencies over multiple alleles, a solution is re-infusion from the foundation colony.

In recent years, monitoring by traditional biochemical and immunological techniques that demonstrate polymorphisms in the proteins encoded by genes, has given way to molecular methods that detect genomic DNA polymorphisms. Animal genomes contain tandemly arranged, highly repetitive DNA sequences near the centromeres or telomeres of chromosomes. These repeating sequences, typically referred to as satellite DNA, are often not transcribed, and they are quite variable. The role of satellite DNA, which may account for up to 50% of the genome in some species, remains obscure. A polymorphism based on differences in the number of tandem repeats at a genomic location is generically referred to as a simple tandem repeat polymorphism (STRP). STRPs are substantially more polymorphic than traditional protein markers because the STRPs do not code for functional proteins and hence, are under less selective pressure. STRPs come in two basic types, i.e., micro and mini. A microsatellite polymorphism has a very short core repeating-unit of 2 to 9 base pairs, whereas a minisatellite has a longer core unit, usually consisting of 10 to 60 base pairs. Microsatellite polymorphisms, referred to as simple sequence length polymorphisms (SSLP), are identified by PCR with locus-specific primers [10].

Minisatellite polymorphisms are identified by the Southern-blot hybridization technique using a multi-locus probe and are referred to as variable number of tandem repeats (VNTR). As minisatellite copies are found throughout the genome, a complex pattern of bands appears on minisatellite Southern blots. This pattern is referred to as a DNA fingerprint. The core-repeat sequences in minisatellites are not unique to a species. Multilocus VNTR probes of human origin have typically been used to produce DNA fingerprints of inbred mouse and rat strains [4,5]. These band patterns can be analyzed by subdividing the fingerprint into individual size bins for categorization and analysis. These bin frequencies can be used to analyze allele frequencies in a relative fashion.

In the present study, 20 CD rats were sampled from each of six CD production colonies and the CD IGS foundation colony. Microsatellite analysis was performed on all samples; DNA fingerprinting was performed on the samples from three colonies (i.e., 60 samples).

MATERIALS AND METHODS

Microsatellite Analysis: Microsatellite polymorphisms were demonstrated using unique oligonucleotide primer pairs complementary to sequences located immediately upstream and downstream of the following microsatellite loci:

Locus	Chr	Protein
Myl-2	1	Myosin light chain 2
Fibg2	2	Fibrinogen gamma chain 2
Eno-2	4	Enolase 2
Try-1	4	Trypsin 1
Il-6g	4	Interleukin 6
Apa2s	13	ATPase alpha 2 subunit

Following PCR, the sizes of the PCR products were determined by ethidium bromide-agarose electrophoresis.

DNA Fingerprinting: DNA fingerprints were prepared at Charles River Genetic Testing Services. Rat DNA was digested with the restriction enzyme Hae III. Southern blots of the DNA were hybridized with either the OPT-2 or OPT-3 minisatellite probe. Autoradiograms were digitized, that is scanned, into a computer. Based on the distance migrated by the molecular weight sizing standards, proprietary software divided each autoradiogram from top to bottom (i.e., from high to low molecular weight) into sizing "bins". Each hybridization band (or marker) in a lane was assigned to a bin. Results were reported qualitatively by bin as "+" if a band was present. The percentage of specimens positive at each bin was computed.

Test Specimens: Twenty rats were tested from each of the Charles River colonies listed in the following table.

Colony		Collection	Microsatellite Markers Monitored
CRL Facility	Area	Date	
Wilmington	IGS Foundation	6/2000	√
Tsukuba	T21	6/2000	√
France	F24	6/2000	√
Raleigh	R12	6/2000	√
	R04	6/2001	√ *
	R05	6/2001	√ *
	R10	6/2001	√ *

*Minisatellite analysis

RESULTS

Microsatellites: With the exception of FIBG2, all markers had segregating alleles in the colonies evaluated. ENO2 was the most polymorphic, with a total of 5 alleles being found. MYL2, TRY1, IL6G and PA2S were dimorphic. Allele frequencies were largely consistent among the four colonies tested. For example, all CD rats, irrespective of source, were homozygous for the 134bp allele at the FIBG2 marker. In all colonies, the TRY1 137bp allele frequencies were approximately 60 % and the PA2S 124bp allele frequencies were above 70% (Table 1).

Table 1. Comparative Genetic Monitoring of CRL CD Rat Colonies by Analysis of Microsatellite Polymorphisms: Allele Frequencies

Chrom.	Marker	Source:	IGS Foundation		T21		R12		F24	
		Allele[A]	Number	Percent	Number	Percent	Number	Percent	Number	Percent
1	MYL2	184	18	45%	14	35%	22	55%	14	35%
		194	22	55%	26	65%	18	45%	26	65%
		Total:	40		40		40		40	
2	FIBG2	134	40	100%	40	100%	40	100%	40	100%
		Total:	40		40		40		40	
4	ENO2	112	3	8%	0	0%	0	0%	0	0%
		122	17	43%	15	38%	23	58%	0	0%
		132	14	35%	6	15%	11	28%	21	53%
		140	6	15%	17	43%	6	15%	16	40%
		152	0	0%	2	5%	0	0%	3	8%
		Total:	40		40		40		40	
	TRY1	137	26	65%	25	63%	23	58%	27	68%
		174	14	35%	15	38%	17	43%	13	33%
		Total:	40		40		40		40	
	IL6G	167	10	25%	18	45%	26	65%	15	38%
		180	30	75%	22	55%	14	35%	25	63%
		Total:	40		40		40		40	
13	PA2S	124	33	83%	36	90%	38	95%	29	73%
		145	7	18%	4	10%	2	5%	11	28%
		Total:	40		40		40		40	

ALength of allele in number of base pairs(bp) +/- 2bp.

Minisatellites: The bin frequencies for the OPT-2 and OPT-3 DNA fingerprints are shown in Tables 2A and B. The OPT-2 and OPT-3 average bin frequencies were 53% and 34%, respectively. The average (min-max) standard deviations for the OPT-2 and OPT-3 bin frequencies were 14%(0%-55%) and 14% (3%-48%).

Table 2A: Comparative Genetic Monitoring of CRL Raleigh CD Rat Colonies by Analysis of Microsatellite VNTR: "Bin" Frequencies
Probe/Enzyme Combination: OPT-02/Hae III

Source:	R04		R05		R10		All	
Bin	Number	Percent	Number	Percent	Number	Percent	Number	Percent
91	0	0%	0	0%	1	5%	1	2%
77	19	95%	20	100%	18	90%	57	95%
714	0	0%	0	0%	1	5%	1	2%
717	0	0%	1	5%	0	0%	1	2%
719	0	0%	9	45%	19	95%	28	47%
720	0	0%	10	50%	20	100%	30	50%
725	18	90%	6	30%	7	35%	31	52%
726	0	0%	10	50%	0	0%	10	17%
727	0	0%	2	10%	20	100%	22	37%
57	0	0%	1	5%	0	0%	1	2%
58	3	15%	4	20%	0	0%	7	12%
59	2	10%	8	40%	11	55%	21	35%
512	19	95%	20	100%	20	100%	59	98%
515	11	55%	10	50%	8	40%	29	48%
517	9	45%	18	90%	18	90%	45	75%
520	10	50%	3	15%	9	45%	22	37%
522	19	95%	18	90%	17	85%	54	90%
525	5	25%	10	50%	3	15%	18	30%
528	18	90%	20	100%	20	100%	58	97%
530	19	95%	18	90%	5	25%	42	70%
531	13	65%	19	95%	17	85%	49	82%
533	4	20%	0	0%	0	0%	4	7%
534	9	45%	8	40%	15	75%	32	53%
535	19	95%	20	100%	20	100%	59	98%
537	0	0%	1	5%	0	0%	1	2%
538	19	95%	19	95%	20	100%	58	97%
32	19	95%	20	100%	20	100%	59	98%
33	15	75%	4	20%	19	95%	38	63%
34	4	20%	16	80%	1	5%	21	35%
35	0	0%	1	5%	0	0%	1	2%
36	19	95%	20	100%	20	100%	59	98%
38	1	5%	1	5%	1	5%	3	5%
39	19	95%	20	100%	20	100%	59	98%
311	17	85%	20	100%	20	100%	57	95%
312	17	85%	18	90%	19	95%	54	90%
314	19	95%	20	100%	20	100%	59	98%
315	19	95%	20	100%	19	95%	58	97%
316	0	0%	1	5%	1	5%	2	3%
317	19	95%	19	95%	20	100%	58	97%
318	0	0%	0	0%	2	10%	2	3%
	Average:	48%		54%		56%		53%

Table 2B: Comparative Genetic Monitoring of CRL Raleigh CD Rat Colonies by Analysis of Microsatellite VNTR: "Bin" Frequencies
Probe/Enzyme Combination: OPT-03/Hae III

Source:	R04		R05		R10		All	
Bin	Number	Percent	Number	Percent	Number	Percent	Number	Percent
134	0	0%	5	25%	4	20%	9	15%
86	0	0%	10	50%	19	95%	29	48%
74	8	40%	10	50%	5	25%	23	38%
79	0	0%	3	15%	0	0%	3	5%
714	0	0%	10	50%	15	75%	25	42%
722	0	0%	0	0%	4	20%	4	7%
723	4	20%	7	35%	9	45%	20	33%
724	4	20%	6	30%	9	45%	19	32%
52	2	10%	6	30%	5	25%	13	22%
53	1	5%	0	0%	12	60%	13	22%
54	0	0%	5	25%	5	25%	10	17%
55	0	0%	2	10%	0	0%	2	3%
511	4	20%	7	35%	9	45%	20	33%
518	19	95%	16	80%	15	75%	50	83%
522	0	0%	0	0%	1	5%	1	2%
527	8	40%	8	40%	15	75%	31	52%
530	0	0%	0	0%	2	10%	2	3%
533	4	20%	3	15%	1	5%	8	13%
535	0	0%	9	45%	0	0%	9	15%
537	19	95%	16	80%	15	75%	50	83%
538	0	0%	2	10%	3	15%	5	8%
539	0	0%	0	0%	1	5%	1	2%
33	12	60%	14	70%	10	50%	36	60%
37	1	5%	7	35%	6	30%	14	23%
38	1	5%	4	20%	1	5%	6	10%
39	0	0%	5	25%	1	5%	6	10%
310	15	75%	12	60%	14	70%	41	68%
311	12	60%	14	70%	14	70%	40	67%
313	9	45%	9	45%	16	80%	34	57%
314	6	30%	15	75%	7	35%	28	47%
316	17	85%	16	80%	18	90%	51	85%
317	0	0%	0	0%	1	5%	1	2%
318	15	75%	10	50%	16	80%	41	68%
319	17	85%	16	80%	18	90%	51	85%
	Average:	26%		36%		40%		34%

DISCUSSION AND CONCLUSIONS

The microsatellite allele frequencies were remarkably consistent in the IGS foundation and production colonies. This supports the conclusion that genetic divergence among CD rat colonies, by this analysis, is minimal. The DNA fingerprint bin frequencies for the colonies were generally similar, but as with any arbitrary subdivision of continuous data, provided difficulties in analysis. Obviously, differences observed among DNA fingerprints may be artificial because the band patterns are often very complex. With the minisatellite probes employed in this study, the numbers of bands that appear on CD rat fingerprints are substantially fewer than those observed on fingerprints of other species and the percentage of band sharing is higher. For these reasons, DNA fingerprinting is not an especially sensitive method for detecting genetic drift among CD rat colonies, but can provide a reasonable assessment of the general similarity of colonies. Unfortunately, the technique is both time consuming and expensive, which limits the number of animals that can reasonably be done on a frequent basis.

The microsatellite panel consists of just 6 loci on 4 chromosomes. Additional loci represented by a much greater number of microsatellites could be used to further refine the analysis of allele frequencies and is currently being pursued as a method for regular monitoring of genetic drift. Analysis such as these coupled with phenotypic data and sharing of control data through mechanisms such as the IGS study group further enables the ongoing assessment of the CD IGS production colonies.

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REFLECTIONS

REFLECTIONS OF THE CD(SD)IGS STUDY GROUP

A history of the founding of the CD(SD)IGS Study Group is described in the preface of the article, Biological Reference Data on CD(SD)IGS Rats -1998. Approximately eight years have past since the founding of this Study Group was planned. For the past five years, the Study Group has been active as an official organization during which time four reference data books were published. I would like to extend my appreciation to the officers of the Study Group, the bureau, editors, secretariat and printing office staff for their continuous effort and support and to the domestic or overseas members for their invaluable contributions and articles.

It was a privilege and honor to have had the opportunity to have numerous scientific exchanges with the many people involved in toxicology of this Study Group.

The reference data books are the compilation of control data from repeated-dose, reproductive & developmental toxicity and carcinogenicity studies to verify the production of a new strain of rats. The original purpose of the data books is considered to be achieved by publishing the last data book on carcinogenicity studies, a most time-consuming task, though personally, somewhat later data collection was hopefully expanded to include data from pharmacology/drug efficacy and metabolism data. As described above, this data set predominantly consists of control data. Therefore, I think the data set should have somewhat include data of animals treated with well-known substances in view of drug response. As a reference, please see "Chemical toxicity database" in a website of Ministry of Health, Labor and Welfare [URL: http://wwwdb.mhlw.go.jp/ginc/html/db1-j.html] and also the National Institute of Health Sciences website. This database contains data of 200 or more compounds from single dose toxicity, preliminary reproduction toxicity screening, combined repeat dose and reproductive/developmental toxicity and twenty-eight day repeat dose toxicity studies using IGS rats.

At present, CD(SD)IGS rat has become routinely employed in Japan and the activity of this Study Group was instrumental in this utilization.

Lastly, I would like to express my profound gratitude to Charles River Japan, Inc., the sponsor of this Study Group.

Hiroyuki Inoue, Ph.D. (ex-Chairman).

REFLECTIONS OF THE CD(SD)IGS STUDY GROUP

A prehistory until publication of the first edition of CD (SD) IGS database of 1998 are described in the "Preface". The purpose and organization of the CD (SD) IGS Study Group should be clearly understood since these were described in the previous reviews. It was of immense benefit that researchers contributed not only from Japan but also Europe and the United States. I profoundly appreciate their cooperation.

My only regret is that I feel that artifactual data were not presented accurately or realistically. However, this may be due to the inherent nature of such findings in outbred rats.

Dr. Keenan in North America initiated the establishment of a control animal database, though this effort was not coordinated with our Study Group. I would like to pay more attention to such future activities.

As a user of rat animal models, I anticipate that the concept of IGS will expand to other strains such as Wistar and F344 rats in addition to CD(SD)IGS rats. If feed and feeding data are also integrated, it would help greatly benefit of animal welfare.

I wish to express my deepest respect and gratitude to the Chairman, members of editorial board and other Study Group members in addition to the secretarial and support staff and the sponsor, Charles River Japan, Inc. who devoted their time and effort during 1998-2000 when I took charge of vice-Chairman and Editor-in-chief.

Titles of review references are as follows:

1. Consideration of various factors relating to biological reference data on albino laboratory rats (1998).
2. Technical factors affecting clinical chemistry values in laboratory rats (1999).
3. The importance of urinalysis and effects of urine collection techniques in toxicity studies with rats (1999).
4. Strain differences in behavior and nervous system, immune response, and male fertility in the laboratory rat (2000).
5. Gender-specific factors in the differential responsiveness of rats to chemicals and stress (2003).

Reference

1. Keenan, C., Hughes-Earle, A., Case, M., Stuart, B., Lake, S., Mahrt, C., Halliwell, W., Westhouse, R., Elwell, M., Morton, D., Morawietz, G., Rittinghausen, S., Deschl, U. and Mohr, U. 2002. The North American control animal database: A resource based on standardized nomenclature and diagnostic criteria. Toxicol. Pathol. 30: 75-79.

Lastly, I sincerely desire that our fellow researchers continue in this endeavor.

In the summer of 2003

Toshiaki Matsuzawa, Ph.D. (ex-viceChairman &Editor-in-chief).,

REFLECTIONS OF THE GENERAL TOXICOLOGY WORKING GROUP

The General Toxicology Working Group of CD(SD)IGS Study Group was established in July 1997 at Toranomon Pastral Hotel (Tokyo). It was at that time when the momentum to utilize CD(SD)IGS rats, rather than the traditional CD(SD) rats, in safety studies was started and background data of IGS rats began to accumulate in various laboratories. These efforts came to fruition as the "Biological Reference Data on CD(SD)IGS Rats-1998" in which data from 18 general toxicity studies were employed. Data included body weight, food consumption, urinalysis, ophthalmology, hematology, blood chemistry, gross pathology and organ weight data obtained from 2-, 4-, 13- and 26-week repeated dose toxicity studies. Adequate histopathology data were not available at that time. Comparison data between the traditional CD(SD) and the new CD(SD)IGS rat accounted for approximately half of the reports (9 reports). These comparisons demonstrated that there were no appreciable differences between the two strains, though some minor differences were evident. Seven reports described the comparison between low protein (18%) and normal protein diets (23-25%). As would be expected, decreased body weight gain and increased food consumption were evident in the low protein diet (18%). A similar tendency was noted in the following year data book (1999), which contained 16 reports of general toxicity studies with more extensive histopathology data.

In 2000, although the number of reports contributed decreased to 11, the content was expanded to include more specifics such as hormone levels. Literature references were also included.

The number of reports further decreased to 11 and 7 in subsequent data books, which included new investigational methods with CD(SD)IGS rats and characteristic pathological findings. Many of these were literature references.

The main activity of the board of the General Toxicology Working Group was to request the contribution of data and to introduce the contents of contributed papers at the meeting of the Study Group. Since regular meetings of the General Toxicology Working Group were not scheduled, there was little chance of inter-group communication. However, a total of 63 reports were presented in the reference data books as a result of cooperative efforts of members of this Working Group. I wish to express my deepest respect and gratitude to the members of this Working Group and to Drs. Maeda, Okazaki, Naeshiro, Yagi, Kojima, Ikuse and Yasuba who have continually supported us and served as the editorial board for this Working Group.

Masaharu Hashimoto, Ph.D.

REFLECTIONS OF THE REPRODUCTION TOXICOLOGY WORKING GROUP

The most critical factor for accurate evaluations of toxicity studies of drugs is the experimental animals. In particular, genetically stable and readily available healthy experimental animals are imperative. Charles River Japan, Inc. has contributed to much fulfill the researcher's needs. To meet these demands, Charles River Japan, Inc. developed the IGS rat produced by the worldwide-scope manufacturing system. When we heard of this proposal, we immediately felt the need to review historical data that we had accumulated up to that time as well as the possibility of global usage of study data. Since historical data are indispensable for evaluations of reproductive toxicity studies as well as in other areas of toxicity, we felt a sense of urgency. In order to respond to such an important and pressing assignment, the CD(SD)IGS Study Group was established in 1997 through the efforts of Dr. Inoue and Dr. Matsuzawa and the collaboration of Charles River Japan, Inc. At the time of establishment, secretaries of the Reproduction Toxicology Working Group consisted of Mr. Furuhashi (Nihon Bioresearch Inc.), Mr. Shibano (Ina Research Inc.), Dr. Sanbuissho (Sankyo Co., Ltd.) and Mr. Inoue (Taisho Pharmaceutical Co., Ltd.). All of the secretaries energetically conducted laboratory experiments and supported activities of the Working Group. Consequently, the first edition of "Biological Reference Data on CD(SD)IGS Rats", printed in 1998, had 22 contributors, which exceeded our expectations. As a result of such a successful beginning, research activities for the next 5 years increased. The Working Group not only accumulated a multitude of historical data but also made significant contributions of scientifically superior research during that period. We concluded that the reproductive and developmental characteristics of IGS rats were resolved and the mission of the Working Group was complete during the 6 years of research activities. I believe that the research achievements accomplished by this Working Group contributed substantially to the evaluations of reproductive and developmental toxicity studies of medicinal drugs.

Lastly, I am deeply grateful to the staff of Charles River Japan, Inc., and to Messrs. Takura, Morimura, Chazono and Ii, who operated the Reproduction Toxicology Working Group.

Michio Fujiwara, Ph.D.

REFLECTIONS OF THE CARCINOGENICITY WORKING GROUP

The Carcinogenicity Working Group of the CD(SD)IGS Study Group was established in 1997 with Messes. K. Shibuya, M. Takeuchi, H. Maeda, H. Kandori and I as the original group members. Data of carcinogenicity studies are not available for evaluation for nearly three years from initiation. Therefore, this Working Group was somewhat relaxed at the time of establishment unlike serving on General Toxicology and Reproduction Toxicology Working Groups which require much less time to reach conclusions and recommendations. The first priority of the Working Group was to prepare questionnaires to collect data and to generate data acquisition files. The group members met to discuss policy and exchange ideas and opinions at the Annual Meetings of Japanese Society of Toxicologic Pathology. It proved difficult to collect data on carcinogenicity studies due to time and cost restrains. The data on CD(SD)IGS rat carcinogenicity studies became available from 1999 and peaked in 2000 by which time 12 studies had been reported. Several other studies have been reported since then. In addition to routine data including mortality, body weight, food consumption, hematology and neoplastic findings, data of low protein diets and dietary restriction were also collected. International comparisons as well as comparisons with traditional CD(SD) strain rats were possible since data were also reported from UK (5 studies) and USA (1 study). The primary concerns in the utilization of CD(SD)IGS rats prior to the survey were spontaneous abnormalities and unusual tumors in the reproductive system as compared to the traditional CD(SD) rats. However, this survey revealed a high incidence of the spontaneous occurrence of pituitary and other endocrine organ and mammary gland tumors, similar to that of the traditional SD rat. The survival rates of CD(SD)IGS rats were slightly longer than the traditional Japanese CD(SD) rats. There were no appreciable differences between the Japanese and foreign data, suggesting genetic uniformity of the CD(SD)IGS rat.

In closing, I would like to express my heartfelt gratitude to Dr. H. Miyajima who reviewed the data from the various Japanese facilities as well as to Messes. H. Satoh and Y. Tanakamaru who served diligently as new members of the Carcinogenicity Working Group.

Hijiri Iwata, D.V.M., Ph.D.

AFTERWORD

The CD(SD)IGS Study Group was planned in the autumn in 1995 and established in 1997 by several individuals including Dr. Matsuzawa as the leading advocate. Several reference data books (vol.1 to vol. 4) totaling over 1000 pages were published. With completion of this monumental task, the CD(SD)IGS Study Group will dissolve following publication of data book vol. 5.

The activities of our members over the past eight years have produced significant contributions to the scientific community both in Japan and abroad as did the Fisher Rat Study Group in the past. We believe that the results of our efforts, published in the 5 data books, will be of great benefit as future reference data.

I deeply appreciate the devotion of our executive officers including Dr. Hiroyuki Inoue (ex-Chairman) and Dr. Toshiaki Matsuzawa (ex-Editor-in-chief) for their efforts in founding of the Study Group and for their long lasting impressions and leadership. I also wish to express our heartfelt appreciation to all the contributors who provided the valuable data for the 5 data book volumes.

We are confronted with new questions and problems every day. Although the CD(SD)IGS Study Group will complete its activities, I hope that you will gain as wonderful relationships as those you obtained through your participation in the CD(SD)IGS Study Group and proceed to new productive activities.

The activities of the CD(SD)IGS Study Group continued for “eight” years. In Japan, “eight (8)” is considered to be a lucky number meaning getting more and more prosperous as time goes on because the shape of a Chinese character of “eight (八)” widens toward the end. Therefore, dissolution of the Study Group after “eight” years from their establishment gives me a premonition of further progress of all of you in your own fields and the opportunity for meeting together again.

Kohichi Kojima, Ph.D. (vice-Chairman)

APPENDIX

Activities of CD(SD)IGS Study Group for 1996-2003

				1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
Study Group	Meetings	Inaugural Meeting			(Arrangement)		Jul.23						
		Meeting for Distribution of Reference Data Book						Feb.4	Jan.21	Jan.14	Jan.19		Dec.19
		Top Dirctors Meeting					Feb.13					Apr.4	Apr.11
		Committee Member Meeting					Feb.19 Nov.5		Apr. 16	Apr.12	Apr.25	Apr.24	May.16
		Editional Board Meeting							May.18 Oct.19	Oct.25			
		Working Group Meeting	General Toxicology										
			Reproduction Toxicology										
			Carinogenicity				Dec.16	Feb.4	Oct.8	Nov.22	Jan.27	Jan.25	
		Account Audit						○	○	○	○	○	○
	Members	Number of Domestic Companies					64	70	72	73	67	68	66
		Number of Overseas Companies					15	15	15	15	15	13	13
		Number of Individual Members					168	179	187	182	169	173	164
Breeding	Introduction of Breeders to CRJ			○									
	Initiation of Distribution from Each Facility				TBC	HBC	ABC						
	Forward Migration								○				○
	Back Migration									○			

TBC : Tsukuba Breeding Center
HBC : Hino Breeding Center
ABC : Atsugi Breeding Center

CD(SD)IGS Study Group / Steering Committee Member 1998-2003

Japan fiscal year	1998	1999	2000	2001	2002/2003
Chairman:	Hiroyuki Inoue	Hiroyuki Inoue	Hiroyuki Inoue	Hiroyuki Inoue	Kazumoto Shibuya
Vice-Chairman:	Toshiaki Matsuzawa		Kazumoto Shibuya	Kazumoto Shibuya	Kohichi Kojima
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