Biological Reference Data on CD(SD) IGS Rats - 2001

CD(SD)IGS Study Group

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Library of Congress Cataloging -in- Publication Data

Biological Reference Data on CD(SD)IGS Rats - 2001 ／ edited by Yasuyuki Maeda and Hiroyuki Inoue

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Biological Reference Data on CD(SD)IGS Rats - 2001

ISBN 4-9980718-6-6

Printed by BEST PRINTING Co. Ltd., Tokyo

PREFACE

I am pleased to present to our membership the annual edition of Biological Reference Data on CD(SD)IGS Rats for 2001.

Since initiation of the study group in 1997, we have now accomplished four editions of Biological Reference Data. The current volume was produced under the auspices of Dr. Yasuyuki Maeda as an editor-in-chief, and was completed on schedule thanks to the tireless efforts of the head and members of the editing committee. Professor Kunitoshi Mitsumori wrote the Introduction. My warmest thanks to all contributors for their efforts.

At the meeting of the steering committee in April, there was the suggestion that not only safety data, but also background data on metabolism and pharmacology/efficacy should be included, however sufficient data could not be collected in time for publication. I invite the efforts of all members to help in the task of providing such data for publication in the next edition.

Regarding IGS breeding methods, both forward migration and then backward migration have been completed, and the so-called second generation of rats is starting to be used in various countries. Investigating any possible differences between the two generations is one of the purposes of the present study group. This year's edition includes only one paper dealing with this matter, and we encourage contribution of more papers concerning this topic for next year's edition.

I have had the honor of serving as a chairman of the study group since its inception. Thinking that the new century deserves a new chairman for the group, I expressed my intention of retiring the position last year. However, I have put off my resignation after being requested to serve another year. It is my earnest hope that next year will find ever greater progress under the leadership of a new chairman. I am sincerely grateful for the cooperation extended by the entire membership through my years of service.

In the fall of 2001

Hiroyuki Inoue, Ph.D., Chairman

PREFACE

The CD(SD)IGS study group was founded in 1997 for the purpose of clarifying the characteristics of CD(SD)IGS rats. To achieve the stated purpose of the study group, one of the major projects of the group has been to publish collections of data on IGS rats.

Under the auspices and thanks to the efforts of Chairman Dr. Hiroyuki Inoue, of the first editor-in-chief Dr. Toshiaki Matsuzawa, and to all the editing committee members, the first edition of this publication saw the light of day in 1998. Since that time, we have succeeded in producing four editions of the book. I humbly thank the many members who have contributed the great number of manuscripts for this purpose.

Dr. Toshiaki Matsuzawa, who served as the first editor-in-chief from the inception of the study group, retired from this position upon completion of the publication of the data collection for the year 2000. At the same time, the membership of the study group was renewed, and several members accepted roles in the steering committee. I am looking forward to the continued fruitful activity of our group.

I have become involved in the editing work from the present 2001 edition of the data collection. Due to my lack of qualification for this job, I must apologize for any shortcomings in my actions, but I will continue to strive to be of service to the members of the study group in producing the data collections concerning IGS rats. Your cooperation and understanding are humbly requested.

Many thanks are due to Dr. Matsuzawa and the other steering committee members and study group members who have been involved in the editing process up to now. As the only study group devoted to IGS rats, I anticipate further continuation of the group's activities, and by gradual accumulation of data collection, further contribution to the elucidation of IGS rat characteristics.

In the fall of 2001

Yasuyuki MAEDA, D.V. M., Editor-in-Chief

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ACKNOWLEDGEMENTS

We received numerous papers for the 2001 edition of the data collection. Our warmest thanks to those who contributed this valuable information.

Appreciation is also due to the members of the editing committee (Drs. S. Okazaki, K. Kojima, N. Hoshino, T. Inoue, H. Maeda, and H. Satoh) for their careful preparation of the large number of contributed papers for the current edition.

Further, the present publication benefited greatly from the understanding and cooperation of Charles River Laboratories, U.S.A and Charles River Japan, Inc., in the publication of this collection of data. We are especially grateful to Mr. James. C. Foster, president of Charles River

Laboratories, U.S.A., and to Mr. T. Kashiwagi, president of Charles River Japan, Inc. for strong support in the organization of this society.

Additionally, we received the benefit of the labors of Mr. G. Shimaya, Mr. Y. Chazono, and Ms. E. Hattori of Charles River Japan, Inc., who ran the study group's administrative office. 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, Inc., thanks to whose support this volume was able to progress smoothly throughout the edition process.

The present data 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. We acknowledge this, and respectfully request continued cooperation in our endeavors for the future.

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. and Hiroyuki Inoue, Ph.D.

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CHAPTER 1

Introduction

Variability in the Incidence of Spontaneous Tumors in CD (SD) IGS, CD (SD), F344 and Wistar Hannover Rats

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ABSTRACT. References dealing with the incidence of spontaneous tumors in CD (SD), F344 and Wistar Hannover rats that were reared as untreated controls in 2-year carcinogenicity studies were collected, and a comparison of the incidence of spontaneous tumors between these three different strains was made. In addition, the incidence of spontaneous tumors in the original strain of SD rats was compared with that in the new strain of CD (SD) IGS rats. Pituitary and mammary tumors were frequently observed in SD and Wistar Hannover rats while mononuclear cell leukemia and testicular interstitial cell tumors in F344 rats, as expected. Tumors observed in Wistar Hannover rats in the present literature search were generally the same as those that have been reported previously, but myoepitheliomas and sarcomas of the salivary gland were only observed in Wistar Hannover strain. Granular cell tumors in the meninx, pancreatic islet cell tumors and uterine adenocarcinomas were observed in Wistar Hannover rats at relatively higher incidences as compared to those in SD and F344 rats, but the incidences of other tumors in Wistar Hannover rats were generally low. No clear intra-strain difference in the incidence and type of spontaneous tumors was found between the original strain of SD rats and SD-IGS rats, although there were slight intra-strain differences in the incidences of hepatocellular adenomas, adrenal cortical adenomas, thyroid C-cell adenomas, testicular interstitial cell tumors and mammary fibromas. － Key word: spontaneous tumors, CD(SD)IGS, CD(SD), F344, Wistar Hannover

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INTRODUCTION

For the evaluation of carcinogenicity of various chemicals including pharmaceuticals, pesticides, food additives and veterinary drugs, rats and mice have been widely used for many years. From 1997, the ICH, "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals, for Human Use", has proposed a new guideline on carcinogenicity studies [6]. The basic scheme of the new guideline consists of one long-term rodent carcinogenicity study using rodent species that are most relevant to human beings plus one other short-term in vivo carcinogenicity study using genetically engendered mice, neonatal mice and so on. That means that long-term carcinogenicity studies using rats are still essential for the evaluation of carcinogenicity of newly-developed chemicals, since it was concluded by the Expert Working Group of the ICH that mice were extremely susceptible to liver carcinogenesis induced by drug metabolizing enzyme inducers and were not a suitable species for the carcinogenicity evaluation [5]. At present, F344, Wistar and Sprague-Dawley (SD) strains of albino rats have been widely used in long-term carcinogenicity studies of various chemicals [1-3, 7-10, 12, 15-18, 20, 25-32]. However, it has been generally recognized that there are strain differences in the incidence of spontaneous tumors among these strains, and such differences disturb the reproducibility of the results of carcinogenicity studies. The CD (SD) rats produced by Charles River U.S.A., Inc. had a tendency to become over-weight more rapidly, and their survival rates for 2 years were lower than those in other colonies and breeders from 1990 [19, 23]. For this reason, this company prepared a production and supply system for the International Genetic Standard (IGS) rats in their eight colonies existing throughout the world [33], and at the present time, such a so-called old strain of CD (SD) rats cannot be purchased from Charles River company expect for Charles River Japan, Inc. any more. Recently, Wistar Hannover rats have been introduced as the global standards of outbred rat strains from the Global Alliance for Laboratory Animal Standardization (GALAS) [21]. However, there is no report dealing with the differences in the incidences of spontaneous tumors in Wistar Hannover rats, the old strain of CD (SD) rats that was used from 1970's and CD (SD) IGS rats that were introduced from 1990's.

In this manuscript, we have searched references dealing with the incidence of spontaneous tumors in Wistar Hannover GALAS rats (Wistar) that were recently introduced [4], F344 rats [18], and CD (SD) rats originating from Charles River Laboratory in U.S.A. (SD-CRL) [14] and CD (SD) rats originating from Charles River Japan (SD-CRJ) [11, 22] that were reared as untreated controls in 2-year carcinogenicity studies, and made a comparison of the incidence of spontaneous tumors between these three different strains of rats. In addition, the incidence of spontaneous tumors in the old strain of CD (SD) rats was compared with that in the new strain of CD (SD) IGS rats (SD-IGS) [22].

Survival rate

The mean survival rates of the Wistar, F344, SD-CRJ and SD-IGS rats in the control group that were subjected to 2-year carcinogenicity studies were 78, 85, 30 and 38 in males and 76, 90, 25 and 58 in females, respectively. The highest survival was observed in F344 rats. The survival in SD-CRJ rats was extremely lower than that in other strains including SD-IGS rats.

Major spontaneous tumors and their incidences（Tables 1 and 2）

Brain: Granular cell tumors were observed in these different strains of rats. The incidences of them ranged from 0.1 to 1.0 % in males and 0 to 0.1 % in females of F344 and SD rats, but those in Wistar rats were 2.3 % in males and 1.1 % in females, being relatively high as compared to F344 and SD rats.

Gliomas including astrocytomas and oligodendrocytomas were seen in these three different strains. The incidences of them ranged from 0 to 1.8 % in males and 0 to 0.5 % in females of

Wistar, F344 and SD rats, and no marked differences were found among these rats.

Lung: Pulmonary adenomas and adenocarcinomas were seen in these three different strains. The incidences of adenomas ranged from 0.3 to 4.2 % in males and 0 to 2.2 % in females of Wistar, F344 and SD rats. The incidence in F344 rats were 4.2 % in males and 2.2 % in females, being relatively high as compared to Wistar and SD rats. The incidences of adenocarcinomas were from 0 to 0.5 % in males and 0 to 0.1 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats.

Liver: Hepatocellular adenomas and carcinomas were observed in these three different strains. The incidences of adenomas ranged from 0.8 to 3.2 % in males and 0.5 to 3.2 % in females of Wistar, F344 and SD rats. No marked differences in the incidence of hepatocellular adenomas were seen between Wistar and F344 rats, but there was an intra-stain difference in the incidence among SD rats. The incidence of hepatocellular adenomas was slightly high in SD-IGS male rats as compared to the other SD rats. The incidences of hepatocellular carcinomas were from 0.3 to 2.2 % in males and 0 to 1.7 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats.

Salivary gland: Spontaneous tumors were extremely rare in F344 and SD rats, but myoepitheliomas and sarcomas were observed in Wistar male rats and the incidences of myoepitheliomas and sarcomas were 6.4 and 4.3 %, respectively. Since such tumors were seldom seen in Wistar rats other than GALAS rats [1, 17, 24], the occurrence of these tumors are considered to be specific to Wistar GALAS male rats.

Pituitary gland: Pituitary tumors of the pars distalis were the most common spontaneous tumors in these three strains. The incidences of adenomas ranged from 33.6 to 41.1 % in males and 50.3 to 65.8 % in females of Wistar and SD rats. The incidence in F344 rats were 12.4 % in males and 28.2 % in females, being extremely low as compared to Wistar and SD rats. The incidences of adenocarcinomas were from 0 to 0.4 % in males and 0.1 to 3.6 % in females of Wistar, F344 and SD rats, but those in SD-CRJ rats were 3.9 % in males and 6.0 % in females, being relatively high as compared to other SD rats.

Adrenal gland: Cortical tumors including adenomas and carcinomas were observed in these three strains. The incidences of cortical adenomas were from 0.7 to 1.3 % in males and 1.8 to 2.2 % in females of Wistar, F344 and SD rats. On the other hand, there was an intra-strain difference in the incidence in SD rats. The incidence in SD-CRJ rats were 3.3 % in males and 5.2 % in females, being relatively high as compared to other SD rats. The incidences of cortical carcinomas were from 0.1 to 0.9 % in males and females of Wistar, F344 and SD rats, and no marked differences were found among these rats.

With respect to adrenal medullary tumors, pheochromocytomas were seen with a higher incidence in male rats rather than female rats. The incidences of pheochromocytomas ranged from 3.2 to 11.9 % in males and 1.1 to 3.2 % in females of Wistar, F344 and SD rats. The incidence in Wistar male rats was 3.2 % in males, being slightly low as compared to F344 and SD male rats. The incidences of malignant pheochromocytomas were from 0.3 to 1.0 % in males and 0 to 2.3 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats except for SD-CRL females.

Thyroid gland: C-cell tumors were relatively common in Wistar and F344 rats. The incidence ranged from 10.1 to 12.5 % in males and 4.1 to 10.7 % in females of Wistar, F344 and SD rats. There was an intra-strain difference in the incidence in SD rats, the incidence in male SD-CRJ rats (2.9 %) being relatively low as compared to other SD rats. The incidences of C-cell carcinomas were from 0.3 to 1.4 % in males and 0 to 1.3 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats.

Follicular cell tumors were observed in these three strains, but their incidences were rather low as compared to C-cell tumors. The incidences of follicular adenomas were from 0.7 to 3.1 % in males and 0.3 to 1.9 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats except for Wistar males (3.1 %). The incidences of follicular cell carcinomas were from 0.3 to 1.6 % in males and 0 to 1.3 % in females of Wistar, F344 and SD rats, and no marked differences were found among these rats.

Pancreas: The incidences of acinar cell adenomas ranged from 0 to 1.4 % in males and 0 to 0.1 % in females of Wistar, F344 and SD rats, but the incidence in SD-IGS males (3.6 %) were relatively high as compared to the other SD rats. The incidences of acinar cell adenocarcinomas were extremely rare in these three different strains.

With respect to islet cell tumors, islet cell adenomas were seen with a higher incidence in male rats rather than female rats. The incidences of islet cell adenomas ranged from 1.5 to 5.3 % in males and 0.3 to 1.9 % in females of Wistar, F344 and SD rats. The incidences of islet cell carcinomas were rare in these three different strains, the highest incidence being 1.9 % in Wistar males.

Kidney: Renal epithelial tumors including adenomas and carcinomas were extremely rare in these three strains, the incidences of adenomas being 0 to 0.4 % in males and 0 to 0.2 % in females. There was no strain difference in the incidence of these tumors. Nephroblastomas were also extremely rare in these strains, their highest incidence being 0.1 % in F344 and SD-CRJ rats.

Hematopoietic system: Malignant lymphomas were observed in Wistar and SD rats at the incidence of 1.4 to 3.8 % in males and 0.7 to 4.0 % in females. On the other hands, the incidence of F344 rats was 0.7 % in males and 0.3 % in females, being relatively low as compared to Wistar and SD rats. Mononuclear cell leukemia was specific to F344 rats, and the incidence was 23.6 % in males and 21.9 % in females. Histiocytic sarcomas were observed in these three different strains, their incidence being 0.1 to 2.7 % in males and 0.1 to 3.3 % in females. Myelocytic leukemia was extremely rare in these three strains, the highest incidence being 0.2 % in Wistar females.

Thymus: Thymomas were rare tumors in Wistar, F344 and SD rats, the highest incidence being 1.3 % in SD males.

Testis: Interstitial cell tumors were frequently found in F344 rats, their incidence being 74.6 %. On the contrary, their incidence of Wistar and SD rats were 1.8 to 7 %.

Prostate: Prostatic adenomas and adenocarcinomas were extremely rare tumors in these different strains, their incidence

being 0 to 0.5 % in adenomas and 0 to 0.4 % in adenocarcinomas.

Ovary: The incidence of granulosa cell tumors ranged from 0 to 0.9 % in F344 and SD rats. On the contrary, the incidence in Wistar rats was 2.4 %, being higher than that in the other strains.

Uterus: Endometrial stromal polyps were observed in these three different strains, and their incidence ranged from 1.7 to 7.2 % in Wistar and SD rats. On the contrary, the incidence in F344 rats was 12.6 %, being higher than that in the other strains. Adenocarcinomas were extremely rare tumors in F344 and SD rats, while the incidence (3.4 %) in Wistar rats were higher than that in the other strains. Endometrial stromal sarcomas were also rare in these three strains, their incidence being 0 to 1.4 %.

Clitoral gland: Adenomas and adenocarcinomas of the clitoral gland were rare tumors in Wistar and SD rats, their incidence being 0 to 0.5 %. The incidence of these tumors (1.3 %) in F344 rats was slightly high as compared to the other strains.

Mammary gland: Mammary tumors were common in female rats rather than male rats. The incidence of mammary fibroadenomas ranged from 0.8 to 2.5 % in males of Wistar, F344 and SD rats and 16.7 to 44.5 % in females of Wistar and SD rats. In F344 females, the incidence (7.1 %) was rather low as compared to the other strains. Mammary adenomas were also seen in these three strains, and their incidence was 1.6 to 14.2 %. There was an intra-strain difference in the incidence in SD rats, the incidence being higher in SD-CRL than the other SD strains. The incidence of mammary adenocarcinomas ranged from 1.6 to 16.4 % in these three strains, and the incidence in SD-IGS was highest among these strains. Mammary adenomas and adenocarcinomas in males were rare in these three different strains.

Integumentary system: As skin tumors, papillomas, acanthomas, and squamous cell carcinomas were observed in Wistar, F344 and SD rats, and their incidence were less than 2 % in males and 0.9 % in females, except for acanthomas in Wistar (3.6 %) and SD-IGS (4.1 %) rats. As subcutaneous tumors, fibromas were relatively common in males as compared to females, their incidence being 3.3 to 13.7 % in males and 1.1 to 5.0 % in females. The highest incidence was seen in SD-CRL rats. Mammary fibrosarcomas were found in these strains, their incidence being 0.3 to 1.0 % in males and 0.1 to 0.4 % in females. The incidence in SD-IGS rats (3.2 %) was slightly higher than that in the other SD rats.

Abdominal cavity: Lipomas were only observed in Wistar rats, their incidence being 8.4 % in males and 11.4 % in females. Mesotheliomas occurred in Wistar and F344 rats, and the incidence were 2.9 to 3.1 % in males and 1.8 % in females.

DISCUSSION

It has been generally accepted that the survival rate at the end of 2-year carcinogenicity studies in rats was high 344 rats as compared to Wistar and SD rats. In the present surveys of the literatures on untreated rats in 2-year carcinogenicity studies, in a similar manner, the survival rate in F344 rats was highest and then Wistar and SD rats in decreasing order. Among SD rats, the survival rate of SD-IGS rats at week 104 was 38 % in males and 58 % in females, while that in SD-CRJ rats was 30 % in males and 25 % in females [11]. That suggests that the survival in SD-IGS rats is much better than that in SD-CRJ rats.

With respect to spontaneous tumors, it has been recognized that pituitary and mammary tumors are frequently observed in untreated SD and Wistar rats while mononuclear cell leukemia and testicular interstitial cell tumors in F344 rats that were subjected to terminal kill in 2-year carcinogenicity studies [1-3, 7-10, 12, 15-18, 20, 25-32]. In the present surveillance, similar tendency was found, and such a difference was considered to be strain specific variation. In addition, relatively higher incidences of lung adenomas and thyroid C-cell adenomas and lower incidence of malignant lymphomas were seen in male F344 rats as compared to the other strains. These variations were also considered to be a biological feature of F344 rats [3, 7, 8, 10, 16, 18, 25-30].

The incidence of spontaneous tumors in Wistar rats has been already reported [1, 17, 24]. Tumors observed in Wistar Hannover rats in the present literature search were generally the same as those that have been reported previously, except for myoepitheliomas and sarcomas of the salivary gland. Especially, myoepitheliomas are extremely rare tumors in rats, although such tumors are frequently observed in mammary gland of aging dogs [13]. In addition, such tumors were not observed in F344 and SD rats in the present survey. These findings suggest that relatively higher incidences of myoepitheliomas of the salivary gland are characteristic to Wistar Hannover rats. With respect to the other tumors, granular cell tumors in the meninx, pancreatic islet cell tumors and uterine adenocarcinomas were observed in Wistar Hannover rats at relatively higher incidences as compared to those in SD and F344 rats. On the other hand, the incidences of other tumors in Wistar Hannover rats were generally low as compared to those in SD rats. Especially, the incidence of mammary tumors in Wistar Hannover rats was clearly lower than that in SD rats. However, since the data obtained in this review are limited, further data on wistar Hannover rats are necessary for the comparison between SD and Wistar rats.

In the present literature search, we have also conducted a comparison of spontaneous tumors between SD-CRJ, SD-CRL and SD-IGS rats. As a result, there was no marked difference in the types and incidences of spontaneous tumors among them. However, there were slight intra-strain differences in the incidences of hepatocellular adenomas, adrenal cortical adenomas, thyroid C-cell adenomas, testicular interstitial cell tumors and mammary fibromas in males and hepatocellular adenomas, adrenal cortical adenomas, histiocytic sarcomas and mammary tumors in females. The incidences of hepatocellular adenomas and C-cell adenomas in males and mammary adenocarcinomas in females of SD-IGS rats were higher than those in SD-CRJ or SD-CRL rats in spite of the fact that the body weight in SD-IGS rats were lower than in SD-CRJ rats [11]. The reasons why the incidences of these tumors were higher in SD-IGS rats are not clarified.

In conclusion, no clear intra-strain difference in the incidence and type of spontaneous tumors was found between SD-CRJ or SD-CRL and SD-IGS rats. However, since the historical data of spontaneous tumors in SD-IGS rats are limited at the present time, the accumulation of such historical data is absolutely necessary for the clarification of these unknown points. In this

respect, it is recommended that further studies to accumulate the historical control data on spontaneous tumors in SD-IGS rats should be performed in many laboratories for the better understanding of SD-IGS rats.

Table 1. Incidence of spontaneous tumors in different strains of untreated male rats in 2-year carcinogenicity studies

Organ	Type of tumors	Incidence (%)				
		IGS a) (CRJ)	SD b) (CRJ)	SD c) (CRL)	F344 d) (CRJ)	Wistar e) (GALAS)
Brain	Granular cell tumor	0.5	0.2	1.0	0.1	2.3
	Glioma	0	1.8	0	0.3	0.7
Lung	Adenoma	1.4	0.3	1.0	4.2	0.4
	Adenocarcinoma/Carcinoma	0	0.2	0	0.5	0.2
Liver	Hepatocellular adenoma	3.2	0.8	1.3	2.6	2.0
	Hepatocellular carcinoma	0.9	2.2	0.7	0.3	0.3
Salivary gland	Myoepithelioma	0	0	0	0	6.4
	Sarcoma	0	0.1	0	0	4.3
Pituitary gland	Adenoma pars distalis	33.6	38.1	35.7	12.4	41.1
	Adenocarcinoma pars distalis	0	3.9	0.3	0.4	0
Adrenal gland	Cortical adenoma	1.4	3.3	0.7	0.7	1.3
	Cortical carcinoma	0.9	0.3	0.7	0.1	0.2
	Pheochromocytoma	10.0	6.0	11.3	11.9	3.2
	Malignant pheochromocytoma	0.5	0.3	1.0	0.3	0.8
Thyroid gland	C-cell adenoma	10.5	2.9	5.0	12.5	10.1
	C-cell carcinoma	0.5	1.1	0.3	0.4	1.4
	Follicular cell adenoma	0.9	1.3	0.7	0.8	3.1
	Follicular cell carcinoma	0.5	1.6	0.3	0.4	0.8
Pancreas	Acinar cell adenoma	3.6	1.1	0	0.3	1.4
	Acinar cell adenocarcinoma	0	0	0	0.0	0.4
	Islet cell adenoma	3.6	4.1	4.0	1.5	5.3
	Islet cell carcinoma	0.5	1.6	1.0	0.0	1.9
Kidney	Adenoma	0	0.4	0	0	0.3
	Adenocarcinoma	0	0.1	0.3	0	0.1
	Nephroblastoma	0	0.1	0	0.1	0.1
Hematopietic system	Malignant lymphoma	1.4	3.2	3.3	0.7	3.8
	Mononuclear cell leukemia	0	0	0.7	23.6	0
	Histiocytic sarcoma	2.7	0.8	3.3	0.1	1.4
	Myelocytic leukemia	0	0	0	0	0
Thymus	Thymoma	0	0.2	1.3	0.3	1.1
Testis	Interstitial cell tumor	1.8	6.2	7.0	74.6	4.3
Prostate	Adenoma	0.5	0	0	0.2	0.2
	Adenocarcinoma	0.0	0.4	0.3	0.1	0.1
Mammary gland	Fibroadenoma	1.4	2.5	1.3	0.8	1.2
	Adenoma	0.0	0.2	0.3	0.4	0.2
	Adenocarcinoma	0.5	0.7	0.3	0.1	0.5
Integumentary system	Papilloma	0.9	0.7	2.0	2.0	1.3
	Acanthoma	4.1	1.4	1.3	0.8	3.6
	Squamous cell carcinoma	0.5	0.4	0.7	1.4	0.9
	Fibroma	8.2	3.3	13.7	7.2	4.2
	Fibrosarcoma	3.2	1.0	1.0	0.7	0.3
Abdominal cavity	Lipoma	0	0	0	0	8.4
	Mesothelioma	0	0	0	2.9	3.1

Number of animals examined
a:n=110 b:n=150 c:n=1260 d:n=960 e:n=2280

Table 2. Incidence of spontaneous tumors in different strains of untreated female rats in 2-year carcinogenicity studies

Organ	Type of tumors	Incidence (%)				
		IGS a) (CRJ)	SD b) (CRJ)	SD c) (CRL)	F344 d) (CRJ)	Wistar e) (GALAS)
Brain	Granular cell tumor	0	0	0	0.1	1.1
	Glioma	0	0.3	0.3	0.4	0.5
Lung	Adenoma	0.5	0	0.7	2.2	0.1
	Adenocarcinoma/Carcinoma	0	0	0	0.1	0.1
Liver	Hepatocellular adenoma	0.5	2.0	0.7	1.7	3.2
	Hepatocellular carcinoma	0	1.7	0.7	0	0.6
Salivary gland	Myoepithelioma	0	0	0	0	0
	Sarcoma	0	0	0	0	0
Pituitary gland	Adenoma pars distalis	56.8	57.0	50.3	28.2	65.8
	Adenocarcinoma pars distalis	3.6	6.0	2.7	0.7	0.1
Adrenal gland	Cortical adenoma	1.8	5.2	1.7	2.2	1.8
	Cortical carcinoma	0.9	0.5	0	0.2	0.1
	Pheochromocytoma	2.3	1.1	2.3	3.2	1.3
	Malignant pheochromocytoma	0.5	0.3	2.3	0	0.3
Thyroid gland	C-cell adenoma	5.0	4.1	5.7	8.2	10.7
	C-cell carcinoma	0	1.2	1.0	0.2	1.3
	Follicular cell adenoma	0.9	0.5	1.7	0.3	1.9
	Follicular cell carcinoma	0	1.3	0	0.2	0.3
Pancreas	Acinar cell adenoma	0	0.1	0	0	0.1
	Acinar cell adenocarcinoma	0	0	0	0	0
	Islet cell adenoma	1.4	1.4	0.3	0.2	1.9
	Islet cell carcinoma	0	0.7	0.3	0	0.6
Kidney	Adenoma	0	0.1	0	0	0.2
	Adenocarcinoma	0	0.1	0.3	0	0
	Nephroblastoma	0	0	0	0	0
Hematopietic system	Malignant lymphoma	1.8	1.8	0.7	0.3	4.0
	Mononuclear cell leukemia	0	0	0	21.9	0
	Histiocytic sarcoma	1.4	0.6	3.3	0.1	0.8
	Myelocytic leukemia	0	0	0	0	0.2
Thymus	Thymoma	0	1.0	1.0	0.1	1.0
Ovary	Granulosa cell tumor	0	0.9	0.3	0.4	2.4
Uterus	Polyp	3.2	4.8	1.7	12.6	7.2
	Adenocarcinoma	0.5	0.2	0.3	0.2	3.4
	Stromal sarcoma	1.4	0.2	0	0.9	0.6
Clitoral gland	Adenoma/carcinoma	0.5	0	0	1.3	0
Mammary gland	Fibroadenoma	44.5	33.9	16.7	7.1	30.2
	Adenoma	5.5	4.1	14.0	6.2	1.6
	Adenocarcinoma	16.4	2.5	7.0	1.0	5.1
Integumentary system	Papilloma	0	0.4	0.3	0.6	0.2
	Acanthoma	0.9	0.5	0	0.2	0
	Squamous cell carcinoma	0	0.4	0	0.8	0.3
	Fibroma	5.0	1.4	3.3	1.1	1.1
	Fibrosarcoma	2.7	0.4	0.3	0.1	0.1
Abdominal cavity	Lipoma	0	0	0	0	11.4
	Mesothelioma	0	0	0	0	1.8

Number of animals examined
a:n=110 b:n=150 c:n=1263 d:n=959 e:n=2256

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CHAPTER 2

General Toxicology Related To

Baseline Data Comparing CD®(SD)IGS Rats Supplied From Charles River Japan, Charles River UK and Charles River USA.

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ABSTRACT. CD®(SD)IGS rats were provided from CRJ (Japan), CRUK (UK) and CRL Raleigh (USA) to the CR Sierra Biomedical test facility in California, for this study of potential differences among different CRL colonies. The animals were supplied from various facilities in the same week and at the same age, 5-weeks old. After one week of acclimation, the rats were 6-weeks old when the 6 week long study began and 11-weeks old when it ended. During the course of the study, body weight gains, food consumption rates, glucose tolerance test values, hematological values and blood chemical values were measured and analyzed. As the results, no statistically significant differences were observed among all animals from the three different geographic sources from Japan, UK and USA. The early stage differences of larger body weight from the CRL(UK) animals was attributed to the larger size of the rats at 5-weeks old, prior to the start of the experiment. — Key words: Crj:CD(SD)IGS, Baseline Data

CD(SD)IGS-2001: 9-18

INTRODUCTION

The CD®(SD)IGS rats were produced using the Charles River International Genetic Standard breeding system (IGS). The main objective of the IGS system is to minimize genetic differences among CD® rat colonies within the Charles River group worldwide. The baseline data presented in this article were obtained from rats supplied from Charles River Japan (Tsukuba Breeding Center), Charles River UK and Charles River USA (Raleigh, NC) and were collected under a single study protocol performed at the Sierra Biomedical, a Charles River Company, La Jolla, California.

MATERIALS AND METHODS

The testing protocol was performed at Sierra Biomedical, a Charles River Company, La Jolla, California, USA.

1. Date experiment performed:
   July − September, 2
2. Sources of animal used:
   CD®(SD)IGS rats supplied
   From: CR Japan, Tsukuba plant (CRJ)
   CR UK plant CRL(UK)
   CR USA, Raleigh plant CRL(R)
3. Number of animal used:
   2 males and 2 females each, at 5-weeks old, (total of 12) were shipped from the above three different geographic facilities to the CR Sierra Biomedical facility in La Jolla, California.
4. Age of animal used
   All animals were provided a period of observation and acclimation for one week after arrival at Sierra Biomedical facility. The experiment was started at 6-weeks old; and terminated when the rats were 11-weeks old.
5. Housing conditions:
   All animals were housed in wire-bottom cages from arrival to the end of the experiment. Animal room temperature was kept between 18℃ − 26℃, with 1 % fresh air supplied at 1 air changes per hour. The lighting cycle was 12 hours light, 12 hours dark. Filtered drinking water from bottles; and Certified Rodent Diet 5 2 (Purina) were supplied to the animals *ad libitum* during the experiment.
6. Experimental method:
   General clinical observations were made on a daily basis. Body weight gains and food consumption rates were measured weekly. Blood samples were collected at ages 6, 9 and 11-weeks old from the tail vein after fasting. EDTA was added to blood samples for hemato-logical analysis. Sera were also obtained for a routine panel of serum chemistries. Glucose tolerance tests were also performed at 8-weeks old after 12-hours fasting period by oral administration of a 5 % dextrose solution at 2g/kg. Blood glucose values were measured from tail vein blood samples taken 15min., 3 min., 6 min. and 12 min. after dextrose administration.
7. Statistical calculations:
   Group means, with standard deviations, were calculated on male and female groups. Significant differences among different facilities were identified by using the ANOVA method on combined data from males and females.

RESULTS

1. General clinical observations:
   One male rat died at 8-weeks old from the CRL(UK) group. The death was attributed to be a dosing accident. All other animals were observed normal during the entire course of the experiment. (Table 1 and Figure 1)
2. Body weights:
   CRL(UK) animals were observed to be larger at 5-weeks old and statistically larger than other groups at 6-weeks old. Despite this observation, no significant differences in the body weight gain rate were observed among all groups. (Table 2)
3. Food consumption rates:
   Except a transient significant increase in the consumption rate at 6-7 weeks old, there was no significant difference

observed among the groups during the period of the experiment. (Table 3)

4. Glucose tolerance testing values:
   No significant difference in the ΣGTT value and blood glucose values from Omin. to 12 min. was observed. The GTT values obtained from the CRL(USA, Raleigh) group were excluded because of technical error during measurement. (Table 4)
5. Hematological values:
   The eryhtrocyte parameters of the CRJ animals had some statistically significant lower values when compared with those of CRL(Raleigh) animals; also the HCT values of CRJ animals were lower than those of the CRL(UK) animals. (Tables 5, 6 and 7)
6. Serum chemistry values:
   No significant differences were observed in any parameter during the course of the experiment, between 6 and 11 -weeks old. (Tables 8, 9 and 1)

DISCUSSION

The statistically significant larger size observed in the CRL(UK) animals was due mainly to the fact that these animals were larger on arrival than those supplied from CRJ and CRL(R). Due to the fact that no significant difference was observed in the actual rate of weight gain among all three groups, it was concluded that there was no significant difference in body weight increase among the animals from these three different geographic facilities. As to the differences in the hematological values observed in CRJ animals, both erythrocyte parameters (RBC and HCT) were within the normal physiological ranges and thus should not be considered as significant differences due to geographic facility differences.

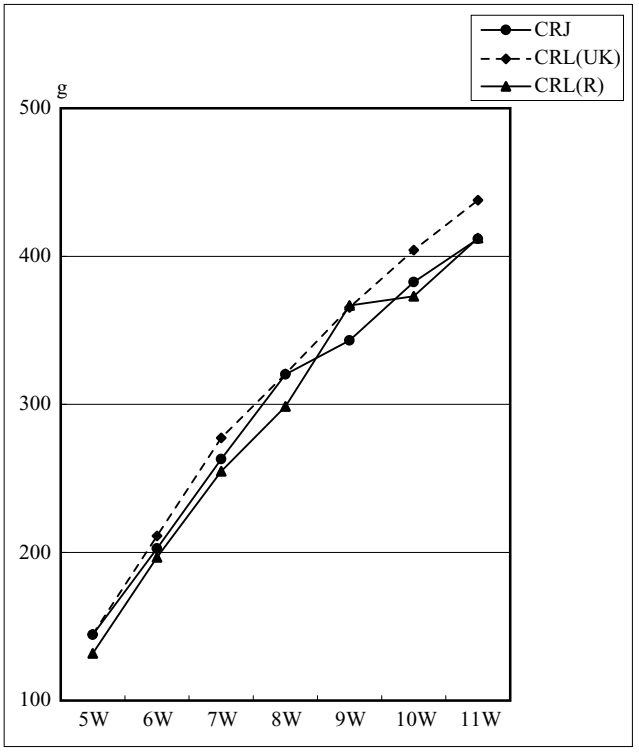

Figure 1-1. Body weight changes(♂)

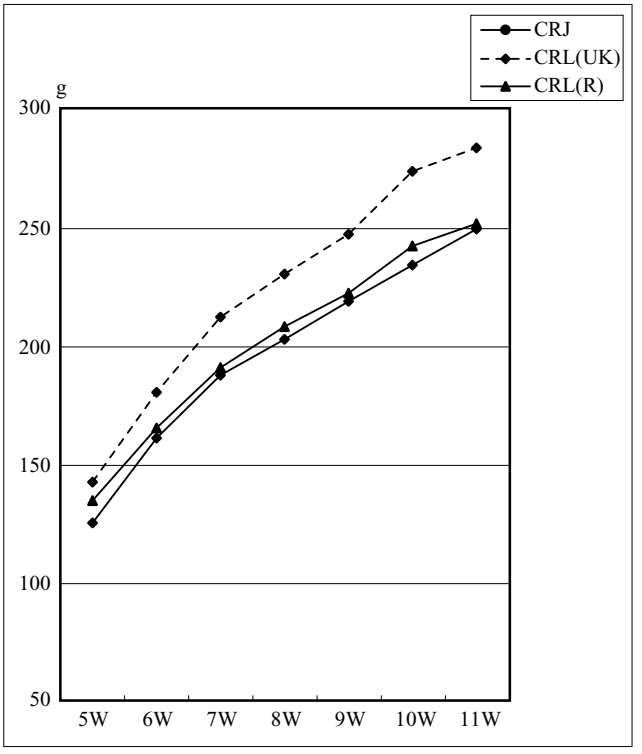

Figure 1-2. Body weight changes(♀)

CRJ; Charles River Japan
CRL(UK); Chales River United Kingdam
CRL(R); Charles River USA (Raleigh, NC)

Table 1. Body weight values

		5W	6W	7W	8W	9W	10W	11W
♂	CRJ	144.4±8.9[a)]	202.6±17.1	263.0±17.9	320.2±22.2	343.1±26.2	382.5±29.1	411.7±34.9
		N=20	N=20	N=20	N=10	N=20	N=20	N=20
	CRL(UK)	144.6±13.1	211.0±16.1	277.3±19.3	320.6±23.5	365.5±24.5	404.2±37.4	437.8±32.2
		N=20	N=20	N=20	N=10	N=19	N=19	N=19
	CRL(R)	131.7±13.9	196.6±15.3	254.8±18.2	298.5±18.7	336.7±22.3	373.0±21.6	412.2±32.4
		N=20	N=20	N=20	N=10	N=20	N=20	N=20
♀	CRJ	125.7±6.3	161.5±10.6	188.1±15.1	203.3±22.6	219.3±19.2	234.6±19.9	249.8±23.4
		N=20	N=20	N=20	N=10	N=20	N=20	N=20
	CRL(UK)	142.9±11.5	180.8±12.7	212.6±18.7	230.8±17.6	247.7±23.9	274.2±25.4	284.2±33.8
		N=20	N=20	N=20	N=10	N=20	N=20	N=20
	CRL(R)	135.0±8.6	165.8±8.6	191.3±10.0	208.6±11.8	222.8±13.3	242.7±26.9	252.1±16.4
		N=20	N=20	N=20	N=10	N=20	N=20	N=20
Combined	CRJ	135.0±12.2	182.1±25.1	225.5±41.3	261.7±63.8	281.2±66.7	308.6±78.8	330.8±87.1
		N=40	N=40	N=40	N=20	N=40	N=40	N=40
	CRL(UK)	143.8±12.2	195.9±21.0	244.9±37.7	275.7±50.3	305.1±64.3	337.6±72.9	359.1±84.4
		N=40	N=40	N=40	N=20	N=39	N=39	N=39
	CRL(R)	133.4±11.5	181.2±19.8	233.1±35.3	253.6±48.5	279.7±60.5	307.9±70.3	332.2±84.9
		N=40	N=40	N=40	N=20	N=40	N=40	N=40
	P	CRJ VS UK: <0.01 ↓	CRJ VS UK: <0.05 ↓	CRL(R) VS UK: <0.01 ↓				
		CRL (R) VS UK: <0.001 ↓	CRL (R) VS UK: <0.05 ↓					

(g)
CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 2. Body weight gain rates

		5～6W	6～7W	7～8W	8～9W	9～10W	10～11W
♂	CRJ	7.3±1.4[a)]	8.6±1.3	8.2±1.5	5.1±0.9	5.6±0.9	4.2±2.3
		N=20	N=20	N=10	N=10	N=20	N=20
	CRL(UK)	8.3±1.1	9.5±1.5	7.1±0.8	5.9±1.0	5.5±2.7	4.8±3.1
		N=20	N=20	N=10	N=10	N=19	N=19
	CRL(R)	8.1±0.6	8.3±0.8	6.6±0.6	5.5±0.8	5.2±1.9	5.6±3.9
		N=20	N=20	N=10	N=10	N=20	N=20
♀	CRJ	4.5±1.0	3.8±1.0	2.3±0.6	1.9±0.8	2.2±1.0	2.2±0.8
		N=20	N=20	N=10	N=10	N=20	N=20
	CRL(UK)	4.7±0.7	4.5±1.1	2.6±0.7	2.9±0.9	3.8±2.4	1.4±4.5
		N=20	N=20	N=10	N=10	N=20	N=20
	CRL(R)	3.8±0.6	3.6±0.6	2.5±0.8	2.3±0.6	2.8±3.2	1.3±3.1
		N=20	N=20	N=10	N=10	N=20	N=20
Combined	CRJ	5.9±1.9	6.2±2.7	5.3±3.2	3.5±1.8	3.9±2.0	3.2±2.0
		N=40	N=40	N=20	N=20	N=40	N=40
	CRL(UK)	6.5±2.0	7.0±2.8	4.9±2.4	4.4±1.8	4.6±2.6	3.1±4.2
		N=40	N=40	N=20	N=20	N=39	N=39
	CRL(R)	6.0±2.2	6.0±2.5	4.6±2.2	3.9±1.7	4.0±2.9	3.5±4.1
		N=40	N=40	N=20	N=20	N=40	N=40

(g)
CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 3. Food consumption rates

		6W～7W	7W～8W	8W～9W	9W～10W	10W～11W
♂	CRJ	208.3±15.4[a)]	212.9±28.5	219.2±24.6	220.3±19.2	221.0±26.0
		N=20	N=9	N=20	N=20	N=20
	CRL(UK)	219.8±22.9	215.5±19.4	233.7±17.1	234.3±26.3	227.0±26.2
		N=20	N=10	N=19	N=19	N=19
	CRL(R)	183.3±12.1	193.1±15.2	200.3±16.0	207.9±17.1	202.1±20.1
		N=20	N=10	N=20	N=20	N=20
♀	CRJ	142.5±15.2	139.9±20.6	149.6±19.5	167.0±44.8	153.6±23.9
		N=20	N=10	N=20	N=19	N=20
	CRL(UK)	151.3±16.3	152.4±15.3	154.1±18.5	159.8±18.1	158.1±30.1
		N=20	N=10	N=20	N=20	N=20
	CRL(R)	140.3±8.7	147.3±10.5	149.3±10.8	157.7±14.0	151.5±14.1
		N=20	N=10	N=20	N=20	N=20
Combined	CRJ	175.4±36.6	174.5±44.4	184.4±41.5	194.4±43.2	187.3±42.1
		N=40	N=19	N=40	N=39	N=40
	CRL(UK)	185.5±39.8	183.9±36.6	192.9±44.0	196.1±43.7	191.7±44.7
		N=40	N=20	N=39	N=39	N=39
	CRL(R)	161.8±24.1	170.2±26.7	174.8±29.1	182.8±29.7	176.8±30.8
		N=40	N=20	N=40	N=40	N=40
	P	CRL(R) VS UK: <0.01 ↓				

(g)
CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 4. Glocose tolerance testing values

		0min.	15min.	30min.	60min.	120min.	ΣGTT
♂	CRJ	89.9±11.0[a)]	138.9±25.8	167.8±16.4	150.2±7.2	97.4±8.7	644.2±43.5
N=10	CRL(UK)	70.6±5.2	156.0±20.1	181.6±22.3	135.4±14.2	102.9±5.4	646.5±40.5
♀	CRJ	86.1±7.0	155.1±30.6	189.5±25.7	145.1±19.5	100.0±9.6	675.8±54.7
N=10	CRL(UK)	73.3±8.3	172.3±35.4	176.3±18.7	119.4±13.2	101.1±10.7	642.4±51.6
Combined	CRJ	88.0±9.2	147.0±28.8	178.7±23.8	147.7±14.5	98.7±9.0	660.1±50.8
N=20	CRL(UK)	72.0±6.9	164.6±29.6	178.8±20.1	127.0±15.6	101.9±8.4	644.3±45.4

(mg/dl)
CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
a)Mean±S.D.

Table 5-1. Blood hematological values(6-weeks old)

		WBC(/nl)	RBC(/pl)	HGB(g/dl)	HCT(%)	MCV(fl)	MCH(pg)	MCHC(g/dl)	Platelets(nl)
♂	CRJ	12.9±2.5[a)]	6.6±0.4	13.2±0.8	40.6±2.5	62.1±2.2	20.3±0.9	32.5±0.9	948.7±119.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	14.7±3.9	6.7±0.4	13.3±0.6	42.0±1.9	62.8±1.7	19.9±0.6	32.0±0.4	987.3±155.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	15.0±2.8	6.7±0.3	13.3±0.8	42.1±2.5	62.4±2.1	19.8±0.9	31.8±0.7	1013.6±119.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	11.8±3.4	6.8±0.3	13.4±0.8	40.9±2.0	60.5±1.7	19.6±0.6	32.7±0.6	1124.2±226.2
		N=20	N=19	N=19	N=19	N=20	N=19	N=19	N=20
	CRL(UK)	12.8±2.4	7.4±0.4	14.5±0.7	43.3±1.8	58.3±1.8	19.3±0.8	33.4±0.6	1078.7±165.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	14.3±4.0	7.3±0.4	14.3±0.7	43.9±2.0	60.2±1.6	19.7±0.7	32.5±0.6	1236.6±346.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	12.3±3.0	6.7±0.4	13.3±0.8	40.8±2.3	61.3±2.1	19.9±0.8	32.6±0.7	1036.5±199.4
		N=40	N=39	N=39	N=39	N=40	N=39	N=39	N=40
	CRL(UK)	13.7±3.3	7.1±0.5	13.9±0.8	42.6±1.9	60.5±2.8	19.6±0.7	32.7±0.9	1033.0±164.7
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(R)	14.7±3.4	7.0±0.5	13.8±0.9	43.0±2.4	61.3±2.2	19.7±0.8	32.1±0.7	1125.1±279.4
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P	CRJ VS CRL(R): <0.01 ↓	CRJ VS UK: <0.001 ↓ CRJ VS CRL(R): <0.01 ↓	CRJ VS UK: <0.01 ↓ CRJ VS CRL(R): <0.05 ↓	CRJ VS UK: <0.01 ↓ CRJ VS CRL(R): <0.001 ↓			CRJ VS CRL(R): <0.05 ↑ CRL(R) VS UK: <0.01 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 5-2. Blood hematological values(6-weeks old)

		Seg neut(%)	Bands(%)	Lymph(%)	Mono(%)	Eos(%)	Baso(%)	Meta(%)
♂	CRJ	15.5±8.1[a)]	0.0±0.0	79.8±7.9	4.1±2.4	0.7±1.0	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	20.9±6.4	0.0±0.0	75.1±6.1	3.8±2.0	0.3±0.6	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	11.3±5.5	0.0±0.0	82.8±5.9	5.2±2.5	0.7±0.9	0.1±0.2	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	17.3±5.2	0.0±0.0	77.9±5.9	3.6±1.6	1.2±1.2	0.1±0.3	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	12.9±4.8	0.0±0.0	82.6±5.9	3.4±2.0	1.1±0.8	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	9.1±5.3	0.0±0.0	86.5±7.2	3.9±2.5	0.6±0.6	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	16.4±6.8	0.0±0.0	78.8±7.0	3.8±2.0	1.0±1.1	0.1±0.2	0.0±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(UK)	16.9±6.9	0.0±0.0	78.8±7.0	3.6±2.0	0.7±0.8	0.0±0.0	0.0±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(R)	10.2±5.4	0.0±0.0	84.6±6.8	4.6±2.6	0.7±0.7	0.0±0.2	0.0±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P	CRJ VS CRL(R): <0.001 ↑ CRL(R) VS UK: <0.001 ↓		CRJ VS CRL(R): <0.01 ↓ UK VS CRL(R): <0.01 ↓				

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 6-1. Blood hematological values(9-weeks old)

		WBC(/nl)	RBC(/pl)	HGB(g/dl)	HCT(%)	MCV(fl)	MCH(pg)	MCHC(g/dl)	Platelets(nl)
♂	CRJ	13.0±3.9[a)]	7.8±0.3	14.7±0.5	44.9±1.6	57.7±1.7	19.1±0.7	32.9±0.7	1023.2±151.8
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	14.0±3.4	8.0±0.4	15.2±0.5	46.8±1.8	58.8±1.3	19.1±0.7	32.6±0.8	983.5±143.5
		N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(R)	17.5±4.1	8.1±0.3	15.0±0.6	45.7±1.9	56.6±1.2	18.6±0.7	32.7±0.8	1057.0±160.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	12.5±2.1	7.9±0.5	14.8±1.0	44.4±3.0	56.0±1.6	18.7±0.7	33.3±0.7	967.1±211.0
		N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(UK)	11.8±3.2	8.3±0.5	15.3±0.7	45.7±2.2	55.3±1.7	18.5±0.9	33.5±0.9	878.1±182.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	13.2±4.2	8.1±0.4	15.0±0.5	44.9±2.0	55.3±1.3	18.5±0.6	33.6±0.8	963.0±177.2
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	12.8±3.1	7.9±0.4	14.8±0.8	44.7±2.4	56.9±1.8	18.9±0.7	33.1±0.8	995.9±182.8
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(UK)	12.9±3.4	8.1±0.4	15.2±0.6	46.3±2.1	57.0±2.3	18.8±0.8	33.1±0.9	929.5±171.0
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(R)	15.3±4.6	8.1±0.4	15.0±0.5	45.3±2.0	55.9±1.4	18.5±0.6	33.2±0.9	1010.0±173.5
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P	CRJ VS CRL(R): <0.05 ↓	CRJ VS UK: <0.05 ↓	CRJ VS UK: <0.01 ↓	CRJ VS UK: <0.01 ↓	CRL(R) VS UK: <0.05 ↓			
		UK VS CRL(R): <0.05 ↓	CRJ VS CRL(R): <0.05 ↓						

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 6-2. Blood hematological values(9-weeks old)

		Seg neut(%)	Bands(%)	Lymph(%)	Mono(%)	Eos(%)	Baso(%)	Meta(%)
♂	CRJ	12.7±4.5[a)]	0.0±0.0	81.7±4.9	4.4±2.4	0.9±1.3	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	16.5±5.9	0.0±0.0	79.0±6.0	3.5±2.0	0.9±1.0	0.0±0.0	0.0±0.0
		N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(R)	8.2±5.2	0.0±0.0	86.9±6.1	4.4±2.2	0.5±0.7	0.1±0.3	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	12.5±5.7	0.0±0.0	84.4±6.3	2.7±1.7	0.5±0.8	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	13.5±7.0	0.0±0.0	80.0±7.3	5.1±2.9	1.5±1.5	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	8.7±4.4	0.0±0.0	86.8±5.3	3.7±2.5	0.9±1.0	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	12.6±5.1	0.0±0.0	83.0±5.7	3.5±2.2	0.7±1.0	0.0±0.0	0.0±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(UK)	14.9±6.6	0.0±0.0	79.5±6.6	4.3±2.6	1.2±1.3	0.0±0.0	0.0±0.0
		N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(R)	8.4±4.7	0.0±0.0	86.9±5.6	4.0±2.3	0.7±0.9	0.1±0.2	0.0±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P	CRJ VS CRL(R): <0.01 ↑	CRJ VS UK: <0.05 ↑					
		CRL(R) VS UK: <0.001 ↓	CRJ VS CRL(R): <0.05 ↓					
			UK VS CRL(R): <0.001 ↓					

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 7-1. Blood hematological values(11-weeks old)

		WBC(/nl)	RBC(/pl)	HGB(g/dl)	HCT(%)	MCV(fl)	MCH(pg)	MCHC(g/dl)	Platelets(nl)
♂	CRJ	12.4±4.3[a)]	8.3±0.4	14.9±0.6	46.9±1.9	56.9±1.8	18.2±0.5	31.7±0.9	945.8±159.5
	N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19	
	CRL(UK)	13.9±4.7	8.5±0.3	15.3±0.5	50.7±1.6	59.6±1.3	18.1±0.5	30.3±0.7	916.7±131.9
		N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(R)	14.0±3.9	8.8±0.4	15.5±0.6	52.3±2.5	59.6±1.3	17.8±0.7	29.7±0.8	934.7±145.5
		N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19
♀	CRJ	9.8±3.5	8.1±0.3	14.8±0.5	47.6±1.6	58.6±2.1	18.3±0.7	31.3±0.6	927.5±224.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	8.0±2.6	8.2±0.4	14.7±0.5	47.2±1.8	57.9±1.5	17.9±0.7	31.4±0.7	911.4±149.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	7.7±2.5	8.2±0.4	15.0±0.7	49.2±2.9	60.2±1.6	18.4±0.6	30.6±0.8	915.8±207.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	11.1±4.1	8.2±0.4	14.9±0.5	47.3±1.8	57.7±2.1	18.2±0.6	31.5±0.8	936.4±193.2
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(UK)	10.9±4.8	8.3±0.4	15.0±0.6	48.9±2.5	58.7±1.7	18.0±0.6	30.9±0.9	913.9±139.3
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(R)	10.8±4.5	8.5±0.5	15.2±0.7	50.7±3.1	59.9±1.5	18.1±0.7	30.1±0.9	925.0±177.7
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	P		CRJ VS CRL(R): <0.05 ↓	CRJ VS CRL(R): <0.05 ↓	CRJ VS UK: <0.05 ↓ CRJ VS CRL(R): <0.001 ↓ UK VS CRL(R): <0.01 ↓	CRJ VS CRL(R): <0.001 ↓ UK VS CRL(R): <0.05 ↓		CRJ VS UK: <0.01 ↑ CRJ VS CRL(R): <0.001 ↑ CRL(R) VS UK: <0.001 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 7-2. Blood hematological values(11-weeks old)

		Seg neut(%)	Bands(%)	Lymph(%)	Mono(%)	Eos(%)	Baso(%)	Meta(%)
♂	CRJ	11.6±5.7[a)]	0.0±0.0	83.4±6.5	3.6±2.0	1.2±1.4	0.0±0.0	0.0±0.0
		N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(UK)	9.3±3.4	0.0±0.0	86.4±3.8	3.3±2.1	0.9±1.0	0.0±0.0	0.0±0.0
		N=19	N=19	N=19	N=19	N=19	N=19	N=19
	CRL(R)	9.3±5.6	0.0±0.0	85.6±6.8	4.8±2.5	0.2±0.4	0.0±0.0	0.0±0.0
		N=19	N=19	N=19	N=19	N=19	N=19	N=19
♀	CRJ	7.9±3.7	0.0±0.0	86.7±3.4	4.8±2.9	0.7±1.2	0.0±0.0	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	10.7±8.2	0.0±0.0	87.0±8.9	1.5±1.7	0.9±0.9	0.1±0.2	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	8.5±4.5	0.0±0.0	88.2±5.0	2.7±2.2	0.7±0.9	0.1±0.2	0.0±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	9.7±5.1	0.1±0.3	85.1±5.3	4.2±2.5	0.9±1.3	0.0±0.0	0.0±0.0
		N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(UK)	10.0±6.3	0.0±0.0	86.7±6.8	2.4±2.1	0.9±0.9	0.0±0.2	0.0±0.0
		N=39	N=39	N=39	N=39	N=39	N=39	N=39
	CRL(R)	8.9±5.0	0.0±0.0	86.9±6.0	3.7±2.5	0.4±0.7	0.0±0.2	0.0±0.0
	N=39	N=39	N=39	N=39	N=39	N=39	N=39	
	P			CRJ VS UK: <0.01 ↑ UK VS CRL(R): <0.05 ↓				

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA（Raleigh, NC)
a)Mean±S.D.

Table 8-1. Blood chemical values(6-weeks old)

		TP(g/dl)	ALB(g/dl)	Globulin(g/dl)	Glucose(mg/dl)	T-cho(mg/dl)	TG(mg/dl)	BUN(mg/dl)	CRE(mg/dl)
♂	CRJ	5.4±0.2[a)]	2.8±0.2	2.6±0.1	158.8±13.6	82.6±14.3	75.0±20.0	11.0±1.8	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	5.5±0.2	3.0±0.3	2.5±0.3	167.5±13.1	82.3±11.4	60.6±21.6	9.7±3.6	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	5.5±0.2	2.9±0.1	2.6±0.1	157.7±16.2	83.6±10.4	93.5±39.8	12.0±1.6	0.4±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	5.8±0.4	3.3±0.3	2.5±0.3	159.4±9.5	95.4±10.9	43.4±13.5	9.6±2.3	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	6.0±0.3	3.5±0.3	2.5±0.2	159.6±18.7	98.7±14.6	61.0±13.8	12.3±2.4	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	6.0±0.3	3.3±0.2	2.7±0.1	162.7±15.7	89.5±11.7	55.5±27.3	13.2±1.8	0.4±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	5.6±0.4	3.1±0.4	2.5±0.2	159.1±11.6	89.0±14.1	59.2±23.2	10.3±2.2	0.4±0.1
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(UK)	5.8±0.4	3.2±0.4	2.5±0.3	163.5±16.4	90.5±15.4	60.8±17.9	11.0±3.3	0.4±0.1
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(R)	5.7±0.3	3.1±0.3	2.7±0.1	160.2±15.9	86.5±11.3	74.5±38.8	12.6±1.8	0.4±0.0
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P			CRJ VS CRL(R): <0.05 ↓ UK VS CRL(R): <0.05 ↓				CRJ VS CRL(R): <0.001 ↓ UK VS CRL(R): <0.05 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 8-2. Blood chemical values(6-weeks old)

		BIL(mg/dl)	ALT(IU/l)	ALP(IU/l)	Pho(mg/dl)	Sodium(mmol/l)	Potassium(mmol/l)	Chloride(mmol/l)	Calcium(mg/dl)
♂	CRJ	0.3±0.1[a)]	69.9±7.5	430.4±72.3	8.9±1.1	142.1±2.4	6.1±0.6	104.4±2.0	10.1±0.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	0.3±0.2	63.6±7.5	463.4±107.9	9.7±0.6	142.1±2.6	6.2±0.6	103.8±2.7	10.2±0.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	0.3±0.1	56.2±7.1	415.9±107.1	9.9±0.7	140.8±1.1	6.1±0.7	101.9±1.3	10.0±0.2
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	0.3±0.1	55.5±7.4	293.9±44.5	8.6±0.6	143.0±2.9	5.9±0.5	105.7±2.9	10.3±0.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	0.3±0.1	48.7±5.5	314.1±66.3	8.9±0.6	143.8±3.6	6.0±0.9	104.7±4.0	10.5±0.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	0.3±0.0	45.3±4.4	274.2±70.6	8.8±0.7	140.9±1.4	5.7±0.7	102.8±1.8	10.2±0.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	0.3±0.1	62.7±10.3	362.1±91.0	8.8±0.9	142.5±2.7	6.0±0.6	105.0±2.6	10.2±0.4
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(UK)	0.3±0.1	56.1±10.0	388.7±116.3	9.3±0.7	142.9±3.2	6.1±0.8	104.2±3.4	10.4±0.4
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(R)	0.3±0.1	50.7±8.0	345.0±114.7	9.3±0.9	140.9±1.2	5.9±0.7	102.4±1.6	10.1±0.3
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P		CRJ VS UK: <0.05 ↑ CRJ VS CRL(R): <0.001 ↑ CRL(R) VS UK: <0.05 ↓		CRJ VS UK: <0.05 ↓ CRJ VS CRL(R): <0.05 ↓	CRJ VS CRL(R): <0.05 ↑ CRL(R) VS UK: <0.01 ↓		CRJ VS CRL(R): <0.001 ↑ CRL(R) VS UK: <0.01 ↓	CRL(R) VS UK: <0.05 ↓

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 9-1. Blood chemical values(9-weeks old)

		TP(g/dl)	ALB(g/dl)	Globulin(g/dl)	Glucose(mg/dl)	T-cho(mg/dl)	TG(mg/dl)	BUN(mg/dl)	CRE(mg/dl)
♂	CRJ	6.0±0.3[a)]	3.2±0.3	2.7±0.1	151.5±27.2	76.9±11.7	122.3±36.7	15.3±1.6	0.4±0.0
		N=20	N=19	N=19	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	6.2±0.3	3.3±0.1	2.9±0.3	147.6±13.9	76.6±13.0	119.6±39.4	13.1±1.7	0.4±0.1
		N=18	N=18	N=18	N=18	N=18	N=19	N=18	N=18
	CRL(R)	6.1±0.1	3.4±0.2	2.8±0.2	150.2±17.2	79.8±11.9	176.6±71.0	15.1±2.1	0.4±0.0
		N=19	N=19	N=19	N=19	N=19	N=20	N=19	N=19
♀	CRJ	6.3±0.2	3.4±0.3	2.8±0.2	156.6±12.8	87.4±10.7	55.2±18.3	13.3±1.3	0.4±0.0
		N=18	N=16	N=16	N=18	N=18	N=19	N=17	N=18
	CRL(UK)	6.5±0.3	3.5±0.2	3.0±0.2	155.0±16.4	99.9±12.5	64.6±24.4	11.8±1.4	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	6.5±0.3	3.8±0.3	2.7±0.2	158.7±16.9	101.7±11.1	86.3±30.6	14.6±1.6	0.4±0.0
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	6.1±0.3	3.3±0.3	2.8±0.2	153.9±21.5	81.9±12.3	89.6±44.6	14.4±1.8	0.4±0.0
		N=38	N=35	N=35	N=38	N=38	N=39	N=37	N=38
	CRL(UK)	6.4±0.4	3.4±0.2	3.0±0.3	151.4±15.5	88.8±17.2	91.4±42.5	12.4±1.7	0.4±0.1
		N=38	N=38	N=38	N=38	N=38	N=39	N=38	N=38
	CRL(R)	6.3±0.3	3.6±0.3	2.7±0.2	154.5±17.4	91.0±15.8	131.4±70.7	14.8±1.9	0.4±0.0
		N=39	N=39	N=39	N=39	N=39	N=40	N=39	N=39
	P	CRJ VS UK: <0.001 ↓	CRJ VS CRL(R): <0.001 ↓	CRJ VS UK: <0.001 ↓		CRJ VS CRL(R): <0.05 ↓	CRJ VS CRL(R): <0.01 ↓	CRJ VS UK: <0.001 ↑	
		CRJ VS CRL(R): <0.01 ↓	UK VS CRL(R): <0.01 ↓	CRL(R) VS UK: <0.001 ↓			UK VS CRL(R): <0.01 ↓	UK VS CRL(R): <0.001 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 9-2. Blood chemical values(9-weeks old)

		BIL(mg/dl)	ALT(IU/l)	ALP(IU/l)	Pho(mg/dl)	Sodium(mmol/l)	Potassium(mmol/l)	Chloride(mmol/l)	Calcium(mg/dl)
♂	CRJ	0.3±0.1[a)]	60.7±13.6	371.9±80.3	8.4±0.7	144.2±2.0	5.4±0.4	104.5±1.7	10.2±0.3
		N=20	N=19	N=19	N=19	N=20	N=19	N=20	N=19
	CRL(UK)	0.2±0.1	54.9±8.6	399.2±81.1	8.5±0.6	144.2±1.0	5.4±0.3	104.0±1.0	10.4±0.3
		N=18	N=18	N=18	N=18	N=18	N=18	N=18	N=18
	CRL(R)	0.3±0.1	52.5±10.5	335.8±98.7	8.8±0.4	146.1±2.6	5.9±0.3	105.3±2.8	10.1±0.3
		N=19	N=19	N=19	N=19	N=19	N=19	N=19	N=19
♀	CRJ	0.3±0.1	48.7±7.4	246.8±45.0	7.2±0.7	143.5±1.5	5.1±0.4	105.3±2.0	10.3±0.3
		N=17	N=17	N=16	N=17	N=17	N=17	N=18	N=17
	CRL(UK)	0.3±0.0	45.9±7.6	240.5±45.1	7.6±0.4	144.2±2.1	5.1±0.4	104.9±2.2	10.7±0.3
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	0.3±0.1	47.1±11.7	214.3±65.3	7.1±0.9	146.1±2.4	5.3±0.4	108.1±2.2	10.6±0.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	0.3±0.1	55.0±12.5	314.7±91.2	7.9±0.9	143.9±1.8	5.3±0.4	104.9±1.9	10.3±0.3
		N=37	N=36	N=35	N=36	N=37	N=36	N=38	N=36
	CRL(UK)	0.3±0.1	50.2±9.2	315.7±102.6	8.0±0.7	144.2±1.6	5.3±0.4	104.4±1.8	10.5±0.3
		N=38	N=38	N=38	N=38	N=38	N=38	N=38	N=38
	CRL(R)	0.3±0.1	49.7±11.4	273.5±102.6	7.9±1.1	146.1±2.4	5.6±0.4	106.7±2.9	10.4±0.4
		N=39	N=39	N=39	N=39	N=39	N=39	N=39	N=39
	P	UK VS CRL(R): <0.01 ↓				CRJ VS CRL(R): <0.001 ↓	CRJ VS CRL(R): <0.01 ↓	CRJ VS CRL(R): <0.01 ↓	CRJ VS UK: <0.01 ↓
						UK VS CRL(R): <0.001 ↓	UK VS CRL(R): <0.01 ↓	UK VS CRL(R): <0.001 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 10-1. Blood chemical values(11-weeks old)

		TP(g/dl)	ALB(g/dl)	Globulin(g/dl)	Glucose(mg/dl)	T-cho(mg/dl)	TG(mg/dl)	BUN(mg/dl)	CRE(mg/dl)
♂	CRJ	6.3±0.3[a)]	3.2±0.2	3.1±0.1	190.0±60.8	67.2±9.5	129.3±49.0	17.5±1.8	0.5±0.1
		N=20	N=20	N=20	N=20	N=18	N=20	N=20	N=20
	CRL(UK)	6.6±0.3	3.4±0.2	3.2±0.1	235.8±68.4	70.4±10.5	149.3±40.3	16.4±1.7	0.5±0.1
		N=19	N=19	N=19	N=19	N=18	N=19	N=19	N=19
	CRL(R)	6.5±0.3	3.4±0.2	3.1±0.2	214.2±48.7	71.9±13.7	208.5±117.8	15.2±3.3	0.5±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
♀	CRJ	6.8±0.3	3.6±0.3	3.2±0.1	211.1±40.7	82.7±11.3	86.6±38.2	16.2±2.2	0.5±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	7.0±0.3	3.9±0.3	3.1±0.1	197.1±43.6	88.5±14.6	86.4±41.8	15.4±2.5	0.5±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(R)	6.9±0.4	3.9±0.3	3.0±0.1	173.2±24.3	87.0±12.8	148.0±90.7	14.5±2.3	0.4±0.1
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	6.5±0.4	3.4±0.4	3.1±0.1	200.5±52.1	75.3±13.0	108.0±48.5	16.8±2.1	0.5±0.1
		N=40	N=40	N=40	N=40	N=38	N=40	N=40	N=40
	CRL(UK)	6.8±0.4	3.6±0.3	3.1±0.1	215.9±59.6	79.9±15.6	117.0±51.6	15.8±2.2	0.5±0.1
		N=39	N=39	N=39	N=39	N=38	N=39	N=39	N=39
	CRL(R)	6.7±0.4	3.6±0.4	3.1±0.2	193.7±43.3	79.4±15.1	178.2±108.2	14.8±2.8	0.5±0.1
		N=40	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	P	CRJ VS UK: <0.05 ↓	CRJ VS UK: <0.05 ↓ CRJ VS CRL(R): <0.05 ↓				CRJ VS CRL(R): <0.001 ↓ UK VS CRL(R): <0.01 ↓	CRJ VS CRL(R): <0.01 ↑	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Table 10-2. Blood chemical values(11-weeks old)

		BIL(mg/dl)	ALT(IU/l)	ALP(IU/l)	Pho(mg/dl)	Sodium(mmol/l)	Potassium(mmol/l)	Chloride(mmol/l)	Calcium(mg/dl)
♂	CRJ	0.3±0.5[a)]	64.8±13.6	286.1±67.1	10.6±1.3	146.6±1.7	7.6±1.1	98.7±1.7	11.1±0.5
		N=17	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	0.3±0.3	60.8±11.1	339.1±84.3	11.2±1.3	145.7±1.7	8.7±1.4	98.3±1.6	11.1±0.5
		N=15	N=19	N=19	N=19	N=19	N=17	N=19	N=19
	CRL(R)	0.2±0.1	62.3±28.4	259.5±71.2	12.0±2.0	144.9±2.9	9.0±1.9	96.5±1.7	11.2±0.2
		N=16	N=20	N=20	N=20	N=20	N=18	N=20	N=20
♀	CRJ	0.3±0.2	53.9±9.2	193.5±38.8	9.8±1.2	146.1±1.4	7.6±0.7	100.3±2.0	11.3±0.6
		N=19	N=20	N=20	N=20	N=20	N=20	N=20	N=20
	CRL(UK)	0.3±0.1	50.0±9.9	213.5±51.4	10.7±1.5	145.0±1.5	10.4±1.1	100.8±1.2	11.2±0.4
		N=20	N=20	N=20	N=20	N=20	N=18	N=20	N=20
	CRL(R)	0.2±0.1	49.0±10.6	163.2±68.6	10.0±0.8	144.6±1.3	8.8±1.1	98.9±2.0	11.2±0.4
		N=20	N=20	N=20	N=20	N=20	N=20	N=20	N=20
Combined	CRJ	0.3±0.3	59.3±12.7	239.8±71.6	10.2±1.3	146.3±1.6	7.6±0.9	99.5±2.0	11.2±0.5
		N=36	N=40	N=40	N=40	N=40	N=40	N=40	N=40
	CRL(UK)	0.3±0.2	55.3±11.8	274.7±93.4	11.0±1.4	145.4±1.6	9.6±1.5	99.6±1.9	11.2±0.5
		N=35	N=39	N=39	N=39	N=39	N=35	N=39	N=39
	CRL(R)	0.2±0.1	55.6±22.2	211.4±84.5	11.0±1.8	144.7±2.2	8.9±1.5	97.7±2.2	11.2±0.3
		N=36	N=40	N=40	N=40	N=40	N=38	N=40	N=40
	P			CRL(R) VS UK: <0.01 ↓		CRJ VS CRL(R): <0.001 ↑	CRJ VS UK: <0.001 ↓ CRJ VS CRL(R): <0.001 ↓	CRJ VS CRL(R): <0.001 ↑ CRL(R) VS UK: <0.001 ↓	

CRJ;Charles River Japan
CRL(UK);Charles River United Kingdom
CRL(R);Charles River USA (Raleigh, NC)
a)Mean±S.D.

Body Weight, Food Consumption, Clinical Laboratory Test and Organ Weight Data in Crj:CD(SD)IGS Rats Before and After Forward Migration

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ABSTRACT. The Crj:CD(SD)IGS(IGS hereafter) rat strain was developed by Charles River, Inc. for the global supply of test animals with a uniform genetic character, as a part of an internationalization program. The forward migration was done on August, 1999 by Charles River Japan, Inc. Atsugi breeding center to uniform the genetic character. The body weight gain, food consumption, clinical laboratory test and organ weight data in IGS rats were compared before and after the forward migration in An-Pyo Center. There were no differences between the results before and after the forward migration. — Key words: CD(SD)IGS, rat, forward migration, historical control data

CD(SD)IGS-2001: 19-23

The Crj:CD(SD)IGS(IGS hereafter) rat strain was developed by Charles River, Inc. for the global supply of test animals with a uniform genetic character, as a part of an internationalization program. The IGS rats are widely used in acute, subchronic, chronic and oncogenecity/carcinogenecity studies of chemicals in An-Pyo Center. The forward migration was done on August, 1999 by Charles River Japan, Inc. Atsugi breeding center to uniform the genetic character. The body weight gain, food consumption, hematological examination, blood chemistry examination and organ weight data in IGS rats were compared before the forward migration (October, 1997 to May, 1999, 11 studies) and after the forward migration (January, 2000 to May, 2001, 6 studies) in An-Pyo Center.

Animals were purchased at 4 weeks old (birth spread within 3 days) and acclimated to the testing facility for 7 days. Rats were housed in a hygienic barrier system controlled at a temperature of 23±3℃ and humidity of 55±20% under a 12-hr light/12-hr dark cycle. The rats were housed individually in a stainless steel wire mesh cage (W20.0 cm×D28.2 cm×H18.0 cm). The animals had free access to commercial feed (modified NIH open formula rat and mouse ration or CRF-1 sterilized by radiation, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water via an automatic watering nozzle. The rats were observed twice daily for clinical signs, and body weight and food consumption were measured weekly. The data were obtained from the animals aged 5 to 11 weeks old. Clinical laboratory tests (hematology and coagulation, blood chemistry examination) and organ weight measurement were conducted on the half of the animals at the age of 9 weeks and on the remaining half of the animals at the age 11 weeks.

The survival ratio in IGS rats was 100% and no abnormal clinical signs were observed during the rearing period.

There were no differences in the mean body weight (Fig. 1) or mean food consumption (Fig. 2) before and after the forward migration for either sex.

There were no differences in any item of the hematological and coagulation examinations (Table 1, 2) and blood chemistry examinations (Table 3, 4).

There were no differences in either absolute or relative organ weight of any organ of IGS rats (Table 5, 6).

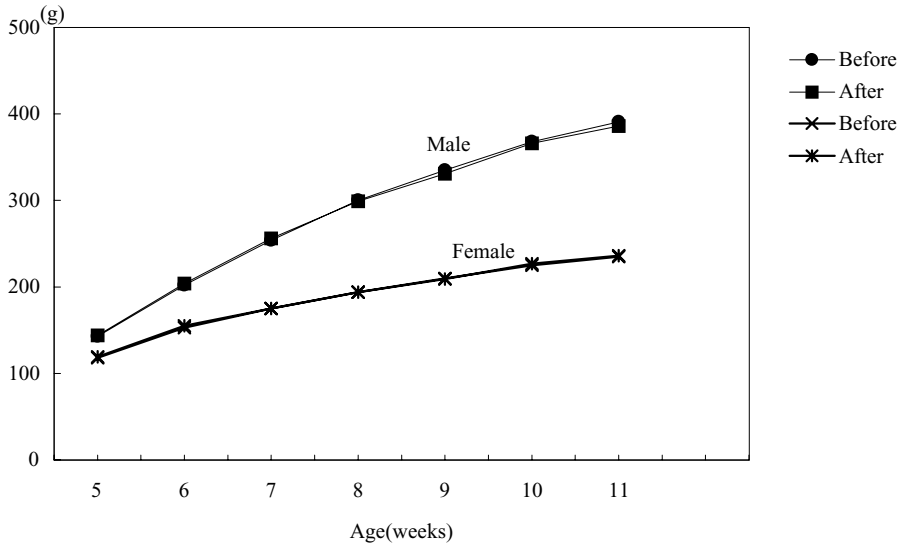

Fig. 1 Mean body weight in IGS rats.

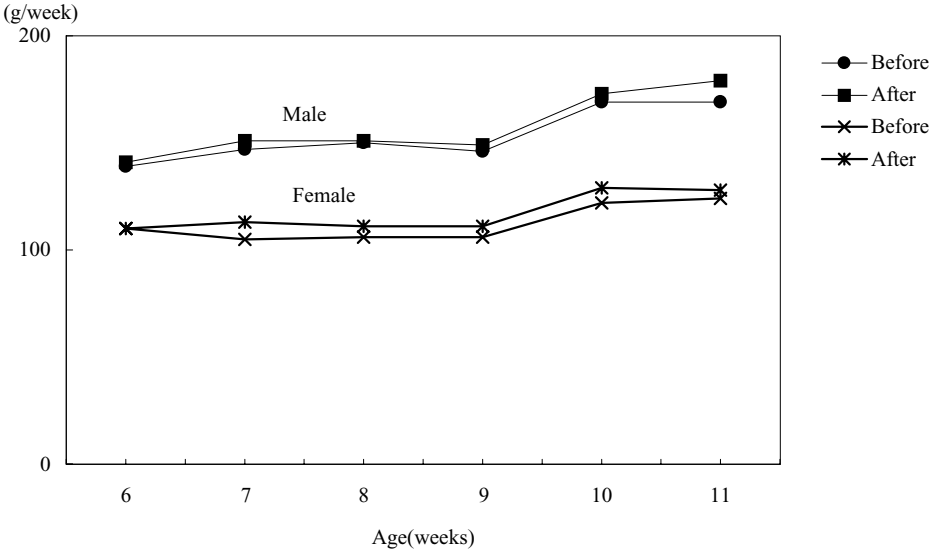

Fig. 2 Mean food consumption in IGS rats.

Table 1. Historical control data of hematology and coagulation in IGS rats aged 9 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
HCT(%)	45.3±1.6	45.8±1.4	43.6±2.3	44.1±1.4
HGB(g/dL)	15.2±0.4	15.2±0.5	15.0±0.6	15.1±0.5
RBC($x10^6/mm^3$)	7.76±0.31	7.82±0.26	7.64±0.34	7.62±0.30
MCV(μm^3)	58.4±2.0	59.0±1.4	57.1±1.8	57.9±1.4
MCH(pg)	19.6±0.5	19.6±0.5	19.7±0.5	19.9±0.4
MCHC(%)	33.6±0.9	33.3±0.5	34.6±0.9	34.3±0.6
PLT($x10^3/mm^3$)	1190±168	1203±109	1219±151	1258±124
WBC($x10^3/mm^3$)	10.7±3.1	9.6±2.2	6.5±2.1	6.3±1.9
Differential leukocyte counts(%)				
NEUT	10±3	11±4	11±4	12±4
LYMPH	87±3	86±5	85±4	85±4
MONO	2±1	2±1	2±1	2±1
EOSN	1±0	1±0	1±1	1±1
BASO	0±0	0±0	0±0	0±0
LUC	1±1	1±1	1±1	1±1
PT(sec.)	14.5±0.8	15.6±0.7	14.6±0.6	15.2±0.8
APTT(sec.)	26.2±1.6	20.6±1.2	22.3±1.8	18.4±0.6
Fibrinogen(mg/dL)	223±17	252±23	185±14	198±23

NEUT: Neutrophil, LYMPH: Lymphocyte, MONO: Monocyte, EOSN: Eosinophil, BASO: Basophil, LUC: Large unstained cells, PT: Prothrombin time, APTT: Activated partial thromboplastin time
Values are expressed as Mean±S.D.

Table 2. Historical control data of hematology and coagulation in IGS rats aged 11 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
HCT(%)	45.7±2.0	45.6±1.1	43.3±1.6	43.7±1.5
HGB(g/dL)	15.5±0.5	15.4±0.4	15.3±0.5	15.3±0.6
RBC($x10^6/mm^3$)	8.25±0.38	8.32±0.30	7.94±0.32	7.97±0.37
MCV(μm^3)	55.4±1.8	54.8±1.6	55.5±1.8	54.9±1.1
MCH(pg)	18.8±0.6	18.6±0.6	19.2±0.6	19.2±0.4
MCHC(%)	34.0±0.8	33.9±0.5	35.3±0.6	35.0±0.6
PLT($x10^3/mm^3$)	1111±114	1186±122	1170±121	1232±134
WBC($x10^3/mm^3$)	11.6±3.2	10.5±3.3	7.0±2.5	6.2±1.8
Differential leukocyte counts(%)				
NEUT	10±3	11±3	10±3	12±4
LYMPH	86±4	86±3	85±3	84±4
MONO	2±1	2±1	2±1	2±1
EOSN	1±0	1±0	1±1	1±0
BASO	0±0	0±0	0±0	0±0
LUC	1±0	1±1	1±1	1±0
PT(sec.)	14.6±0.7	15.9±0.8	14.8±0.6	15.9±0.6
APTT(sec.)	26.7±1.6	22.1±1.7	22.0±1.4	17.6±1.2
Fibrinogen(mg/dL)	218±20	242±23	179±18	191±19

NEUT: Neutrophil, LYMPH: Lymphocyte, MONO: Monocyte, EOSN: Eosinophil, BASO: Basophil, LUC: Large unstained cells, PT: Prothombin time, APTT: Activated partial thromboplastin time
Values are expressed as Mean±S.D.

Table 3. Historical control data of blood chemistry in IGS rats aged 9 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
Glucose(mg/dL)	144±18	142±19	124±16	123±16
T.cholesterol(mg/dL)	51±10	52±10	54±12	52±12
Triglyceride(mg/dL)	34.3±14.0	32.5±7.7	14.4±8.0	12.5±4.6
BUN(mg/dL)	11.2±2.1	11.4±2.2	15.5±3.2	15.0±2.7
Creatinine(mg/dL)	0.22±0.03	0.22±0.03	0.27±0.04	0.27±0.04
T.bilirubin(mg/dL)	0.03±0.02	0.03±0.01	0.04±0.02	0.03±0.01
T.protein(g/dL)	5.56±0.23	5.58±0.23	5.66±0.27	5.75±0.23
Sodium(mmol/L)	143.3±1.2	144.0±1.2	143.1±1.2	143.2±1.1
Potassium(mmol/L)	4.57±0.38	4.49±0.27	4.46±0.39	4.33±0.32
Chloride(mmol/L)	106.8±1.5	108.1±1.3	109.4±1.3	109.6±1.5
Calcium(mg/dL)	9.80±0.33	9.91±0.25	9.72±0.29	9.87±0.27
I.phosphorus(mg/dL)	8.27±0.56	8.05±0.52	6.42±0.72	6.67±0.68
AST(U/L)	75±11	70±7	76±16	73±8
ALT(U/L)	26±4	27±6	22±7	23±4
ALP(U/L)	760±162	763±118	473±112	471±151
γ -GTP(U/L)	0.4±0.2	0.5±0.2	0.6±0.3	0.7±0.3

Values are expressed as Mean±S.D.

Table 4. Historical control data of blood chemistry in IGS rats aged 11 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
Glucose(mg/dL)	152±16	155±18	133±16	123±13
T.cholesterol(mg/dL)	54±12	53±15	65±15	63±8
Triglyceride(mg/dL)	38.5±19.0	40.3±26.9	22.5±11.3	19.4±13.1
BUN(mg/dL)	13.3±1.7	14.0±2.2	15.7±2.3	17.6±4.1
Creatinine(mg/dL)	0.23±0.03	0.25±0.03	0.28±0.04	0.32±0.05
T.bilirubin(mg/dL)	0.04±0.01	0.05±0.01	0.06±0.02	0.07±0.02
T.protein(g/dL)	5.75±0.26	5.82±0.24	5.97±0.27	6.07±0.36
Sodium(mmol/L)	143.2±1.4	143.6±1.3	142.5±1.4	142.6±1.4
Potassium(mmol/L)	4.62±0.45	4.55±0.35	4.53±0.40	4.40±0.32
Chloride(mmol/L)	106.1±1.5	107.3±1.5	108.3±1.8	110.0±1.5
Calcium(mg/dL)	9.86±0.38	9.95±0.28	9.67±0.24	9.92±0.24
I.phosphorus(mg/dL)	7.48±0.52	8.05±0.52	5.90±0.66	5.77±0.80
AST(U/L)	70±8	67±9	76±17	67±10
ALT(U/L)	25±4	28±5	24±8	25±8
ALP(U/L)	557±122	643±156	310±64	330±92
γ -GTP(U/L)	0.3±0.1	0.5±0.2	0.6±0.2	0.7±0.3

Values are expressed as Mean±S.D.

Table 5. Absolute and relative organ weights of IGS rats aged 9 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
Absolute organ weight				
Brain(g)	2.04±0.07	2.03±0.09	1.92±0.08	1.91±0.10
Liver(g)	9.87±1.31	9.77±1.10	6.02±0.57	6.18±0.79
Kidneys(g)	2.50±0.27	2.56±0.26	1.61±0.15	1.61±0.17
Spleen(g)	0.59±0.09	0.60±0.10	0.43±0.07	0.43±0.07
Adrenals(mg)	52±9	51±7	63±8	64±8
Testes(g)	2.94±0.24	2.94±0.18	—	—
Ovaries(mg)	—	—	79±12	83±15
Thymus(mg)	507±116	530±108	454±86	451±130
Relative organ weight				
Brain(%)	0.616±0.057	0.631±0.052	0.923±0.056	0.934±0.083
Liver(%)	2.954±0.214	3.019±0.217	2.885±0.160	2.999±0.177
Kidneys(%)	0.750±0.050	0.777±0.061	0.772±0.061	0.798±0.053
Spleen(%)	0.176±0.021	0.186±0.020	0.207±0.029	0.208±0.027
Adrenals(%)	0.016±0.002	0.016±0.002	0.030±0.004	0.031±0.004
Testes(%)	0.887±0.093	0.914±0.075	—	—
Ovaries(%)	—	—	0.038±0.006	0.040±0.006
Thymus(%)	0.152±0.033	0.164±0.030	0.217±0.038	0.217±0.050

Values are expressed as Mean±S.D.

Table 6. Absolute and relative organ weights of IGS rats aged 11 weeks

Item	Male		Female	
	Before	After	Before	After
No. of animals	55	30	55	30
Absolute organ weight				
Brain(g)	2.10±0.08	2.11±0.10	1.97±0.08	1.97±0.07
Liver(g)	10.82±1.68	10.93±1.81	6.26±0.72	6.47±0.87
Kidneys(g)	2.83±0.35	2.82±0.38	1.77±0.19	1.75±0.16
Spleen(g)	0.66±0.11	0.67±0.13	0.46±0.08	0.49±0.08
Adrenals(mg)	55±9	54±10	66±10	66±8
Testes(g)	3.11±0.23	3.09±0.25	—	—
Ovaries(mg)	—	—	81±12	84±14
Thymus(mg)	458±110	430±92	399±90	400±92
Relative organ weight				
Brain(%)	0.543±0.059	0.566±0.061	0.842±0.066	0.860±0.082
Liver(%)	2.761±0.224	2.898±0.219	2.664±0.156	2.798±0.143
Kidneys(%)	0.728±0.115	0.750±0.066	0.753±0.051	0.762±0.055
Spleen(%)	0.169±0.031	0.180±0.029	0.197±0.023	0.214±0.029
Adrenals(%)	0.014±0.003	0.015±0.002	0.028±0.004	0.029±0.003
Testes(%)	0.804±0.096	0.832±0.107	—	—
Ovaries(%)	—	—	0.035±0.005	0.036±0.004
Thymus(%)	0.117±0.025	0.115±0.018	0.170±0.038	0.174±0.036

Values are expressed as Mean±S.D.

Comparison of Urinary Parameters between Crj:CD(SD)IGS and Crj:CD(SD) rats

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ABSTRACT. Urinary parameters of Crj:CD(SD)IGS (IGS) rats were collected and compared with those of Crj:CD(SD) (CD) rats that had been accumulated in our institute. The male and female IGS rats, four-week-old, were purchased and maintained in our facility by 31 weeks of age. Urine samples were collected at 8, 17 and 30 weeks of age and examined. The results obtained indicated that both IGS and CD rats had similar data in almost all the parameters examined. However, the incidence of basic urine was higher than that of CD rats; in contrast, the incidences of protein and urobilinogen in urine were lower than those of CD rats. The urinary excretions of sodium, potassium and chloride were larger than those of CD rats. The urinary parameters in IGS rats obtained in the present study may be useful for toxicity studies. — Key words: Crj:CD(SD)IGS, Urinalysis, Rat

CD(SD)IGS-2001: 24-28

INTRODUCTION

The international genetic standard system, which has been developed by Charles River Laboratories, is a new breeding procedure of laboratory rats. The system has been expected to produce uniform laboratory rats by its genetic ramification control. Crj:CD(SD)IGS (IGS) rats have been produced through the international genetic standard system and begun to be used widely in various toxicity studies. The IGS rats are anticipated to be suitable for internationalization of research and development of new drugs. At present, however, background data of urinary parameters of IGS rats have not yet been fully accumulated. Therefore, we investigated urinary parameters of IGS rats, and the results obtained were compared with those of Crj:CD(SD) (CD) rats.

MATERIALS AND METHODS

Animals: A total of 90 male and 90 female IGS rats, four-week-old, were purchased from Charles River Japan, Inc. (Tsukuba Breeding Center, Ibaraki, Japan). The animals were acclimated to laboratory animal facilities at our institute for 12 days. The general conditions of all rats were a good state of health during the acclimatization period. The animals were housed individually in a stainless wire cage (21W x 35D x 20H cm) during the acclimatization and study periods. The animal room was maintained at a temperature of 21-23 °C and a relative humidity of 30-63% during the acclimatization and study periods. The room was ventilated more than 10 times per hr (all fresh air), and the illuminated for 12 hr per day (from 07:00 to 19:00). Animals were allowed free access to the diet (CR-LPF, Oriental Yeast Co., Tokyo, Japan) sterilized by radiation of 30 kGy and tap water.

Urinalysis: Thirty male and 30 female rats were examined using metabolic cage at 8, 17 and 30 weeks of age, respectively. Fresh urine was collected for five hours under the fasting. Urinary specific gravity, pH, glucose, bilirubin, ketone, protein, urobilinogen and occult blood in IGS rats were measured by a test paper method (N-Multistix SG, Bayer-Sankyo Co., Ltd., Tokyo, Japan) using an automated urine analyzer (CLINITEK 100, Bayer-Sankyo Co., Ltd.). In CD rats, the parameters were measured by the same method in IGS rats using an automated urine analyzer (CLINITEK model 5500, Miles-Sankyo Co., Ltd., Tokyo, Japan). Urinary sediments were stained with Sternheimer-Malbin stain and examined microscopically. After fresh urine was collected, the rat was given diet. Urine for the determination of volume and electrolytes was collected for 24 hours. After urine volume was determined by measuring cylinder, urine sample was centrifuged at 1500 x g for 5 min (4 °C) and, an upper layer was collected and stored at -70 °C until analysis. Sodium, potassium and chloride were determined by an ion-selective electrode method using an automated analyzer (Model STAX-1, Techno Medica Co., Ltd., Yokohama, Japan) in IGS rats. In CD rats, sodium and potassium were determined by using a flame photometer (Model 205D, Hitachi Ltd., Tokyo, Japan) and chloride was measured by a coulometric titration method using a chloride counter (Model CL-7, Hiranuma Sangyo Co., Ltd., Ibaraki, Japan). The results obtained by urinalysis of IGS rats at 8, 17 and 30 weeks of age were compared with those of CD rats at 8 - 12, 20 - 24 and 32 - 36 weeks of age, respectively.

Statistical Analysis: In urine volume and electrolytes, data were analyzed by Welch's *t*-test between the IGS and CD rats. In other parameters, data were analyzed by Wilcoxon rank test between the IGS and CD rats. Differences were considered significant at $p<0.05$.

RESULTS

Urinary data in the male and female IGS rats determined in the present study and in the male and female CD rats that have been determined on the other occasions at our institute are shown in Tables 1 - 4.

In male IGS rats at eight weeks of age, the incidences of white blood cells (WBC) in urinary sediments and high specific gravity of urine were significantly higher than those of their CD counterparts; conversely, the incidences of protein and urobilinogen in urine were significantly lower than those of CD rats. The values of sodium, potassium and chloride were significantly higher than the corresponding values of CD rats; conversely, the value of urine volume was significantly lower in IGS rats. In male IGS rats at 17 weeks of age, the incidences of WBC in urinary sediments and high specific gravity of urine were significantly higher

than the corresponding parameters of CD rats; on the other hand, the incidence of urobilinogen in urine was significantly lower than that of CD rats. The values of sodium, potassium and chloride were significantly higher in IGS rats than in CD rats. In male IGS rats at 30 weeks of age, the incidences of casts and epithelial cells in urinary sediments were significantly higher than those of their CD counterparts; in contrast, the incidence of urobilinogen in urine was significantly lower than that of CD rats. The values of sodium, potassium and urine volume were significantly higher in IGS rats than in CD rats (Tables 1 and 3).

In female IGS rats at eight weeks of age, the incidences of bilirubin and ketone in urine, casts in urinary sediments and basic urine were significantly higher than those of their CD counterparts; conversely, the incidence of protein in urine was significantly lower than that of CD rats. The values of sodium, potassium and chloride were significantly higher than the corresponding values of CD rats. In female IGS rats at 17 weeks of age, the incidences of ketone in urine, high specific gravity of urine and basic urine were significantly higher than the corresponding parameters of CD rats; on the other hand, the incidences of protein and urobilinogen in urine were significantly lower than those of CD rats. The values of sodium and potassium were significantly higher in IGS rats than in CD rats. In female IGS rats at 30 weeks of age, the incidences of casts in urinary sediments and basic urine were significantly higher than those of their CD counterparts; in contrast, the incidences of protein and urobilinogen in urine were significantly lower than those of CD rats. The values of sodium, potassium and urine volume were significantly higher in IGS rats than those in CD rats (Tables 2 and 4).

DISCUSSION

The results obtained in the present study revealed that some of urinary parameters of IGS rats differed significantly from those of CD rats. At 8, 17 and 30 weeks of age in male and/or female IGS rats, the incidences of basic urine and the values of sodium, potassium and chloride were higher than those of CD rats; in contrast, the incidences of protein and urobilinogen in urine were lower than those of CD rats.

The urinary excretions of sodium, potassium and chloride in both sexes of IGS rats were higher than those of CD rats. In CD rats, sodium and potassium were determined by using a flame photometer and chloride was measured by the coulometric titration method. In IGS rats, on the other hand, those parameters were measured by the ion-selective electrode method. It was considered that the higher values of sodium, potassium and chloride in IGS rats might be ascribed mainly to a difference in measuring method. The urinary excretions of sodium, potassium and chloride have reported in Sprague-Dawley (SD) rats [1]. In their reference data, the urinary excretions of sodium, potassium and chloride were higher than those in the urinalysis data of IGS rats and our background data of CD rats.

Owen and Heywood [2] and Neuhaus and Flory [3] have reported the reference range data of urine volume in Wistar and SD rats. The urine volume in IGS rats obtained in the present study was in their range. The urinary reference data of in F344/Yit, F344/DuCrj and Slc:Wistar rats at 19 weeks of age have also been presented [4]. When compared with their data, the urinary glucose and ketone of IGS rats at 17 weeks of age were almost comparable to their data except for pH and protein. Because the incidences of acidic urine and urinary protein in IGS rats were lower than those of CD rats, it was considered that basic urine and low urinary protein were characterized in IGS rats. Age-related changes in urinary parameters in SD rats have been well documented by Neuhaus and Flory [3] and Weaver *et al*. [5]. According to their report, the excretion of urinary protein for one day increased with age. In the present study, age-related increase was observed in the incidence of urinary protein in IGS rats.

In conclusion, urinary parameters of IGS rats were substantially consistent with those of CD rats. The urinary information on the IGS strain presented here may be used as the reference data for toxicity studies.

ACKNOWLEDGMENTS

We are grateful to Messrs. Y. Amano, T. Fujii and K. Sato for animal care, and Mr. K. Nakajima and Ms. K. Miyadera for technical assistance in urinalysis.

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Table 1. Urinalysis in male IGS and CD rats

Parameters		8[a] (8-12[b]) weeks of age							17 (20-24) weeks of age							30 (32-36) weeks of age						
		N	−	±	+	++	+++	++++	N	−	±	+	++	+++	++++	N	−	±	+	++	+++	++++
Glucose	IGS	30	30	0	0	0	0		30	30	0	0	0	0		30	30	0	0	0	0	
	CD	90	89	1	0	0	0		35	35	0	0	0	0		30	30	0	0	0	0	
Bilirubin	IGS	30	27	-	3	0	0		30	29	-	1	0	0		30	27	-	3	0	0	
	CD	90	85	-	5	0	0		35	35	-	0	0	0		30	26	-	4	0	0	
Ketone	IGS	30	5	22	3	0	0		30	2	17	11	0	0		30	2	19	9	0	0	
	CD	90	18	41	29	2	0		35	10	13	12	0	0		30	4	19	7	0	0	
Protein	IGS	30	14	10	6	0	0	*	30	4	10	13	3	0		30	5	4	12	9	0	
	CD	90	9	42	37	2	0		35	3	9	21	2	0		30	4	11	12	3	0	
Occult blood	IGS	30	30	0	0	0	0		30	28	0	2	0	0		30	25	1	2	1	1	
	CD	90	83	2	3	1	1		35	26	9	0	0	0		30	25	3	2	0	0	
Urinary sediments																						
WBC	IGS	30	22	8	0	0	0	0	*	30	23	7	0	0	0	0	*	30	22	7	1	0	0	0
	CD	66	63	3	0	0	0	0		35	33	2	0	0	0	0		30	27	3	0	0	0	0
RBC	IGS	30	30	0	0	0	0	0		30	29	0	1	0	0	0		30	28	0	1	1	0	0
	CD	66	66	0	0	0	0	0		35	35	0	0	0	0	0		30	30	0	0	0	0	0
Casts	IGS	30	27	21	1	0	0	0		30	18	8	4	0	0	0		30	20	7	3	0	0	0	*
	CD	66	59	6	1	0	0	0		35	24	6	5	0	0	0		30	28	2	0	0	0	0	
Epitherial cells	IGS	30	9	15	6	0	0	0		30	0	14	12	3	0	1		30	4	12	10	4	0	0	*
	CD	66	32	28	6	0	0	0		35	0	20	12	3	0	0		30	11	11	6	1	1	0	
		N	0.1	1.0	2.0	4.0	≥ 8.0		N	0.1	1.0	2.0	4.0	≥ 8.0		N	0.1	1.0	2.0	4.0	≥ 8.0	
Urobilinogen (EU/dL)	IGS	30	28	2	0	0	0	*	30	27	3	0	0	0	*	30	22	8	0	0	0	*
	CD	90	53	37	0	0	0		35	18	17	0	0	0		30	8	22	0	0	0	
		N	≤1.005	1.010	1.015	1.020	1.025	≥1.030	N	≤1.005	1.010	1.015	1.020	1.025	≥1.030	N	≤1.005	1.010	1.015	1.020	1.025	≥1.030
Specific gravity	IGS	30	0	1	0	11	14	4	*	30	0	0	3	18	8	1	*	30	0	0	4	13	11	2
	CD	90	0	3	27	37	13	10		35	0	6	14	10	5	0		30	0	0	0	14	10	6
		N	6.0	6.5	7.0	7.5	8	8.5	≥ 9.0	N	6.0	6.5	7.0	7.5	8	8.5	≥ 9.0	N	6.0	6.5	7.0	7.5	8	8.5	≥ 9.0
pH	IGS	30	0	0	0	0	14	16	0	30	0	0	0	0	0	24	6	30	0	0	0	0	4	25	1
	CD	90	2	-	11	-	40	-	37	35	0	-	2	-	18	-	15	30	1	-	3	-	15	-	11

a: IGS rats.
b: CD rats.
N: Number of rats examined.
*: Significantly different from CD rats at $p<0.05$.

Table 2. Urinalysis in female IGS and CD rats

Parameters		8[a] (8-12[b]) weeks of age							17 (20-24) weeks of age							30 (32-36) weeks of age						
		N	−	±	+	++	+++	++++	N	−	±	+	++	+++	++++	N	−	±	+	++	+++	++++
Glucose	IGS	30	29	1	0	0	0		30	30	0	0	0	0		30	30	0	0	0	0	
	CD	90	90	0	0	0	0		35	35	0	0	0	0		29	29	0	0	0	0	
Bilirubin	IGS	30	25	-	5	0	0	*	30	27	-	3	0	0		30	26	-	4	0	0	
	CD	90	87	-	3	0	0		35	31	-	4	0	0		29	28	-	1	0	0	
Ketone	IGS	30	15	14	1	0	0	*	30	15	13	2	0	0	*	30	18	11	1	0	0	
	CD	90	69	21	0	0	0		35	26	8	1	0	0		29	24	5	0	0	0	
Protein	IGS	30	21	5	4	0	0	*	30	23	5	1	1	0	*	30	21	4	4	1	0	*
	CD	90	32	36	20	2	0		35	16	9	9	1	0		29	8	11	6	1	3	
Occult blood	IGS	30	30	0	0	0	0		30	30	0	0	0	0		30	29	0	0	0	1	
	CD	90	88	0	1	1	0		35	34	1	0	0	0		29	28	1	0	0	0	
Urinary sediments																						
WBC	IGS	30	25	5	0	0	0	0	30	29	1	0	0	0	0	30	25	4	1	0	0	0
	CD	66	60	6	0	0	0	0	35	35	0	0	0	0	0	29	28	1	0	0	0	0
RBC	IGS	30	30	0	0	0	0	0	30	30	0	0	0	0	0	30	30	0	0	0	0	0
	CD	66	66	0	0	0	0	0	35	35	0	0	0	0	0	29	29	0	0	0	0	0
Casts	IGS	30	24	4	2	0	0	0	*	30	25	4	1	0	0	0	30	24	4	2	0	0	0	*
	CD	66	63	3	0	0	0	0		35	30	3	2	0	0	0	29	29	0	0	0	0	0	
Epitherial cells	IGS	30	9	12	7	0	1	1		30	7	18	3	2	0	0		30	7	18	2	2	0	1	
	CD	66	20	38	7	1	0	0		35	5	25	4	1	0	0		29	14	10	2	0	1	2	
		N	0.1	1.0	2.0	4.0	≥8.0		N	0.1	1.0	2.0	4.0	≥8.0		N	0.1	1.0	2.0	4.0	≥8.0	
Urobilinogen (EU/dL)	IGS	30	23	7	0	0	0		30	26	4	0	0	0	*	30	26	4	0	0	0	*
	CD	90	53	37	0	0	0		35	15	20	0	0	0		29	14	15	0	0	0	
		N	≤1.005	1.010	1.015	1.020	1.025	≥1.030	N	≤1.005	1.010	1.015	1.020	1.025	≥1.030	N	≤1.005	1.010	1.015	1.020	1.025	≥1.030
Specific gravity	IGS	30	0	1	0	11	14	4	30	0	0	3	17	6	4	*	30	0	0	1	17	8	4	
	CD	90	0	6	14	35	17	18	35	1	7	12	9	3	3		29	0	1	2	12	10	4	
		N	6.0	6.5	7.0	7.5	8	8.5	≥9.0	N	6.0	6.5	7.0	7.5	8	8.5	≥9.0	N	6.0	6.5	7.0	7.5	8	8.5	≥9.0
pH	IGS	30	0	0	0	2	10	18	0	*	30	0	2	1	0	1	24	2	*	30	0	1	1	2	2	22	2	*
	CD	90	9	-	14	-	50	-	17		35	7	-	9	-	11	-	8		29	2	-	5	-	14	-	8	

a: IGS rats.
b: CD rats.
N: Number of rats examined.
*: Significantly different from CD rats at $p<0.05$.

Table 3. Urine volume and erectrolytes of male IGS and CD rats

Parameters		8[a] (8-12[b]) weeks of age		17 (20-24) weeks of age		30 (32-36) weeks of age	
		N	(mmol/24h)	N	(mmol/24h)	N	(mmol/24h)
Sodium	IGS	30	1.61±0.28*	30	1.81±0.25*	30	2.02±0.41*
	CD	42	1.35±0.62	35	0.90±0.61	30	1.24±0.76
Potassium	IGS	30	1.90±0.33*	30	2.04±0.31*	30	2.23±0.47*
	CD	42	1.66±0.54	35	1.50±0.67	30	1.36±0.51
Chloride	IGS	30	1.47±0.34*	30	1.60±0.23*	30	1.62±0.38
	CD	22	1.06±0.65	35	1.14±0.92		N.E.
		N	(mL)	N	(mL)	N	(mL)
Urine volume	IGS	30	13±3*	30	16±3	30	18±5*
	CD	80	17±8	35	16±5	30	12±4

Each value represents mean±S.D..
a: IGS rats.
b: CD rats.
N: Number of rats examined.
N.E.: Not examined
*: Significantly different from CD rats at $p<0.05$.

Table 4. Urine volume and erectrolytes of female IGS and CD rats

Parameters		8[a] (8-12[b]) weeks of age		17 (20-24) weeks of age		30 (32-36) weeks of age	
		N	(mmol/24h)	N	(mmol/24h)	N	(mmol/24h)
Sodium	IGS	30	1.15±0.21*	30	1.22±0.32*	30	1.15±0.29*
	CD	43	0.72±0.45	35	0.81±0.54	29	0.68±0.37
Potassium	IGS	30	1.35±0.27*	30	1.48±0.38*	30	1.33±0.38*
	CD	43	1.00±0.49	35	1.12±0.58	29	0.88±0.51
Chloride	IGS	30	1.27±0.25*	30	1.19±0.33	30	1.01±0.26
	CD	20	0.63±0.54	34	1.14±0.73		N.E.
		N	(mL)	N	(mL)	N	(mL)
Urine volume	IGS	30	10±3	30	12±4	30	13±6*
	CD	78	11±4	35	11±6	29	10±4

Each value represents mean±S.D..
a: IGS rats.
b: CD rats.
N: Number of rats examined.
N.E.: Not examined
*: Significantly different from CD rats at $p<0.05$.

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 12 Months

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ABSTRACT. Male rats were given CR-LPF or CRF-1 diet *ad libitum* or restrictedly for 12 months to examine how dietary restriction and low protein diet affect general signs, body weight, food consumption, blood parameters, and pathological findings.
Lower 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 group. The distinctive quality of CRF-LPF is considered to have been exhibited in this finding. In the blood chemical analysis, lower Glu and TG were noted in the CRF-1 and CR-LPF restricted feeding groups compared with the CRF-1 and CR-LPF *ad libitum* feeding groups. It can be said that these findings were caused by the dietary restriction. There were no apparent differences in food consumption, hematology, organ weights, necropsy findings, or histopathological findings between the CR-LPF and the CRF-1 dietary restriction. — Key words: Dietary restriction, low protein diet, Crj: CD (SD) IGS

CD(SD)IGS-2001: 29-39

INTRODUCTION

Male rats were given CR-LPF diet or CRF-1 diet *ad libitum* or restrictedly for 12 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 hour. 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 x D: 380 x 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; 18.4%, Oriental Yeast Co., Ltd.) was given to a group of animals *ad libitum*, which was named the CR-LPF *ad libitum* feeding group, and to another group of animals restrictedly, which was named the CR-LPF restricted feeding group. Similarly, CRF-1 (protein content; 23.1%, Oriental Yeast Co., Ltd.) was given to a group of animals *ad libitum* (the CRF-1 *ad libitum* feeding group), and to another group of animals restrictedly (the CRF-1 restricted feeding group). Each animal of the restricted feeding groups was given about 18 g 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 12 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 (RET; by Brecher's method) were counted 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.

Table 1. 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 dehydrogenase.

Organ Weight Measurement

The animals, whose blood had been collected, were sacrificed by bleeding via the abdominal aorta, and the following organs were weighed (paired 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 for H.E. staining, and the 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 group, and then for the *ad libitum* and 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%).

Significance 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 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 t test.

RESULTS

General Signs

No abnormalities were noted either in the CRF-1 or CR-LPF *ad libitum* feeding group or in the CRF-1 or CR-LPF restricted feeding group except that subcutaneous mass was detected by palpation in 1 animal of the CRF-1 *ad libitum* feeding group on the 351st day of test feeding and thereafter.

No animals died up to the 372nd day of test feeding.

Body Weight

Body weight changes are shown in Fig. 1 and Tables 2 and 3. 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 groups.

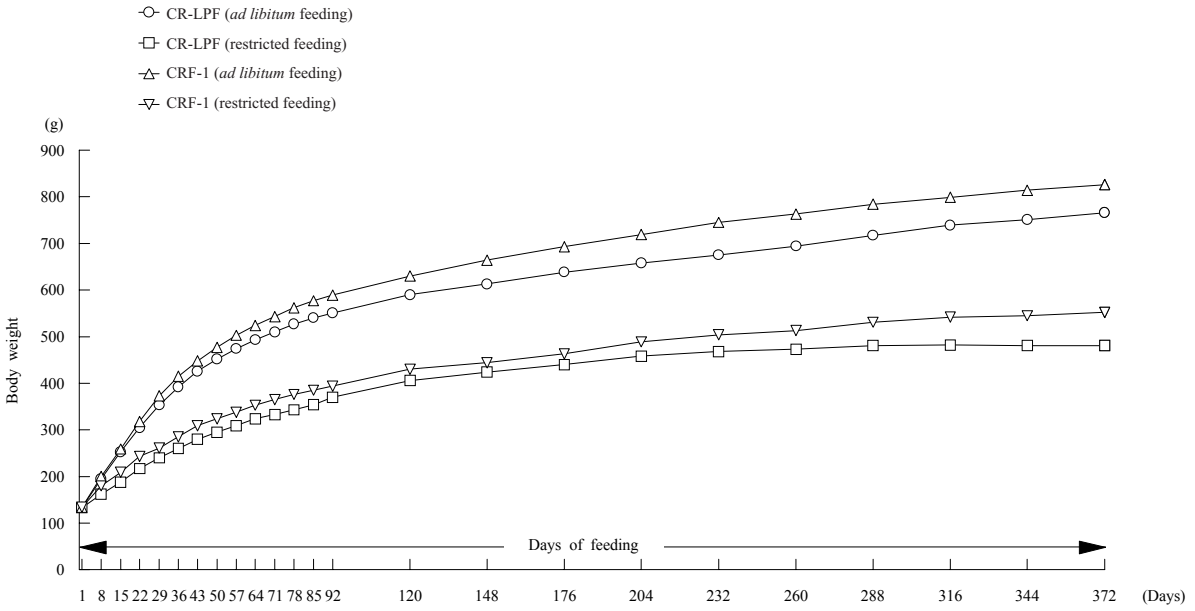

Fig 1. Body weighrs of male rats.

Table 2. 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)

Each value shows mean (g) ± S.D.
**: P<0.01, Significantly different from CR-LPF (*ad libitum* feeding) group.
##: P<0.01, Significantly different from CRF-1 (*ad libitum* feeding) group.
Figures in parentheses indicate number of males.

Table 3. 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)

Each value shows mean (g) ± S.D.
*: P<0.05, **: P<0.01, Significantly different from CRF-1 (*ad libitum* feeding) group.
##: P<0.01, Significantly different from CRF-1 (restricted feeding) group.
Figures in parentheses indicate number of males.

Food Consumption

Changes in food consumption are shown in Fig. 2 and Tables 4 and 5. Food consumption in the CR-LPF *ad libitum* group tended to be slightly higher than that in the CRF-1 *ad libitum* group. There were no abnormal changes in food consumption either in the CRF-1 or CR-LPF *ad libitum* group or in the CRF-1 or CR-LPF restricted feeding group.

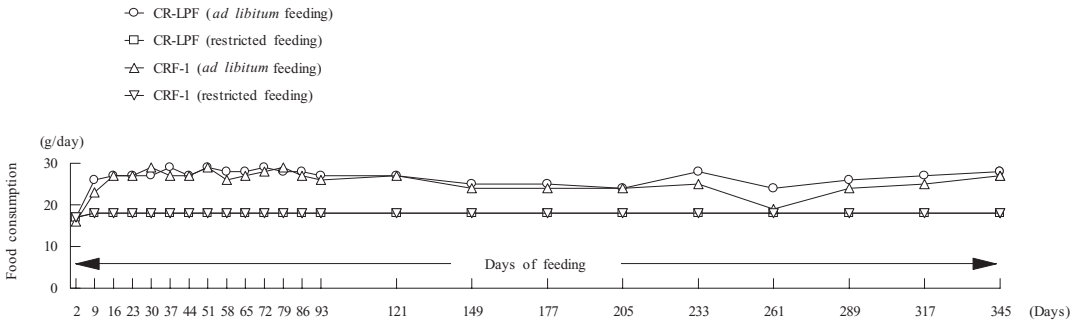

Fig 2. Food consumption of mele rats.

Table 4. 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	17±1	16±1	17±1
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)

Each value shows mean (g/day) ± S.D.
Figures in parentheses indicate number of males.

Table 5. 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*	17±1	17±1
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)

Each value shows mean (g/day) ± S.D.
*: P<0.05, **: P<0.01, Significantly different from CRF-1 (*ad libitum* feeding) group.
Figures in parentheses indicate number of males.

Hematology

Group mean values of hematological parameters are shown in Tables 6 and 7. Regarding the groups which had been given CRF-1, higher MCH and more prolonged APTT were noted in the restricted feeding group compared with the *ad libitum* feeding group. Regarding the groups which had been given CR-LPF, higher MCH and lymphocytic ratio and lower neutrophile ratio were noted in the restricted feeding group compared with the *ad libitum* feeding group. There were no differences 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.

Table 6. Hematological finding in 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)	717±229	741±115	746±218	812±34
HGB	(g/dL)	12.7±4.1	13.9±1.9	13.0±4.2	15.1±0.4
HCT	(%)	39.3±10.9	41.7±5.0	40.5±11.1	45.0±1.5
MCV	(fL)	56.0±4.5	56.6±2.7	54.7±1.8	55.4±2.0
MCH	(pg)	17.7±0.4	18.8±0.5**	17.2±1.0	18.6±0.6#
MCHC	(g/dL)	31.8±2.4	33.3±1.0	31.6±2.6	33.6±0.3
PLT	($10^4/\mu$ L)	114.4±32.3	102.1±17.4	115.3±30.6	92.3±14.0
RET	(‰)	94±144	39±36	79±108	24±4
PT	(sec.)	14.5±0.4	15.0±0.4	14.6±0.8	14.6±0.7
APTT	(sec.)	24.5±3.8	25.3±2.2	23.2±1.8	26.6±1.8#
FIB	(mg/dL)	253±82	222±42	294±76	213±10
WBC	($10^2/\mu$ L)	92±96	47±18	98±78	52±16
Differential leukocyte (%)					
Lymphocyte		81.6±7.4	91.2±1.3*	85.4±12.4	90.6±3.9
Neutrophil		17.4±7.6	7.6±1.5*	14.0±12.5	8.4±3.4
Eosinophil		0.2±0.4	0.6±0.5	0.4±0.5	0.6±0.5
Basophil		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Monocyte		0.8±0.4	0.6±0.5	0.2±0.4	0.4±0.5

Each value shows mean ± S.D.
*: P<0.05, **: P<0.01, Significantly different from CR-LPF (*ad libitum* feeding) group.
#: P<0.05, Significantly different from CRF-1 (*ad libitum* feeding) group.

Table 7. Hematological finding 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
RBC	($10^4/\mu$ L)	746±218	717±229	812±34	741±115
HGB	(g/dL)	13.0±4.2	12.7±4.1	15.1±0.4	13.9±1.9
HCT	(%)	40.5±11.1	39.3±10.9	45.0±1.5	41.7±5.0
MCV	(fL)	54.7±1.8	56.0±4.5	55.4±2.0	56.6±2.7
MCH	(pg)	17.2±1.0	17.7±0.4	18.6±0.6	18.8±0.5
MCHC	(g/dL)	31.6±2.6	31.8±2.4	33.6±0.3	33.3±1.0
PLT	($10^4/\mu$ L)	115.3±30.6	114.4±32.3	92.3±14.0	102.1±17.4
RET	(‰)	79±108	94±144	24±4	39±36
PT	(sec.)	14.6±0.8	14.5±0.4	14.6±0.7	15.0±0.4
APTT	(sec.)	23.2±1.8	24.5±3.8	26.6±1.8	25.3±2.2
FIB	(mg/dL)	294±76	253±82	213±10	222±42
WBC	($10^2/\mu$ L)	98±78	92±96	52±16	47±18
Differential leukocyte (%)					
Lymphocyte		85.4±12.4	81.6±7.4	90.6±3.9	91.2±1.3
Neutrophil		14.0±12.5	17.4±7.6	8.4±3.4	7.6±1.5
Eosinophil		0.4±0.5	0.2±0.4	0.6±0.5	0.6±0.5
Basophil		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Monocyte		0.2±0.4	0.8±0.4	0.4±0.5	0.6±0.5

Each value shows mean ± S.D.

Blood Chemistry

Group mean values of blood chemistry parameters are shown in Tables 8 and 9. Regarding the groups which had been given CRF-1, lower ALP, α 1-glb, β -glb, and Glu and higher alb and A/G ratio were noted in the restricted feeding group compared with the *ad libitum* feeding group. Regarding the groups which had been given CR-LPF, lower Glu, TG, and Ca and higher Cl were noted in the restricted feeding group compared with the *ad libitum* feeding group. There were no differences in the blood chemistry parameters between the CR-LPF *ad libitum* feeding group and the CRF-1 *ad libitum* feeding group. Lower UN and Glu and higher T-cho were noted in the CR-LPF restricted feeding group compared with the CRF-1 restricted feeding group.

Table 8. 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)	107.7±24.1	94.6±27.7	143.0±40.5	112.6±41.2
ALT	(IU/L)	52.9±16.4	35.4±8.7	64.0±14.9	44.3±14.1
ALP	(IU/L)	300.4±74.0	230.3±78.8	316.3±36.7	183.8±35.2##
TP	(g/dL)	6.0±0.4	5.6±0.2	5.8±0.2	5.6±0.2
Alb	(g/dL)	2.73±0.39	2.63±0.33	2.34±0.36	2.72±0.10
Protein fraction (%)					
alb		45.3±5.3	47.2±5.6	40.3±5.8	48.9±1.2#
α 1-glb		22.9±2.6	22.4±1.3	25.6±2.1	22.9±1.2#
α 2-glb		6.9±1.2	6.7±0.5	6.5±0.7	6.4±0.3
β -glb		20.6±3.1	18.1±3.2	21.9±3.4	17.6±0.9#
γ -glb		4.4±0.8	5.6±1.2	5.7±2.7	4.2±0.5
A/G		0.84±0.17	0.91±0.18	0.69±0.16	0.96±0.05#
T-Bil	(mg/dL)	0.10±0.04	0.14±0.03	0.12±0.04	0.14±0.03
UN	(mg/dL)	13.6±2.5	13.5±1.7	15.1±1.9	15.5±0.9
CRE	(mg/dL)	0.38±0.07	0.36±0.07	0.37±0.06	0.37±0.08
Glu	(mg/dL)	174.4±30.3	117.5±8.5*	165.3±23.4	134.1±10.8#
T-Cho	(mg/dL)	107.8±13.9	95.9±8.5	95.8±13.2	83.6±7.3
TG	(mg/dL)	102.1±38.5	28.6±8.6*	85.8±60.0	40.5±11.9
Na	(mEq/L)	147.0±1.1	147.5±1.8	147.2±1.3	147.7±1.5
K	(mEq/L)	4.78±0.13	4.67±0.62	4.77±0.44	4.43±0.22
Cl	(mEq/L)	105.8±2.0	108.2±1.1*	106.5±1.6	107.4±0.8
Ca	(mg/dL)	9.9±0.2	9.4±0.2**	9.7±0.1	9.6±0.2
IP	(mg/dL)	6.4±1.1	5.9±1.7	5.9±0.6	6.0±0.4

Each value shows mean ± S.D.
*: P<0.05, **: P<0.01, Significantly different from CR-LPF (*ad libitum* feeding) group.
#: P<0.05, ##: P<0.01, Significantly different from CRF-1 (*ad libitum* feeding) group.

Table 9. 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)	143.0±40.5	107.7±24.1	112.6±41.2	94.6±27.7
ALT	(IU/L)	64.0±14.9	52.9±16.4	44.3±14.1	35.4±8.7
ALP	(IU/L)	316.3±36.7	300.4±74.0	183.8±35.2	230.3±78.8
TP	(g/dL)	5.8±0.2	6.0±0.4	5.6±0.2	5.6±0.2
Alb	(g/dL)	2.34±0.36	2.73±0.39	2.72±0.10	2.63±0.33
Protein fraction (%)					
alb		40.3±5.8	45.3±5.3	48.9±1.2	47.2±5.6
α 1-glb		25.6±2.1	22.9±2.6	22.9±1.2	22.4±1.3
α 2-glb		6.5±0.7	6.9±1.2	6.4±0.3	6.7±0.5
β -glb		21.9±3.4	20.6±3.1	17.6±0.9	18.1±3.2
γ -glb		5.7±2.7	4.4±0.8	4.2±0.5	5.6±1.2
A/G		0.69±0.16	0.84±0.17	0.96±0.05	0.91±0.18
T-Bil	(mg/dL)	0.12±0.04	0.10±0.04	0.14±0.03	0.14±0.03
UN	(mg/dL)	15.1±1.9	13.6±2.5	15.5±0.9	13.5±1.7#
CRE	(mg/dL)	0.37±0.06	0.38±0.07	0.37±0.08	0.36±0.07
Glu	(mg/dL)	165.3±23.4	174.4±30.3	134.1±10.8	117.5±8.5#
T-Cho	(mg/dL)	95.8±13.2	107.8±13.9	83.6±7.3	95.9±8.5#
TG	(mg/dL)	85.8±60.0	102.1±38.5	40.5±11.9	28.6±8.6
Na	(mEq/L)	147.2±1.3	147.0±1.1	147.7±1.5	147.5±1.8
K	(mEq/L)	4.77±0.44	4.78±0.13	4.43±0.22	4.67±0.62
Cl	(mEq/L)	106.5±1.6	105.8±2.0	107.4±0.8	108.2±1.1
Ca	(mg/dL)	9.7±0.1	9.9±0.2	9.6±0.2	9.4±0.2
IP	(mg/dL)	5.9±0.6	6.4±1.1	6.0±0.4	5.9±1.7

Each value shows mean ± S.D.
#: P<0.05, Significantly different from CRF-1 (restricted feeding) group.

Necropsy

Regarding the groups which had been given CRF-1, decubitus was noted in both hind-limbs of 2 animals of the *ad libitum* feeding group, and no abnormalities were noted in the restricted feeding group. In the CR-LPF *ad libitum* feeding group, dark red mucosa of the glandular stomach, decubitus in both hind-limbs, and that in the left hind-limb were noted in 1 animal each. Dark red mucosa of the glandular stomach was noted in 1 animal of the CR-LPF restricted feeding group.

Organ Weights

Group mean values of the absolute and relative organ weights are shown in Tables 10 and 11. Regarding the groups which had been given CRF-1, lower values of the absolute pituitary, heart, liver, and kidney weights and higher values of the relative lung and adrenal weights were noted in the restricted feeding group compared with the *ad libitum* feeding group. Regarding the groups which had been given CR-LPF, lower values of the absolute pituitary, lung, heart, liver, and kidney weights were noted in the restricted feeding group compared with the *ad libitum* feeding group. Higher values of the relative lung, kidney, and adrenal weights and a lower value of the relative liver weight were also noted in the CR-LPF restricted feeding group compared with the CR-LPF *ad libitum* feeding group. Slightly lower values of the heart, liver, and kidney weights were noted in the CR-LPF restricted feeding group compared with the CRF-1 restricted feeding group. There was no difference in the absolute or relative organ weights between the CR-LPF *ad libitum* feeding group and the CRF-1 *ad libitum* feeding group.

Histopathological Findings

Histopathological findings are summarized in Table 12. The histopathological findings in some organs were noted sporadically in the CR-LPF and CRF-1 *ad libitum* and restricted feeding groups. All of these findings were incidental in nature. Noticeable findings, though the degrees were slight or mild, were confined to the kidneys. Some urinary lesions associated with chronic nephrosis were markedly noted in the CR-LPF and CRF-1 *ad libitum* feeding groups. The renal lesions consisted of focal areas of basophilic tubules in the cortices and chronic nephropathy.

DISCUSSION

Male rats were given CR-LPF diet or CRF-1 diet *ad libitum* or restrictedly for 12 months to examine how dietary restriction and low-protein diet affect general signs, body weight, food consumption, blood parameters, and pathological findings.

Lower 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, respectively; 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. The distinctive quality of CR-LPF is considered to have been exhibited in this finding. A similar tendency for body weight gain to be suppressed was noted in the CR-LPF *ad libitum* feeding group.

Table 10. 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	(g)	772±87	475±17**	823±188	553±6#
Pituitary	(mg)	15.6±2.7	12.3±1.7*	17.2±1.9	13.7±1.2##
	(mg%)	2.0±0.4	2.6±0.3	2.1±0.3	2.5±0.2
Lungs	(g)	1.65±0.15	1.28±0.13**	1.55±0.12	1.39±0.1
	(g%)	0.22±0.02	0.27±0.03**	0.2±0.05	0.25±0.02#
Heart	(g)	1.71±0.29	1.19±0.12**	1.79±0.21	1.37±0.10##
	(g%)	0.22±0.03	0.25±0.03	0.22±0.03	0.25±0.02
Liver	(g)	20.02±2.52	10.26±0.95**	20.57±6.29	11.83±1.06#
	(g%)	2.59±0.17	2.16±0.15**	2.47±0.32	2.14±0.19
Spleen	(g)	1.05±0.37	0.78±0.10	1.20±0.39	0.77±0.03
	(g%)	0.14±0.05	0.16±0.02	0.15±0.06	0.14±0.01
Kidneys	(g)	3.36±0.28	2.37±0.15**	3.89±0.67	2.74±0.26##
	(g%)	0.44±0.05	0.50±0.02*	0.48±0.05	0.50±0.05
Adrenals	(mg)	45.9±6.4	38.3±1.9	41.2±8.2	40.8±6.4
	(mg%)	5.9±0.7	8.0±0.4**	5.1±0.8	7.4±1.1##

Each value shows mean ± S.D.
*: P<0.05, **: P<0.01, Significantly different from CR-LPF (*ad libitum* feeding) group.
#: P<0.05, ##: P<0.01, Significantly different from CRF-1 (*ad libitum* feeding) group.

Table 11. 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	(g)	823±188	772±87	553±6	475±17##
Pituitary	(mg)	17.2±1.9	15.6±2.7	13.7±1.2	12.3±1.7
	(mg%)	2.1±0.3	2.0±0.4	2.5±0.2	2.6±0.3
Lungs	(g)	1.55±0.12	1.65±0.15	1.39±0.10	1.28±0.13
	(g%)	0.20±0.05	0.22±0.02	0.25±0.02	0.27±0.03
Heart	(g)	1.79±0.21	1.71±0.29	1.37±0.10	1.19±0.12#
	(g%)	0.22±0.03	0.22±0.03	0.25±0.02	0.25±0.03
Liver	(g)	20.57±6.29	20.02±2.52	11.83±1.06	10.26±0.95#
	(g%)	2.47±0.32	2.59±0.17	2.14±0.19	2.16±0.15
Spleen	(g)	1.20±0.39	1.05±0.37	0.77±0.03	0.78±0.10
	(g%)	0.15±0.06	0.14±0.05	0.14±0.01	0.16±0.02
Kidneys	(g)	3.89±0.67	3.36±0.28	2.74±0.26	2.37±0.15#
	(g%)	0.48±0.05	0.44±0.05	0.50±0.05	0.50±0.02
Adrenals	(mg)	41.2±8.2	45.9±6.4	40.8±6.4	38.3±1.9
	(mg%)	5.1±0.8	5.9±0.7	7.4±1.1	8.0±0.4

Each value shows mean ± S.D.
#: P<0.05, ##: P<0.01, Significantly different from CRF-1 (restricted feeding) group.

Table 12. Histopathological findings in male rats

Group	CR-LPF(*ad libitum* feeding)					CRF-1(*ad libitum* feeding)					CR-LPF(restricted feeding)					CRF-1(restricted feeding)				
Number of males	5					5					5					5				
Grade	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+
Findings																				
Pituitary																				
Cyst, Rathke's pouch	4	0	1	0	0	5	0	0	0	0	4	0	1	0	0	4	0	1	0	0
Hyperplasia, anterior lobe	4	0	1	0	0	4	0	1	0	0	4	0	1	0	0	4	1	0	0	0
Cyst, 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, focal	3	2	0	0	0	4	0	1	0	0	5	0	0	0	0	3	2	0	0	0
Fibrosis, focal	5	0	0	0	0	3	0	2	0	0	5	0	0	0	0	4	1	0	0	0
Lung																				
Accumulation, foam cell	5	0	0	0	0	3	2	0	0	0	3	2	0	0	0	4	0	1	0	0
Granulation tissue	5	0	0	0	0	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0
Liver																				
Proliferation, bile duct	2	3	0	0	0	2	3	0	0	0	3	2	0	0	0	4	1	0	0	0
Altered hepatocellular focus	3	2	0	0	0	5	0	0	0	0	5	0	0	0	0	4	1	0	0	0
Necrosis, hepatocyte, focal	4	1	0	0	0	3	2	0	0	0	5	0	0	0	0	4	1	0	0	0
Microgranuloma	5	0	0	0	0	5	0	0	0	0	3	2	0	0	0	5	0	0	0	0
Hematopoiesis, extramedullary	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0
Spleen																				
Hematopoiesis, extramedullary	2	2	0	1	0	2	2	0	1	0	3	2	0	0	0	5	0	0	0	0
Kidney																				
Pyelitis	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0
Dilatation, urinary tubule	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0	4	1	0	0	0
Basophlic change, tubular epithelium	4	1	0	0	0	3	2	0	0	0	5	0	0	0	0	5	0	0	0	0
Nephropathy, chronic	3	1	1	0	0	4	0	1	0	0	5	0	0	0	0	5	0	0	0	0
Cellular infiltration, lymphoid cell, focal	5	0	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0
Adrenal																				
Hyperplasia, cortex, focal	3	2	0	0	0	4	1	0	0	0	5	0	0	0	0	5	0	0	0	0

Grade of histopathological findings: −: no abnormality detected, ±: slight, +: mild, 2+: moderate, 3+: marked.

No abnormal feeding conditions were noted either in the CRF-1 or CR-LPF *ad libitum* feeding group or in the CRF-1 or CR-LPF restricted feeding. Slightly lower food consumption was sporadically noted in the CR-LPF *ad libitum* group compared with the CRF-1 *ad libitum* group. This finding is, however, considered to be an incidental change, since it was not reflected in the body weight of the CR-LPF *ad libitum* feeding group. Otherwise, this finding is considered to have been caused by dietary components of CR-LPF.

In the hematological examination, higher MCH and more prolonged APTT were noted in the CRF-1 restricted feeding group compared with the CRF-1 *ad libitum* feeding group. The causes of these findings remain unknown. MCH in the CR-LPF restricted feeding group was also higher than that in the CR-LPF *ad libitum* feeding group. The cause of this finding also remains unknown. The higher lymphocytic ratio and lower neutrophile ratio noted in the CR-LPF restricted feeding group are considered to have been due to decubitus. There were no differences in the values of the hematological parameters either between the restricted feeding groups or between the *ad libitum* feeding groups.

In the blood chemical analysis, lower Glu and TG were noted in the CRF-1 and CR-LPF restricted feeding groups compared with the CRF-1 and CR-LPF *ad libitum* feeding groups. It can be said that these findings were caused by the dietary restriction. The causes of the other findings remain unknown. The changes noted when the CRF-1 *ad libitum* feeding group was compared with CR-LPF *ad libitum* feeding group are considered to have been due to the abnormal data obtained in the CRF-1 *ad libitum* feeding group.

Decubitus, which was noted at necropsy in the CRF-1 and CR-LPF *ad libitum* feeding groups, are considered to be a change accompanying the changes in body weight. The other findings noted at necropsy are not considered to be changes related to the test feeding, since such findings occur spontaneously in this strain of rats.

The absolute weights of almost all the organs weighed were lower in the CRF-1 and CR-LPF restricted feeding groups compared with the CRF-1 and CR-LPF *ad libitum* feeding groups, respectively. These findings are considered to have been related to the lower body weight in the restricted feeding groups. On the other hand, it remains unknown what the lower or higher relative organ weights noted in the restricted feeding groups mean. The changes in the heart, liver, and kidney weights, which were noted when the CR-LPF restricted feeding group was compared with the CRF-1 restricted feeding group, are considered to be changes accompanying the lower body weight in the restricted feeding group, since no differences were noted between the CRF-1 and CR-LPF *ad libitum* feeding groups.

There were no marked differences in the incidence of each histopathological lesion between the CR-LPF restricted feeding group and the CR-LPF *ad libitum* feeding group, between the CRF-1 restricted feeding group and the CRF-1 *ad libitum* feeding group, between the *ad libitum* feeding groups, or between the restricted feeding groups. However, it is considered that occurrence of chronic nephropathy was, although slightly, suppressed in the CRF-1 and CR-LPF restricted feeding groups.

The present study is being carried out to be completed 24 months after initiation of test feeding. After the study is completed, data will be compiled again to prepare a final report, in which the authors are to have a discussion about the results referring to literature published so far.

Thyroid Hormones and Thyroid-Stimulating Hormone in Crj:CD(SD)IGS Rats --- A preliminary examination ---

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ABSTRACT. The thyroid hormones and thyroid-stimulating hormone of rat serum were evaluated to obtain background data on these hormones in Crj:CD(SD)IGS rats. Under the conditions used to measure the hormone levels, the concentrations of 5 hormones in female rats showed differences at each stage of the estrous cycle. The measurements of free triiodothyronine and free thyroxine along with triiodothyronine, thyroxine, and thyroid-stimulating hormone might be an important means for the evaluation of toxicological effects derived from drugs or endocrine-disrupting substances and other chemicals in the environment. — Key words: Thyroid Hormones, Thyroid-Stimulating Hormon, Crj:CD(SD)IGS

CD(SD)IGS-2001: 40-42

INTRODUCTION

The evaluation of hormones is very important to develop new drugs targeting endocrine organs or to detect endocrine-disrupting effects of environmental chemicals in toxicological assessments. Some chemicals including endocrine-disrupting substances may modulate the levels of sex steroid hormones. Data from some studies have suggested that these hormones may affect the hypothalamic-pituitary-thyroid axis. However, these effects are not well understood. In this study, for the monitoring of thyroid functions, the measurements of thyroid-stimulating hormone (TSH), thyroxine (T4), triiodothyronine (T3), free T4 (FT4), and free T3 (FT3) were evaluated.

The need to define what tests should be used to assess endocrine disrupters has recently become a priority issue in the field of toxicology. One of the recommended parameters is hormone levels, and thus methods that are the most sensitive to detect endocrine-disrupting substances by toxicological protocols and the most reliable among laboratories for the measurement of various hormones are required.

In this study, the assay conditions for hormone measurement of rat samples were evaluated, and preliminary background data on hormones in Crj:CD(SD)IGS rats were collected.

MATERIALS AND METHODS

Crj:CD(SD)IGS rats were supplied by Charles River Japan, Inc. In this study, animals were obtained at 5 weeks of age. The animals, acclimated to the laboratory for two weeks prior to the start of the experiment, were housed individually in stainless steel cages with *ad libitum* access to a commercial diet (CE-2, CLEA Japan Inc., Tokyo, Japan) and tap water. The room temperature was maintained at 21 to 25 ℃; and the humidity, at 40 to 75%. Lighting was controlled automatically to give a cycle of 12 hours of light (7:00 to 19:00) and 12 hours of darkness.

Clinical observations and mortality checks were performed on all animals daily. Body weight and food consumption were measured according to the same time intervals as mentioned previously [1].

Daily vaginal lavage fluid in each female was collected at 10 weeks of age. The stage of the estrous cycle was determined, and blood was drawn from the abdominal aorta of each animal under ether anesthesia.

After centrifugation, the serum was separated and stored at −80℃ until enzyme immunoassays could be performed.

Hormonal parameters reported in this article, TSH, T3, T4, FT3, and FT4, are summarized in Table 1 with the list of reagents used.

Data on thyroid hormones in females analyzed between the first day of diestrus (D1) and proestrus (P) or estrus (E) stages, and between the second day of diestrus (D2) and P or E, by t-test.

RESULTS AND DISCUSSION

T4, T3, and TSH were determined by using ELISA kits; and FT4 and FT3, with RIA kits. These reagent kits were selected after assessing several points of applicability among a few reagent kits for each hormone. The reagent kits displayed marked differences of analytical performance on these analytes. The kits listed in Table 1 appeared to be the most suitable for our purposes at present.

With the reagent kit for rats used here, TSH showed good recovery and a wide range of linearity, as reported previously [1]. The reagent kits developed for human T3 and T4 showed some problems, as also reported earlier [1]. However, T3 and T4 showed an acceptable range of linearity, as did FT3 and FT4, which were also developed for human use. The results on validation of the analytical methods are summarized in Table 2. Each kit required a prescribed time for incubation, as well as some additional sample preparation time for assay and measurement, etc. The FT3 assay was the most difficult to optimize of all of the hormone assays evaluated in this study. Spiking recoveries were based upon measured values of the added analyte. Inter-individual differences in analytical performance were seen for rat TSH and T4. The spiking recovery values for rat T3, FT3, and FT4 were satisfactory. A satisfactory analyte stability was also found. Additionally, Table 2 shows that these assays provide sufficient applicability to assess effects of chemical substances and drugs on the hypothalamic-pituitary-thyroid axis. Table 3 shows significant differences in hormone levels at the various estrous stages, D1, D2, P, and E. These differences also indicate that these methods provide sufficient sensitivity to evaluate stage differences of the estrous cycle.

The normal/reference values found for rat TSH, T3, FT3, T4, and FT4 provide a sufficient performance for further studies. We did not experience any difficulties with standardization of these assay kits.

In recent years, hormone measurement has become an important issue for the evaluation of effects derived from environmental contaminants or endocrine-disrupting substances, etc. Each animal has a specific individual hormonal condition, indicating that the method of hormone measurement including sampling conditions needs to be assessed for its reliability before the animal experiment. Use of these assay kits will allow improvement of studies on the hypothalamic-pituitary-thyroid axis.

ACKNOWLEDGMENTS

We thank our colleagues in the Hatano Research Institute, Food and Drug Safety Center for their advice and support for this study effort.

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Table 1 Abbreviations and methods for measurement of hormonal parameters

Hormone	Code	Kit	Supplier
Thyroid-stimulating hormone	TSH	Rat thyroid stimulating hormone (rTSH) EIA system	Amersham Pharmacia Biotech
Triiodothyronine	T3	Active Total T3 EIA*	Diagnostic Systems Laboratories Inc.
Thyroxine	T4	Active Thyroxine (T4) EIA*	Diagnostic Systems Laboratories Inc.
Free triiodothyronine	FT3	DPC free T3 kit*	Diagnostic Products Corporation
Free thyroxine	FT4	GammaCoat Free T4* (Direct One Step)	DiaSorin Inc.

* EIA for human use

Table 2 Validation of analytical methods

Factors	Analyte				
	T3	FT3	T4	FT4	TSH
Time of analysis (for incubation) (min)	75	180	70	90	270
Working range (unit)*	0.5 – 7.5	0.48 – 44	5 – 500	1.6 – 73	0.26 – 64
Sample volume (μl)	50	100	25	50	50/6 **
Within-day precision (CV%)	1.9 – 4.9	7.8 – 18	2.5 – 6.9	5.1 –7.7	2.7 – 7.6
Between-day precision (CV%)	12 – 15	9.1 – 12	6.1 – 8.1	5.2 – 14	4.3 – 9.3
Spiking recovery (%)	86 – 99	70 – 90	83 – 146	73 – 103	73 – 134
Dilution recovery (%)	75 – 82	NT***	72 – 116	NT	87 – 111
Analyte stability after 4 weeks (CV%)	5.2 – 6.9	12 – 22	3.6 –7.2	NT	6 – 20
Analyte stability after 8 weeks (CV%)	7.3 – 10	19 – 27	6.0 – 13	NT	15 – 18

(unit)* : T3, T4, and TSH are reported as ng/ml; and FT3 and FT4, as pg/ml.
** : 50 μl of 6-fold diluted original serum
*** : Not tested

Table 3 Hormonal parameters for Crj:CD(SD)IGS rats and comparison during the estrous cycle

Parameter (unit)	Male	Female Estrous stage			
		D1	D2	P	E
T3 (ng/ml)	0.75 ± 0.12 (0.45 ～ 0.97)[1)]	0.79 ± 0.11 (0.60 ～ 1.14)	0.78 ± 0.12 (0.55 ～ 1.01)	0.85 ± 0.11 (0.66 ～ 1.12)	0.86*[#] ± 0.10 (0.60 ～ 1.09)
FT3 (pg/ml)	1.14 ± 0.190 (0.831 ～ 1.58)	1.19 ± 0.181 (0.831 ～ 1.50)	1.23 ± 0.238 (0.820 ～ 1.58)	1.37* ± 0.289 (1.04 ～ 2.05)	1.33 ± 0.307 (0.836 ～ 1.95)
T4 (ng/ml)	121 ± 22.5 (86.3 ～ 162)	107 ± 17.3 (78.7 ～ 137)	106 ± 25.1 (75.6 ～ 175)	132**[##] ± 26.5 (96.9 ～ 202)	127**[##] ± 19.2 (89.9 ～ 156)
FT4 (pg/ml)	8.86 ± 1.74 (5.81 ～ 11.9)	7.53 ± 1.61 (4.62 ～ 11.0)	7.97 ± 1.42 (5.09 ～ 10.2)	9.97**[##] ± 2.28 (6.04 ～ 13.2)	10.0**[##] ± 1.56 (7.42 ～ 12.9)
TSH (ng/ml)	26.5 ± 7.71 (14.6 ～ 47.7)	38.4 ± 11.8 (20.0 ～ 65.4)	38.4 ± 14.1 (16.7 ～ 67.2)	43.3 ± 12.4 (23.9 ～ 68.9)	44.3 ± 15.4 (25.6 ～ 86.6)

[1)] : Range (Min.～Max.)
Estrous stage : D1, the first day of diestrus; D2, the second day of diestrus; P, proestrus; E, estrus
Number of animals tested : 20
Significantly different from the D1 value (*: $p<0.05$, **: $p<0.01$)
Significantly different from the D2 value ([#]: $p<0.05$, [##]: $p<0.01$)

Dissection and Weighing of Accessory Sex Glands after Formalin Fixation, and a 5-day Assay Using Young Mature Rats are Reliable and Feasible in the Hershberger Assay

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ABSTRACT. The rodent Hershberger assay has been used predominantly by the pharmaceutical industry to evaluate androgenic and antiandrogenic chemicals for potential therapeutic use. However, this assay has not yet been formally validated and standardized for use in toxicology testing. There are many variations in the protocol used for this assay. The weight of androgen-dependent tissues is a definitive endpoint in the Hershberger assay. To find out the reliable assay protocol with feasibility, although many possible factors may affect assay reliability, the present study consist of a series of 3 separate experiments focused on method of dissection and weighing of accessory sex glands (ASGs: ventral and dorso-lateral prostate, seminal vesicles together with coagulating glands, and Cowper's glands), animal age and number of doses. Furthermore, male pubertal assay, an alternative to the Hershberger assay, was also examined its reliability.

Experiment 1 explored whether reliably accurate ASG weights can be obtained after formalin fixation. The ASGs were collected from castrated male rats (11 weeks of age) treated daily with corn oil, or testosterone propionate (TP, 0.25 mg/kg/day, s.c.) and *p,p'*-DDE (0 or 100 mg/kg/day, p.o.) for 5 days. One day after the final treatment, the ASGs were removed carefully and weighed separately, and then fixed overnight in a 10 % neutral-buffered formalin and weighed again. After that, the tissues were dried overnight in an oven and weighed again. A high correlation between fresh and fixed tissue weights, and a high correlation between fixed and dried tissue weights were noted. The changes in the tissue weight due to fixation were marginal and were proportional to the fresh weights of the individual tissue. Standard deviation of the fixed tissue weight in each group and the magnitude of responses to TP or *p,p'*-DDE in fixed tissues were equivalent to those in fresh or dried tissues. These findings indicate that formalin fixation does not interfere with interpretation of assay results, and this procedure was used in the subsequent experiments.

Experiments 2 and 3 explored whether animal age or treatment duration altered assay sensitivity. In Experiment 2, antiandrogenic effect of *p,p'*-DDE (100 mg/kg/day) was detected after 5-and 10-day treatment irrespective of animal age (7 vs. 11 weeks). In Experiment 3, antiandrogenic effects of flutamide (1 and 10 mg/kg/day) and *p,p'*-DDE (10 and 100 mg/kg/day) were compared between two different protocols, the 10-day assay using peripubertal rats and the 5-day assay using young mature rats. Results demonstrated that both protocols could significantly detect antiandrogenic effects of flutamide and *p,p'*-DDE.

These findings demonstrate that (1) dissection and weighing of ASGs after formalin fixation is reliable in the Hershberger assay, (2) when this procedure is used, the 5-day Hershberger assay using young mature rats, expected to be more feasible assay than the 10-day assay using peripubertal rats, is also reliable as well as the 10-day assay using peripubertal rats. Furthermore, we confirmed that male pubertal assay with use of dissection and weighing of fixed tissues also reliable. — Keywords: Endocrine disruptor; Screening; Hershberger assay; *p,p'*-DDE; (Anti)androgen; Immature/mature rat

CD(SD)IGS-2001: 43-55

INTRODUCTION

Steroid receptors are thought to play crucial roles in normal physiology, development, reproduction, and carcinogenesis. Although there is public concern that certain environmental xenobiotics may have toxic effects in wildlife and humans by interfering with steroid receptor mediated-mechanism(s), the link between such environmental chemicals with hormone-like activity and adverse effects on humans has not been well established. On the other hand, the suitability of the current guideline studies for detection of the endocrine-disrupting potential of chemicals has been questioned. To establish short-term *in vivo* screening systems, evaluation of the reliability and feasibility of existing test methods is in progress within the OECD framework. For mammalian species these methods include the uterotrophic and Hershberger assays and the enhanced OECD test guideline 407.

The rodent Hershberger assay evaluates the ability of a chemical to interfere with androgen-mediated mechanism(s). Accessory sex glands/tissues require androgen stimulation to gain and maintain weight during and after puberty. If male rodents are castrated (*i.e.,* endogenous testicular sources of androgen are removed), exogenous sources of androgen are necessary to increase or maintain the weights of these tissues. In the castrated male rodent, therefore, effects on these tissues are likely to be direct and not a result of pituitary or gonadal secretion. For assessment of androgenicity, test chemicals are administered to castrated males, while for antiandrogenicity, test chemicals are administered to castrated, testosterone-treated males.

The rodent Hershberger assay has been used predominantly by the pharmaceutical industry to evaluate androgenic and antiandrogenic chemicals for potential therapeutic use [5, 6, 8]. However, this assay has not yet been formally validated and standardized for use in toxicology testing. There are many variations in the protocol used for this assay [3, 5, 6, 8, 10−18, 21, 22]. The protocol for further standardization and optimization is under consideration by the OECD. The current proposed OECD protocol for the Hershberger assay includes weighing individual sex accessory tissues, since little is known about the response of individual sex accessory tissues to exogenous chemicals that may have androgenic effects. These organs are the ventral prostate, seminal vesicles together with coagulating glands, glans penis, Cowper's glands, *and levator* ani and *bulbocavernosus* muscles. The need for familiarity with dissection and weighing the different accessory sex tissues is a key issue in transferability of the assay, and in studying the responsiveness of the various tissues to androgens and other steroid hormones. In particular, the tissues including fluid such as seminal vesicles and Cowper's glands must be treated with particular care to prevent variations in fluid loss from tissues during processing. In addition, it is difficult to grossly

Note: This paper is reprinted from original paper (Toxicology 162, 103-119, 2001)

distinguish ventral and dose-lateral lobes in fresh prostate (before fixation).

In this study, we evaluated the reliability and feasibility of dissection and weighing of ASGs after fixation and of the 5-day assay using young mature rats in studies for assessment of (anti)androgens, especially the Hershberger assay. The male pubertal assay (an alternative to the Hershberger assay) [2] was also evaluated using dissection and weighing of ASGs after fixation.

MATERIALS AND METHODS

Test Materials. Materials were obtained from the following manufacturers: testosterone propionate (TP) (Purity:>97%, Catalog No. 207-08431), Wako Pure Chemical Industries (Osaka, Japan); 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) (Purity:99%, Catalog No. 12,389-7), Aldrich Chemical Company (Milwaukee, WI); flutamide (Purity:99%, Catalog No. F9397), Sigma Chemical Company (St. Louis, MO); corn oil (Nakarai Tesque, Inc., Kyoto); and pulverized diet CRF-1, Oriental Yeast Co. (Tokyo, Japan).

Animals and Housing. All experiments were performed in accordance with *The Guide for Animal Care and Use of Sumitomo Chemical Co. Ltd.* Male Crj:CD(SD)IGS rats were purchased from Charles River Japan, Inc. (Shiga, Japan). They were 2 (Experiment 4), 5 (Experiments 2 and 3) or 9 (Experiments 1, 2, and 3) weeks of age upon arrival, and were acclimatized to the laboratory environment for 7 days before use. In Experiment 4, immature rats arrived with dams. After the quarantine period, animals in good health (based on clinical signs and body weights) were selected for the study. In Experiments 1-3, castration was performed via the scrotum under ether anesthesia, and chemical treatment was not commenced until 7 days later to allow for complete recovery from surgical stress.

During the experiment, rats were housed two per cage in stainless steel wire mesh cages under controlled environmental conditions, including a temperature of 24±2 degree centigrade, a relative humidity of 55±10%, a frequency of ventilation of more than 10 air exchanges/hr, and a 12-hr light/dark cycle (lights on, 0800-2000). For pubertal assay, before weaning at 3 weeks of age, 10 immature male rats per cage were housed with a dam in each cage; the wire-mesh front and floor were covered with a stainless steel solid panel and a tray with nesting materials, respectively. Drinking water and pellet rodent diet were available *ad libitum.* Prior to dosing, rats were assigned to groups by the stratified randomization method using a computer program based on body weight and body weight gain during the quarantine period, so that no significant differences in mean body weight were present among the groups. At the commencement of treatment, it was confirmed that no significant differences existed in mean body weight among the groups, since variation in animal body weight may be a source of variation in weight of accessory sex glands [2, 18].

Study Design. For each experiment in the Hershberger assay (Experiments 1-3), 6 males per group were used. For the male pubertal assay (Experiment 4), 10 rats per groups were used based on previous findings [22]. Test and reference substances were dissolved in a test vehicle (corn oil). The daily amounts of administration were 5ml/kg BW for oral gavage and 0.5 ml/kg BW for subcutaneous injections. In the Hershberger assay using young mature rats, the amount administered to each animal was adjusted for body weight on Days 1 and 3 only, since daily change in body weight at this age (11 weeks old) was within only 2 % of body weight based on our background data. For the Hershberger assay using peripubertal rats, the amount administered to each animal was adjusted daily for body weight. In the male pubertal assay, the amount administered to each animal was adjusted for the most recent body weight (body weight was determined once a week). For all experiments, clinical signs and body and liver weights were determined as indices of systemic toxicity.

Experiment 1: Effect of formalin fixation on tissue weights. To determine the effects of formalin fixation on tissue weights, each tissue was weighed before and after fixation. Tissue samples were collected from castrated animals treated daily with vehicle (corn oil) or testosterone propionate (TP, 0.25 mg/kg/day, s.c.) and *p,p'*-DDE (0 or 100 mg/kg/day, p.o.) for 5 days. *p,p'*-DDE was selected as a positive control for antiandrogenicity since it is a known environmental contaminant and is thought to have weak antiandrogenic activity *in vitro* and *in vivo* [3, 7, 9, 12, 18, 23]. One hundred mg/kg/day of *p,p'*-DDE *in utero* exposure induced antiandrogenic responses such as reduction in male anogenital distance, increase in retention of male thoracic nipples, and alteration in expression of AR [9, 23]. A suitable value of TP as reference androgen for assessment of antiandrogenicity of test chemicals was selected based on our previous study [18]. One day after the final administration, all rats were anesthetized with ether, and then euthanized by blood withdrawal from the abdominal aorta (AM 0830-1100). After careful trimming to remove fat and other contiguous tissue in a uniform manner, the ventral prostate, dorso-lateral prostate, seminal vesicles (with coagulating glands), and Cowper's glands were weighed freshly, and then were fixed overnight (about 24 hr) in 10% neutral-buffered formalin and weighed again. The tissues were then dried overnight in an oven (70 degree centigrade) and weighed again.

Experiment 2: Comparison between Hershberger assays using peripubertal and young mature rats. Castrated peripubertal (7 weeks of age) or young mature (11 weeks of age) male rats were administered TP (0.25 mg/kg/day, s.c.) and *p,p'*-DDE (0 or 100 mg/kg/day, p.o.) for 5 and 10 days. One day after the final administration, all rats were anesthetized with ether, and then euthanized by blood withdrawal from the abdominal aorta (AM 0830-1100). After careful removal of the accessory sex gland unit (the ventral prostate, dorso-lateral prostate and seminal vesicles with coagulating glands) and Cowper's glands, they were fixed overnight in 10% neutral-buffered formalin. Then each tissue of the accessory sex gland unit was separated and weighed individually. The glans penis, *levator ani* and *bulbocavernosus* muscles and liver were weighed immediately after careful trimming to remove fat and other contiguous tissue in a uniform manner without fixation.

Experiment 3: Comparison of two different protocols: 10-day assay using peripubertal rats and 5-day assay using young mature rats. Using dissection and weighing of ASGs after fixation, activities with two different protocols of the Hershberger assay, the 10-day assay using peripubertal rats and 5-day assay using young mature rats, were compared. The 10-day assay using peripubertal rats is currently under consideration as a standard protocol by the OECD. Flutamide (1 or 10 mg/kg/day) or *p,p'*-DDE (10 or 100 mg/kg/day) was administered as reference antiandrogen. For the 10-day assay using peripubertal rats, rats aged 7 weeks were treated for 10 consecutive days, while for the 5-day assay using young mature rats, rats aged 11 weeks were treated for only 5 consecutive days. Treatment regime, necropsy and method of tissue weighing were identical to those of Experiment 2.

Experiment 4: Male pubertal assay. Immature male rats (21 days old) were treated with 0 (corn oil) or 100 mg/kg/day of *p,p'*-DDE for 20 days. One day after the final administration, all rats were anesthetized with ether, and then euthanized by blood withdrawal from the abdominal aorta (AM 0830-1100). After careful removal of the accessory sex gland unit (the ventral prostate, dorso-lateral prostate, seminal vesicles with coagulating glands) and Cowper's glands, they were fixed overnight in 10% neutral-buffered formalin, and then separated and weighed individually. The testes, epididymides, *levator ani* and *bulbocavernosus* muscles and liver were weighed freshly after careful trimming to remove fat and other contiguous tissue in a uniform manner.

Statistical Analysis. In Experiments 1, 2, and 4, data were assessed by F-test for homogeneity of variance. If homogeneous, the data were analyzed by Student's t-test; and if not homogeneous, the data were analyzed by the Welch test. In Experiment 3, data were assessed by Bartlett's U test for homogeneity of variance. If homogeneous, the data were analyzed by Dunnett's multiple comparison test; and if not homogeneous, the data were analyzed by Steel's test. The significance of differences from the control group was estimated at probability levels of 1 and 5%.

RESULTS

The Hershberger assay: effects of formalin fixation on weights of accessory sex glands.

Figure 1 shows gross findings for the seminal vesicles (with coagulating glands), ventral prostate and dorso-lateral prostate both before and after fixation. Formalin fixation shaded tissue white, and the dorso-lateral prostate became more white than the ventral prostate. With fresh tissue, as shown in Fig. 2, it appears to be difficult to distinguish the dorso-lateral prostate from ventral prostate. With fixed tissues, however, these two were easily distinguished.

The coefficients of correlation (R^2) between fresh and fixed tissue weights were nearly 1: a high correlation was observed between fresh and fixed tissue weights for each tissue (Fig. 3). These data included tissues with variety of size, since the tissues were collected from castrated, castrated±TP treated, and castrated±TP and *p,p'*-DDE-treated rats. As shown in Fig. 4, seminal vesicle weight was marginally changed by fixation, but the weight of fixed seminal vesicles was generally proportional to the weight of the fresh ones. Ventral and dorso-lateral prostates, and Cowper's glands also revealed same tendency (data not shown). Furthermore, a high correlation between fixed and oven dry weights was also observed (Fig. 5), suggesting that even fixed tissues can represent net response to androgen stimulation but not artifact of fixation (ex. change of water penetration).

To determine if the marginal change in tissue weight caused by fixation interferes with interpretation of results of the Hershberger assay, effects of TP and a weak antiandrogen *p,p'*-DDE were compared before to after fixation and after drying (Table 1). With fresh, fixed or dried tissues, subcutaneous injections of TP at dose levels of 0.25 mg/kg/day significantly increased the weights of all tissues examined except Cowper's glands. Co-administration with 100 mg/kg/day of *p,p'*-DDE significantly attenuated the TP 0.25 mg/kg/day-induced increases in weights of these tissues. The variation and magnitude of responses to TP or *p,p'*-DDE in fixed tissues were comparable to those in fresh or dried tissues. These findings demonstrate that formalin fixation does not interfere with interpretation of results of the Hershberger assay.

The Hershberger assay: comparison between peripubertal and young mature rats. Table 2 shows that effects of *p,p'*-DDE on androgen-dependent tissues in 4 kinds of study protocols. Irrespective of treatment period (5- or 10-day) and animal age (7 or 11 weeks of age), statistically significant reductions of tissue weights by *p,p'*-DDE were observed in all androgen-related tissues examined except in three cases: two were Cowper's glands of peripubertal rats treated for 5 days and mature rats treated for 10 days; the other was *levator ani* and b*ulbocavernosus* muscles of mature rats treated for 5 days. Although statistical significance was not observed, a tendency toward antiandrogenic effect of *p,p'*-DDE on weights of these tissues was observed.

The Hershberger assay: comparison of 10-day assay using peripubertal rats with 5-day assay using young mature rats. Both protocols clearly revealed antiandrogenic responses to 1 and 10 mg/kg/day of flutamide, a potent antiandrogen (Table 3). The glans penis of peripubertal rats treated with 1 mg/kg/day of flutamide for 10 days and Cowper's glands of young mature rats treated with 1 and 10 mg/kg/day of flutamide for 5 days tended to be small but not statistically significant compared to control. For *p,p'*-DDE, a weak antiandrogen, both protocols detected an antiandrogenic effect at 100 mg/kg/day. The 10-day assay using peripubertal rats revealed no responses to 10 mg/kg/day of *p,p'*-DDE, whereas the 5-day assay using young mature rats revealed a tendency toward decrease in tissue weights. For the Cowper's glands, unexpected changes were observed in the 5-day assay using young mature rats: a statistically significant decrease in weight was observed with 10 but not 100 mg/kg/day of *p,p'*-DDE. The reason for this unexpected change is unknown but unfamiliarity with dissection technique for this tissue may be related to it.

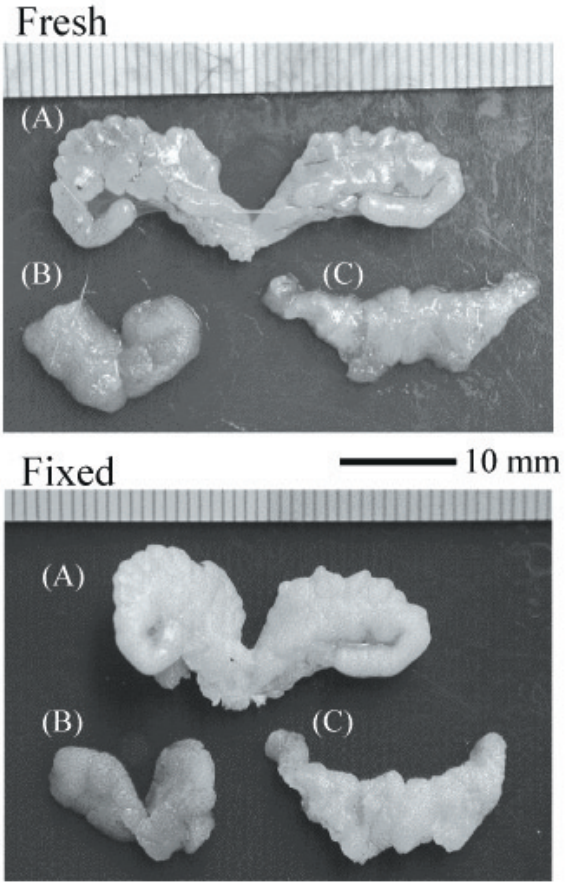

Figure 1. Gross findings for (A) seminal vesicles, (B) ventral and (C) dorso-lateral prostate before and after formalin fixation. Upper panel, before fixation; Lower panel, after fixation. Tissue sample was collected from castrated rats treated with testosterone propionate (0.25 mg/kg/day) by subcutaneous injection for 5 days in Experiment 1.

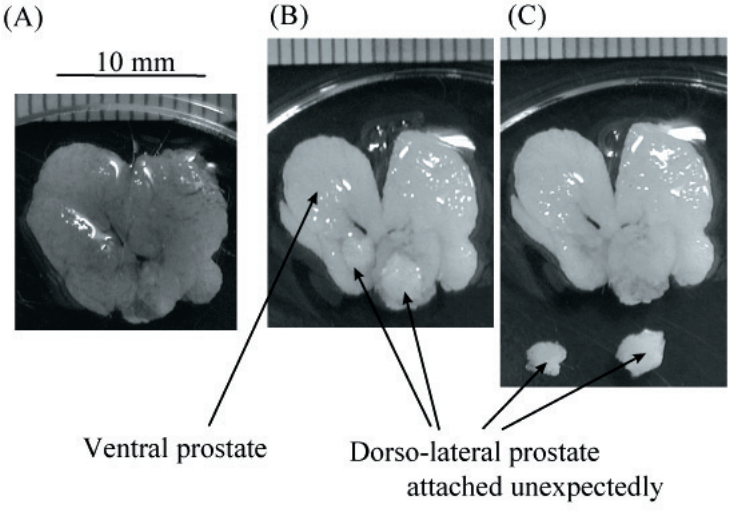

Figure 2. Gross findings for ventral prostate before and after formalin fixation. (A), fresh tissue expected to be ventral prostate. (B), fixed ventral prostate before separation of unexpectedly attached partial dorso-lateral prostate. (C), fixed ventral prostate after separation of unexpectedly attached partial dorso-lateral prostate. Tissue sample was collected from castrated rats treated with testosterone propionate (0.25 mg/kg/day) by subcutaneous injection for 5 days in Experiment 1.

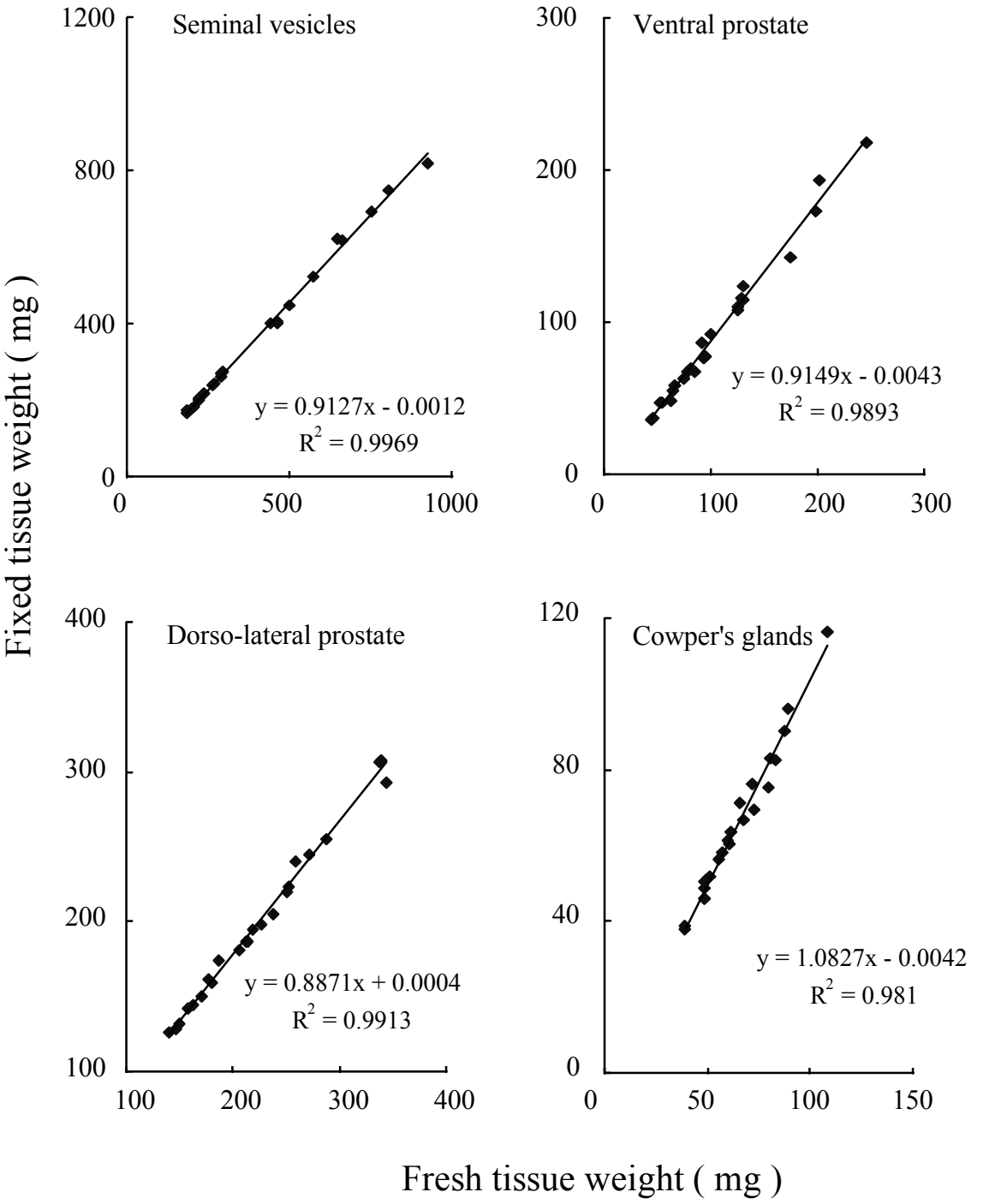

Figure 3. Relationship between fresh and fixed tissue weights. Details of tissue samples are described in Experiment 1 of the Methods section. n=18. y=ax + b, regression equation. R^2, correlation coefficient.

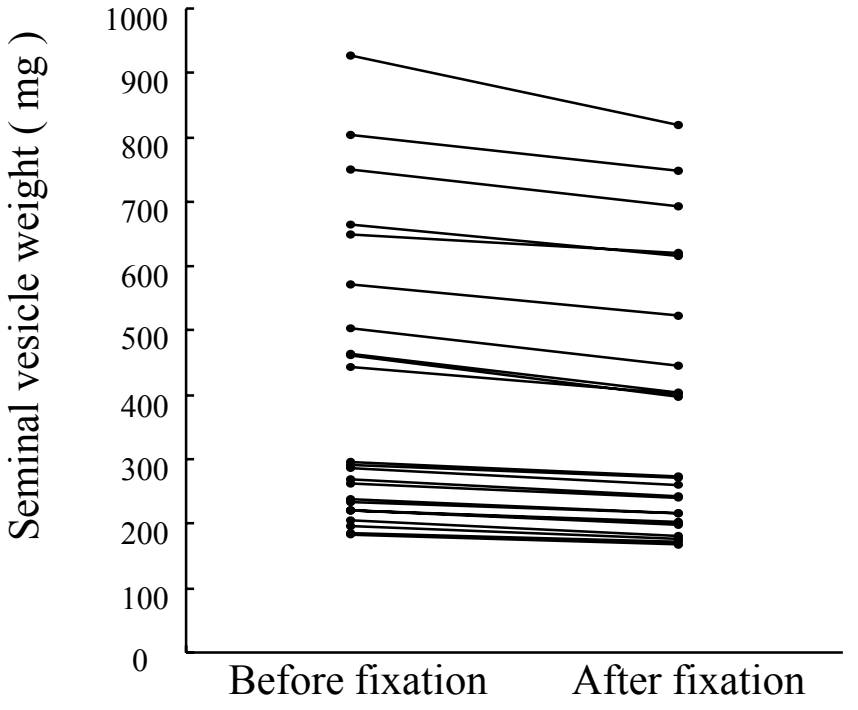

Figure 4. Individual seminal vesicle weights before and after fixation. Details of tissue samples are described in Experiment 1 of the Methods section. n=18.

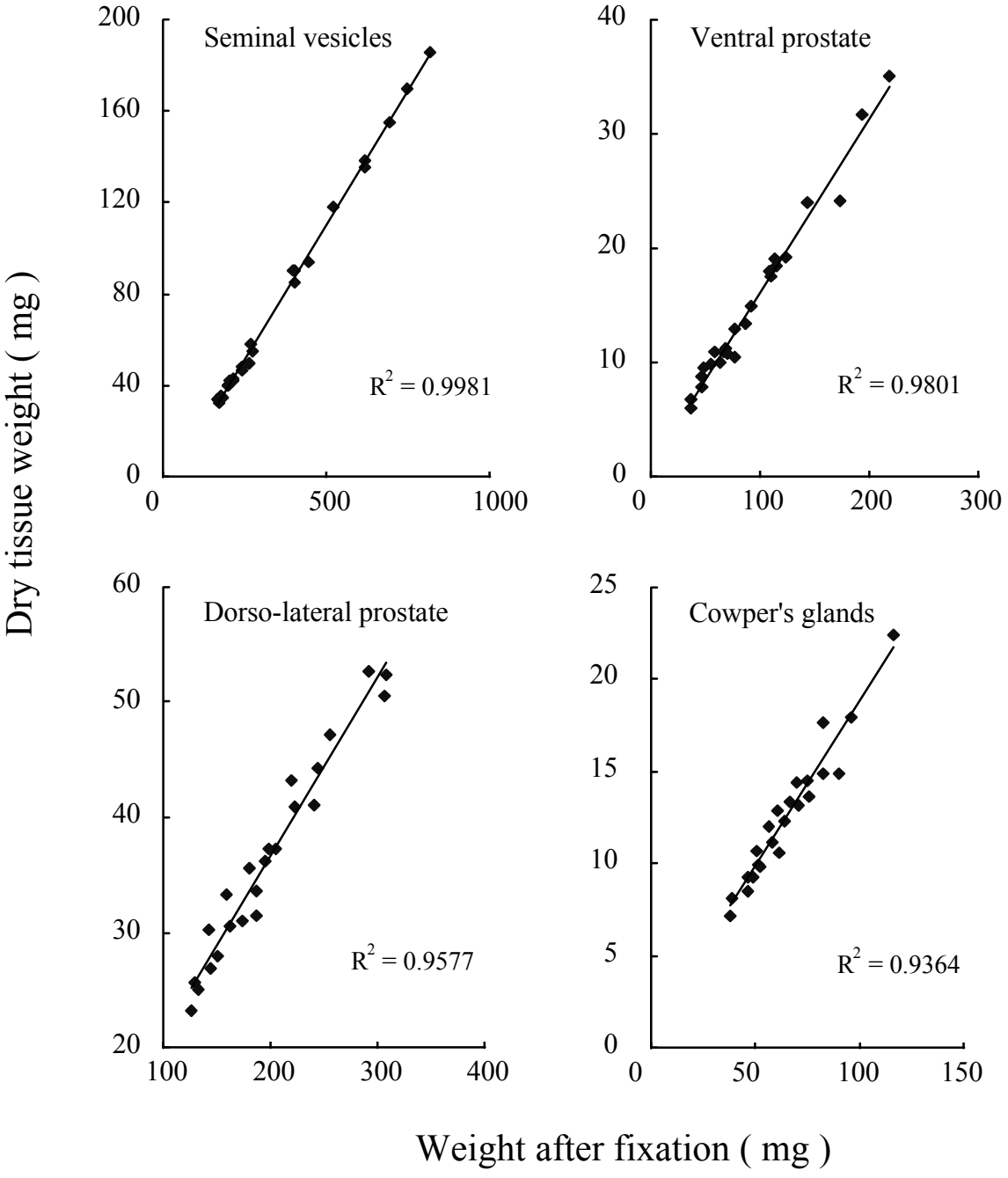

Figure 5. Relationship between dried and fixed tissue weights. Details of tissue samples are described in Experiment 1 of the Methods section. n=18. R^2, correlation coefficient.

Table 1. Effect of Formalin Fixation on Tissue Weight

Group	Seminal vesicles (mg)	Dorso-lateral prostate (mg)	Ventral prostate (mg)	Prostate (combined)[a)] (mg)	Cowper's glands (mg)
Before fixation					
Castrated	247±86.0	177±28.3	67±16.0	244±42.0	60±20.1
Castrated+TP0.25 + *p,p'*-DDE 0	728±127.2** (295%)[b)]	306±38.0** (173%)	179±46.0** (267%)	486±79.4** (199%)	70±15.2 (117%)
Castrated+TP0.25 + *p,p'*-DDE 100	371±93.9$^{\#\#}$ (51%)[c)]	233±19.7$^{\#\#}$ (76%)	112±18.3$^{\#\#}$ (63%)	348±28.2$^{\#\#}$ (72%)	66±16.4 (94%)
After fixation					
Castrated	223±75.5	158±24.7	56±13.1	214±35.5	61±22.1
Castrated+TP0.25 + *p,p'*-DDE 0	670±105.8** (300%)[b)]	275±31.2** (174%)	158±44.2** (282%)	433±71.7** (202%)	70±16.5 (115%)
Castrated+TP0.25 + *p,p'*-DDE 100	3327±6.6$^{\#\#}$ (50%)[c)]	204±17.3$^{\#\#}$ (74%)	101±18.3$^{\#}$ (64%)	307±25.3$^{\#\#}$ (71%)	68±18.0 (97%)
After dry					
Castrated	44±16.4	30±4.7	10±2.0	40±6.1	12±4.2
Castrated+TP0.25 + *p,p'*-DDE 0	150±24.8** (341%)[b)]	48±4.7** (160%)	25±6.8** (250%)	73±10.7** (183%)	14±3.1 (117%)
Castrated+TP0.25 + *p,p'*-DDE 100	71±19.4$^{\#\#}$ (47%)[c)]	37±4.9$^{\#\#}$ (77%)	16±3.4$^{\#}$ (64%)	53±6.4$^{\#\#}$ (73%)	13±3.1 (93%)

Note. Castrated young mature rats (11 weeks of age) were administered with testosterone propionate (0.25 mg/kg/day) by subcutaneous injection and *p,p'*-DDE (0 or 100 mg/kg/day) by oral gavage for 5 days. One day after the final treatment, the ASGs were removed carefully and weighed separately, and then fixed overnight in a 10% neutral-buffered formalin and weghed again. After that, the tissues were dried overnight in an oven and weighed again.
a): Sum of the weights of dorso-lateral plus ventral lobes.
b): Values represent the percentage against the mean values of castrated group.
c): Values represent the percentage against the mean values of castrated + TP 0.25 + *p,p'*-DDE 0 group.
Mean + SD, N=6.
*,**: Significantly different from castrated group (* $p<0.05$, ** $p<0.01$).
#,##: Significantly different from castrated + TP0.25 + *p,p'*-DDE 0 group (# $p<0.05$, ## $p<0.01$).

Table 2. Effect of *p,p'*-DDE on Androgen Relevant Sex Accessory Glands and Tissues in Castrated Male Rats Daily Treated with Testosterone Propionate

Treatment period (days)	Age[a)] (week-old)	Doses (mg/kg/day)	Final Body weight (g)	Seminal vesicles (mg)	Dorso-lateral prostate (mg)	Ventral prostate (mg)	Prostate (combined)[b)] (mg)	LA/BC[c)] (mg)	Grans penis (mg)	Cowper's glands (mg)	Liver (g)
		Control (0)	269±18.2	196±29.9	96±8.8	78±12.6	174±16.1	416±38.9	82±2.6	22±3.7	12.37±1.228
5	7	100	259±11.8	91±7.6**	60±5.6**	44±5.8**	105±9.9**	272±26.6**	71±5.6**	16±6.4	16.96±1.089**
			(96%)[d)]	(46%)	(63%)	(56%)	(60%)	(65%)	(87%)	(73%)	(137%)
		Control (0)	387±10.6	614±78.4	251±27.1	151±24.7	402±49.8	945±125.6	105±5.4	104±25.8	13.50±0.909
5	11	100	377±9.9	381±72.4**	180±7.2**	98±19.6**	278±21.3**	812±65.9	96±4.3*	66±14.8*	17.88±0.982**
			(97%)	(62%)	(72%)	(65%)	(69%)	(86%)	(91%)	(63%)	(132%)
		Control (0)	332±18.8	453±94.7	151±23.3	163±33.1	314±45.4	611±48.3	97±5.9	47±7.7	14.91±1.615
10	7	100	319±18.5	168±47.9**	86±12.2**	78±14.9**	164±21.6**	353±46.0**	81±2.7**	27±5.5**	22.57±2.041**
			(96%)	(37%)	(57%)	(48%)	(52%)	(58%)	(84%)	(57%)	(151%)
		Control (0)	426±20.8	1132±91.5	350±45.7	332±60.3	682±105.4	1128±92.5	110±5.6	95±16.4	15.10±1.840
10	11	100	411±22.3	616±145.2**	233±27.6**	175±19.5**	408±40.7**	828±75.6**	100±5.7**	91±9.2	24.20±2.813**
			(96%)	(54%)	(67%)	(53%)	(60%)	(73%)	(91%)	(96%)	(160%)

Note. Castrated rats administered with testosterone propionate (0.25 mg/kg/day) by subcutaneous injection were administered with *p,p'*-DDE by oral gavage for 5 or 10 days.

a): Age at start dosing

b): Sum of the weights of dorso-lateral plus ventral lobes

c): *Levator ani* + *bulbocavernosus* muscles.

d): Values represent the percentage against the mean values of control group.

Mean ± SD, N=6.

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

**: Significantly different from control ($p<0.01$).

Table 3. Effects of Flutamide and *p,p'*-DDE on Androgen Relevant Accessory Sex Glands and Tissues in Castrated Male Rats Daily Treated with Testosterone Propionate

Protocol[a)]	Doses (mg/kg/day)	Final Body weight (g)	Seminal vesicles (mg)	Dorso-lateral prostate (mg)	Ventral prostate (mg)	Prostate (combined)[b)] (mg)	LA/BC[c)] (mg)	Grans penis (mg)	Cowper's glands (mg)	Liver (g)
10-Day assay using peripubertal rats	Control (0)	323±14.5	388±35.1	139±18.7	121±16.7	260±19.5	570±46.2	92±7.3	40±10.8	14.54±0.702
	Flutamide 1	319±13.0	139±23.7**	75±7.6**	63±6.9**	138±9.6**	378±20.2**	85±4.2	25±4.0**	13.61±1.148
	Flutamide 10	313±8.7	47±7.3**	37±7.1**	23±4.1**	60±9.6**	268±24.5**	63±4.7**	9±2.2*	13.86±1.133
	p,p'-DDE 10	325±14.4	370±43.7	142±14.6	132±27.5	274±36.1	556±40.1	90±8.1	35±6.7	15.69±1.225
	p,p'-DDE 100	318±16.8	143±25.6**	81±16.3**	60±7.3**	142±17.2**	354±33.4**	82±4.4*	22±5.1**	21.81±1.924**
5-Day assay using young mature rats	Control (0)	375±16.8	824±119.7	280±24.6	208±31.1	488±43.3	987±80.9	112±8.3	117±37.4	12.46±1.072
	Flutamide 1	380±19.9	487±47.7**	216±15.4**	112±26.5**	328±39.7**	840±34.4**	100±5.0*	92±24.3	12.69±0.874
	Flutamide 10	378±15.8	250±43.3**	117±16.7**	68±18.2**	185±34.7**	824±85.5**	97±5.6**	82±25.1	13.51±0.745
	p,p'-DDE 10	373±16.1	696±108.7	252±36.5	179±25.8	431±57.4	918±142.2	106±6.0	77±12.3*	13.63±1.243
	p,p'-DDE 100	365±15.4	444±76.1**	201±24.0**	115±18.3**	315±31.9**	815±68.6*	102±4.0*	85±23.4	17.71±0.820**

Note. Castrated rats administered with testosterone propionate (0.25 mg/kg/day) by subcutaneous injection were administered with flutamide or *p,p'*-DDE by oral gavage for 5 or 10 days.
a): Age at start dosing is 7 and 11 weeks for peripubertal and young mature rats, respectively.
b): Sum of the weights of dorso-lateral plus ventral lobes.
c): *Levator ani* + *bulbocavernosus* muscles.
Mean ± SD, N=6.
*: Significantly different from control ($p<0.05$).
**: Significantly different from control ($p<0.01$).

Table 4. Effect of *p,p'*-DDE on Androgen Relevant Sex Accessory Glands and Tissues in Male Pubertal Rats

Doses (mg/kg/day)	Final Body weight (g)	Seminal vesicles (mg)	Dorso-lateral prostate (mg)	Ventral prostate (mg)	Prostate (combined)[a)] (mg)	LA/BC[b)] (mg)	Cowper's glands (mg)	Testes (g)	Epididymides (g)	Liver (g)
Control (0)	220±17.7	140±44.0	72±11.6	132±29.7	204±38.2	320±30.0	23±4.6	1.83±0.200	0.25±0.029	11.72±1.294
100	218±15.8	98±28.4*	61±14.3	112±29.1	173±41.4	300±43.6	18±4.7*	1.81±0.135	0.20±0.024**	16.30±1.191**
	(99%)[c)]	(70%)	(85%)	(85%)	(85%)	(94%)	(78%)	(99%)	(80%)	(139%)

Note. Immature male rats (3 weeks of age) were administered with *p,p'*-DDE by oral gavage for 20 days.
a): Sum of the weights of dorso-lateral plus ventral lobes.
b): *Levator ani* + *bulbocavernosus* muscles.
c): Values represent the percentage against the mean values of control group.
Mean ± SD, N=6.
*: Significantly different from control ($p<0.05$).
**: Significantly different from control ($p<0.01$).

Male pubertal assay using dissection and weighing of ASGs after fixation. As shown in Table 4, treatment with *p,p'*-DDE significantly reduced weights of seminal vesicles, Cowper's glands and epididymides. Prostate weights (dorso-lateral, ventral and both lobes) tended to decrease; the values of weight were 85% of control level, while testis, *levator ani and bulbocavernosus* muscle weights were unaffected (Table 4).

Clinical signs, and body and liver weights. As expected, *p,p'*-DDE increased liver weight in all experiments (Tables 2-4). However, there was no evidence of severe systemic toxicity interfering with endocrine function from clinical signs and body weights.

DISCUSSION

Although the rodent Hershberger assay has been in wide use since the 1950's, it has not been used in toxicology testing. Therefore, most toxicology laboratories are not familiar with it. In the validation and standardization for use of the Hershberger assay as a screening tool for endocrine active chemicals, not only the reliability but also feasibility of use must be demonstrated. Since weight of androgen-related tissue is a definitive endpoint in this assay, the need for familiarity with dissection and weighing of these tissues is one of the key issues in considering transferability of the assay. The findings of the present study demonstrate that dissection and weighing of ASGs after formalin fixation are reliable and feasible in studies assessing androgens and antiandrogens.

Loss of fluid from tissues during dissection and trimming may cause variation in tissue weight or reduction of sample number. Differentiation between the ventral and dorso-lateral lobes of fresh prostate is not easy. In our laboratory, in an effort to avoid large variation in tissue weight, limited persons dissect the specified tissues throughout the necropsy. However, this is time-consuming and requires continuous concentration for long periods of time. To evaluate results precisely, possible confounding factor(s) such as diurnal changes, time lag after dosing, insufficient concentration of persons doing necropsy *etc.* should be reduced. Therefore, rapid necropsy with high accuracy in dissection is required. The present study demonstrated that formalin fixation does not interfere with interpretation of assay results. The procedure used for dissection after fixation completely prevents the loss of fluid from tissues and permits easy differentiation on between the ventral and dorso-lateral lobes of the prostate. The tissues can be weighed one day after necropsy, so necropsy can be performed in a short time; ex. within 3 hours in the morning (AM0830-1130) for 60 animals by about 6 individuals. Therefore, dissection and weighing of ASGs after fixation enhances the capacity of the laboratory performing the Hershberger assay. In this study, we determined the effects of overnight fixation (about 24 hr) on tissue weight. Apart from this, we confirmed that about 48 hr- fixation also does not interfere with interpretation of assay results (only seminal vesicle and ventral prostates were examined, unpublished data). Based on this, the tissues fixed should be weighed within two days after necropsy.

In most reports on the Hershberger assay, tissues have been dissected and weighed before fixation. However, weighing of fresh tissues is not considered best. Dr. Albin and coworkers [1] demonstrated that perforate seminal vesicle weights were less variable than intact seminal vesicle weights. Therefore, they recommended use of perforate seminal vesicle weights. In this case, the technique of perforation should be uniform; if not, it may become another source of variation. When ASGs are dissected and weighed after fixation, no such concern exists because the procedure is very easy to do.

Five days of treatment is not of sufficient duration to detect the antiandorogenic effect of 100 or 200 mg/kg/day of *p,p*'-DDE in the Hershberger assay using peripubertal rats [3]. In contrast, our experiment, in which ASGs were dissected and weighed after fixation, detected the antiandrogenic effect of *p,p'*-DDE at 100 mg/kg/day levels after 5 days of treatment (Table 2). Although other factors such as strain differences *etc*. cannot be ruled out, one of the reasons for enhanced sensitivity may be the procedure used for dissection and weighing of ASGs. A longer treatment period may yield a larger percentage change from control values. However, a shorter period of treatment would be preferred in view of animal welfare and is sufficient for routine screening. In the present study, although the magnitude of response to *p,p'*-DDE (100 mg/kg/day) was larger after 10-day treatment than after 5-day treatment, statistical significance of tissue weight change was observed to the same extent after 5- and 10-day treatments. Therefore, 5-day treatment appears to be reliable when ASGs are dissected and weighed after fixation. To confirm this, further studies of the Hershberger assay using other chemicals are needed.

Several early studies demonstrated that androgen-dependent tissues are more sensitive to the effects of an antiandrogen in immature rats than in mature rats [4, 19, 20]. However, this age differences in sensitivity to antiandrogen may have been overestimated since intact animals were used in those experiments. Since the magnitude of change in hormone levels due to feedback mechanisms of the hypothalamic-pituitary-testis axis is greater in mature rats [4], net response to androgen in tissue level cannot be compared between mature and immature intact animals. According to Lambright *et al*. [11], when linuron, reported to be a weak antiandrogen, was administered for 7 days in the Hershberger assay, the androgen-dependent tissues of the immature animals were more affected than those of adult animals. However, a clear difference in the response to linuron was observed only for seminal vesicle in their study: ventral prostate, 62.5% for immature, 66.5 % for mature; seminal vesicles with coagulating glands, 56.1% for immature, 97.9% for mature; and *levator ani* plus *bulbocavernosus* muscles, 78% for immature, 76% for mature (values in the linuron-treated group are expressed as percent of control value, [11]). In addition, the protocols used for studies of the immature and mature animals were not identical; TP was administered by subcutaneous injection to immature rats, while mature rats received testosterone-filled silastic implants. Therefore, the difference in sensitivity to linuron between immature and mature rats cannot be evaluated by the findings in their study [11]. In the present study, however, we directly compared responses to 100 mg/kg/day of *p,p'*-DDE

in immature and mature rats using the identical protocol. Our findings are consistent with those of several early studies [4, 19, 20] indicating that androgen-related tissues appear to be more sensitive to the effects of antiandrogens in immature rats than in mature rats, however, this age difference in sensitivity is not remarkable. Our findings thus demonstrated that even in young mature rats a weak antiandrogenic effect of *p,p'*-DDE can be detected at 100 mg/kg/day. *In utero* and lactational exposures to 100 mg/kg/day of *p,p'*-DDE induced antiandrogenic responses such as female-like anogenital distance and increase in retention of male thoracic nipples [9, 23]. Our findings suggest that young mature male rats (11 weeks of age) are sufficient to detect weak antiandrogenic effects of test chemicals. Our protocol is more sensitive to the effects of *p,p'*-DDE than another study using mature rats [3]. One of the reasons for enhanced sensitivity may be the procedure used for dissection and weighing of ASGs (we dissected and weighed ASGs after fixation, while Ashby and Lefevre seemed to dissect and weigh ASGs before fixation). Difference in age of animals used for study also cannot be ruled out; we used rats aged 11 weeks, while Ashby and Lefevre used rats aged 18 weeks. Compared to immature rats, the protocol using young mature rats provides a more convenient assay of suitable sensitivity. The organ size of mature rats is larger than that of immature rats, which results in less artifact at trimming of tissues for organ gravimetry. The weight of mature rats is higher than that of immature rats, which increases the accuracy of adjustment of dosing volume by body weight. Body weight gain during the dosing period is smaller in mature than in immature rats, making daily adjustment for body weight unnecessary.

Thirty-four-day administration to pubertal male rats failed to yield a detectable antiandrogenic effect of 100 mg/kg/day of *p,p'*-DDE [2], while our pubertal assay with 20-day dosing using dissection and weighing of ASGs after fixation could detect it at least for the seminal vesicles. Again, although it may not be only primary factor, our procedure of dissection and weighing of ASGs also appear to enhance the accuracy of the male pubertal assay as well as the Hershberger assay. This also provides a usefulness of the male pubertal assay as screening tool for EACs. Validation of the protocol of enhanced OECD 407 guideline is in progress under the OECD framework. In this protocol, animals are necropsied at around 11 weeks of age (7 weeks old at the start of 4-week treatment). ASGs of intact animal at this age have relatively large amounts of fluid. Dissection and weighing after fixation of the prostate (ventral and dorso-lateral lobes separately) and seminal vesicles are also expected to enhance the ability of the enhanced OECD 407 guideline study. In further validation studies using different types of chemicals, we recommend that validation of this procedure be performed for the male pubertal assay and the enhanced OECD 407 guideline as well as the Hershberger assay.

SUMMARY

The findings of the present study suggest that dissection and weighing of ASGs after formalin fixation is reliable and feasible in studies assessing androgens and antiandrogens. Furthermore, we concur that androgen-dependent tissues may be more sensitive to the effects of an antiandrogen in peripubertal rats than in young mature rats. In fact, use of peripubertal rat and 10-day treatment can enhance the sensitivity of the Hershberger assay, compared with use of young mature rats and 5-day treatment. However, excessive sensitivity may result in false-positive responses; and excessively long treatment may result in confounding factors, since longer treatment may enhance liver toxicity such as increase in weight (Table 2). The present study demonstrated that even 5-day assay using young mature rats can detect weak antiandrogenic effects of *p,p'*-DDE at 100 mg/kg/day level when ASGs are dissected and weighed after fixation. We also confirmed this protocol has higher feasibility of use from the technical standpoint. Furthermore, when ASGs are dissected and weighed after fixation, the male pubertal assay also seems to be reliable for assessment of (anti)androgens. Finally, we recommend that, as Ashby and Lefevre [2] mentioned previously, there is an urgent need for international agreement on a list of reference chemicals and their active dose levels, with which to validate individual endocrine disruption assays.

ACKNOWLEDGMENTS

The authors thank Mr. Hisashi Toshi, Ms. Nozomi Tokuhara, Rie Funabiki, and Miki Fujitsuka for their technical assistance. We also thank all members of the Environmental Health Science Laboratory of Sumitomo Chemical Co., Ltd. for their invaluable work.

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Regulation of Prostatic glutathione-Peroxidase (GSH-PO) in Rats Treated with A Combination of Testosterone and 17 β -Estradiol

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ABSTRACT. In order to confirm the relationship between sex hormone administration and glutathione-peroxidase (GSH-PO) in the rat ventral prostate, the levels of GSH-PO mRNA, GSH-PO activity, and lipid peroxide (TBA) value in the ventral prostate were investigated. Male Crj:CD(SD)IGS rats were divided into six experimental groups. Group 1 consisted of intact controls. In group 2, rats were sacrificed two days after castration. In groups 3 and 4, rats were subcutaneously administered 1 mg/animal of testosterone daily for three or seven days after two days of castration, respectively. In groups 5 and 6, rats were subcutaneously administered 1 mg/animal of testosterone plus 0.01 mg/animal of 17 β-estradiol (E_2) daily for three or seven days after two days of castration, respectively. GSH-PO activity of the ventral prostate homogenate for testosterone or testosterone plus E_2 administration to the castrated rat was increased and the TBA value was remarkably decreased. The prostatic GSH-PO mRNA level was diminished in the castrated rat ventral prostate, but was increased by testosterone or testosterone plus E_2 administration. In particular, the GSH-PO mRNA level of testosterone plus E_2-treated animals was higher than that of testosterone-treated animals. These findings strongly suggest that expression of GSH-PO in the rat ventral prostate is considered to be testosterone- or E_2-dependent. Furthermore, it is suggested that the transcription of prostatic GSH-PO mRNA was regulated by testosterone or E_2 and de novo synthesis of GSH-PO would thus be regulated at transcription level by testosterone or E_2.
— Key words: Glutathione-peroxidase (GSH-PO), Prostate, Lipid Peroxidation, mRNA, Testosterone, 17 β-Estradiol

CD(SD)IGS-2001: 56-60

INTRODUCTION

In our previous report [19], immunocytochemical localization of glutathione-peroxidase (GSH-PO), which effectively reduces lipid peroxides was demonstrated in the rat ventral prostate under castration and testosterone administration. As the result, the intensity of GSH-PO staining was decreased by castration and it was clearly recovered by testosterone administration to the castrated rats. Therefore, we postulated that GSH-PO in the glandular epithelial cells of the rat ventral prostate was testosterone-dependent [19].

The development and differentiation as well as the maintenance of structure and secretory functions of the prostates are dependent on androgens, but other steroid hormones, such as estrogen, are also supposed to have an important role in prostate physiology [8,16,21].

In the present study, in order to confirm the relationship between sex hormone administration and the biological significance of GSH-PO in the prostate, we used a biochemical approach (northern blotting, GSH-PO activity and lipid peroxide value) to study the ventral prostate of normal, castrated and testosterone or testosterone plus 17 β-estradiol-administered rats.

MATERIALS AND METHODS

Animal and tissue preparation

Male Crj:CD (SD) IGS rats were purchased from Charles River Japan Inc. (Atsugi, Japan) at the age of seven weeks. The animals were kept in a barrier-sustained animal room, which was maintained at a temperature of 22±2 °C with a relative humidity of 55±15%. The room was ventilated twenty-one times per hr and provided with 12 hr of light (from 8:00 to 20:00). Solid food (CE-2, CLEA Japan Inc.) and tap water were given *ad libitum*. The animals were kept for a one-week acclimation period under laboratory conditions.

Five animals served as controls (Group 1). In group 2, five rats were sacrificed two days after castration. In groups 3 and 4, five rats were subcutaneously administered l mg/animal of testosterone-propionate (testosterone, Sigma Chemical Co., St. Louis, MO) daily for 3 or 7 days after two days of castration, respectively. In groups 5 and 6, five rats were subcutaneously administered l mg/animal of testosterone plus 0.0l mg/animal of 17 β -estradiol (E_2, Teikoku Hormone, Mfg. Co. Ltd., Kawasaki) daily for 3 or 7 days after two days of castration, respectively. Testosterone and E_2 were dissolved in dimethyl sulfoxide. Each rat was killed by decapitation and the ventral prostates were removed immediately.

Biochemical examination

1. Measurement of lipid peroxide levels

One gram of fresh prostate was homogenized with a potter type homogenizer in 10 ml of 0.25M sucrose solution containing 1 mM $MgCl_2$ and 0.7 mM 2-mercaptoethanol. The homogenates were centrifuged at 700 g for 10 min and pellets were removed. Then the lipid peroxide level (TBA value) of the homogenate was determined by the thiobarbituric acid method according to the method of Yagi [28] and expressed in terms of malondialdehyde (n mol/ml).

2. Measurement of GSH-PO activity

One hundred microliters of homogenate obtained were preincubated with 2.5 ml of reaction medium containing 0.1M Tris/borate buffer, pH 8.5, 3 mM EDTA/Tris, 0.12 mM NADPH (Oriental Yeast Co., Tokyo), 0.25 mM reduced glutathione (Sigma Chemical Co., St. Louis, MO) and 2.5 μg of glutathione reductase (Sigma Chemical Co., St. Louis, MO) for 2 min at 37 °C. The reaction was then started by the addition of 0.1 ml of cumen hydroperoxide (Mathen Coleman and Bell Manufacturing Chemist, USA). The decrease in the absorbance at 340 nm was measured after 5 min incubation using a Hitachi spectrophotometer model 210. One unit of enzyme oxidized 1 μ mol NADPH to NADP/min at 37 °C.

Note: This paper is reprinted from original paper (The Journal of Toxicological Sciences, 24(5), 415-420, 1999)

3. Northern blotting

The GSH-PO mRNA was measured in extracts prepared by rapidly homogenizing tissues with guanidinium isothiocyanate solution [5]. Total mRNA was prepared by a standard cesium chloride-ultracentrifugation method [22]. Fifteen micrograms of total RNA were electroseparated and nick-transferred onto nitrocellulose filters. Hybridization was performed, as reported previously, using a [$_{32}$P]-labeled complementary deoxyribonucleic acid (cDNA) probe for GSH-PO [7], while a cDNA probe for chicken beta-actin [4] was used as an internal positive control (house-keeping gene mRNA).

Statistical analysis

The data were expressed as means±SD. Homogeneity of variance was tested by Bartlett's method, and when the assumption of homogeneity of variance was met, one-way layout analysis of variance was performed. When a significant difference was observed, Scheff's or Dunnett's multiple comparative test [30] was performed between the castrated group and the other experimental groups.

RESULTS

Group 1 (Intact control)

TBA value and GSH-PO activity in the ventral prostate homogenates are shown in Table 1. Lipid peroxide levels expressed as TBA values of the ventral prostate homogenates from control rats were 4.97±1.12 nmol/ml. GSH-PO activity was 12.91±0.25 unit/g ventral prostate. The prostatic GSH-PO mRNA levels are shown in Photo 1.

Table 1. Lipid peroxides (TBA) value and glutathione-peroxidase (GSH-PO) activity

Group	Treatment	TBA value (n mol/mL)	GSH-PO activity (unit/g)
1	Intact	4.97±1.12	12.91±0.25
2	Castration	4.07±1.20	7.81±1.01
3	Castration+T 3 days	4.91±0.83	12.88±0.35*
4	Castration+T 7 days	2.73±0.60*	19.82±1.15**
5	Castration+T 3 days+E_2 3 days	4.56±1.88	12.75±0.35*
6	Castration+T 7 days+E_2 7 days	2.79±1.09*	20.05±0.98**

T: Testosterone, E_2: 17 β-estradiol
Values are the mean±S.D. (n=5).
Significantly different from castration (*: $p<0.05$, **: $p<0.01$).

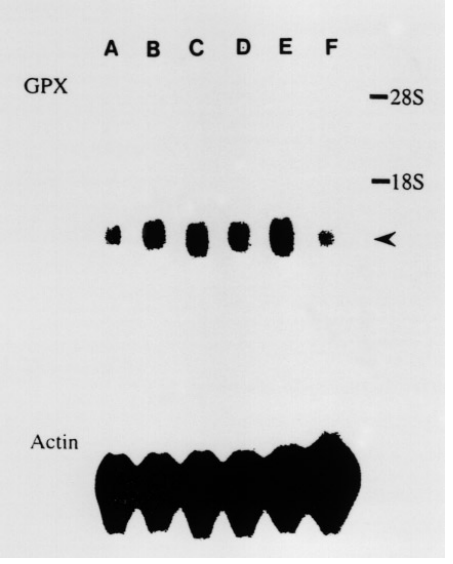

Photo 1. Northern blot analysis of prostatic total RNA. It is hybridized with cDNA for glutathione-peroxidase (GPX) mRNA, or that for chicken β-actin (Actin) mRNA. Lane A: Intact control. Lane B: Castration + Testosterone 3 days. Lane C: Castration + Testosterone 7 days. Lane D: Castration + Testosterone 3 days + E_2 3 days. Lane E: Castration + Testosterone 7 days + E_2 7 days. Lane F: Castration.

Group 2 (Castration)

GSH-PO activity was decreased, but TBA value was similar to that of group 1. The prostatic GSH-PO mRNA level was much lower than that of group 1.

Group 3 (Castration plus Testosterone 3 days)

Both GSH-PO activity and TBA value were similar to those of group 1. The prostatic GSH-PO mRNA level was higher than that of group 1.

Group 4 (Castration plus Testosterone 7 days)

GSH-PO activity was higher than that of group 1. TBA value was much lower, being only about 50% of group 1. The prostatic GSH-PO mRNA level was comparable to that of group 3.

Group 5 (Castration plus Testosterone 3 days and E_2 3 days)

Both GSH-PO activity and TBA value were similar to those of group 3. The prostatic GSH-PO mRNA was comparable to that of group 3.

Group 6 (Castration plus Testosterone 7 days and E_2 7 days)

GSH-PO activity was higher than that of group 4. TBA value was similar to that of group 4. The prostatic GSH-PO mRNA level was higher than that of group 4.

DISCUSSION

In our previous study [19], we demonstrated that the intensity of GSH-PO staining in the glandular epithelial cells of the rat ventral prostate was remarkably decreased after castration, and that it was clearly recovered by testosterone administration to the castrated rats. Therefore, it seemed that GSH-PO in the glandular epithelial cells of the rat ventral prostate could be considered testosterone-dependent, so we further speculated that GSH-PO staining pattern in the glandular epithelial cells of the ventral prostate was thought to be a useful marker for biological testosterone action [19].

In the present study, we found that GSH-PO activity of the ventral prostate homogenate from testosterone or testosterone plus E_2 administration to the castrated rat was increased and the TBA value remarkably decreased. In addition, the prostate GSH-PO mRNA level was diminished in the castrated rat ventral prostate, but increased by testosterone or testosterone plus E_2 administration. In particular, the GSH-PO mRNA level of testosterone plus E_2-treated animals was higher than that of testosterone-treated animals.

It is well known that E_2 alone will induce glandular atrophy of the prostate in normal and castrated animals [1,2]. The suppressive effects of E_2 on the male genital organs have been attributed not only to gonadotropin suppression, but also to the direct effect of E_2 on the gonads and their accessories [1,3]. On the other hand, it is generally accepted that E_2 exerts a synergistic effect with androgen in promoting prostatic growth in the castrated dog, a fact of considerable importance in understanding the pathogenesis of prostatic hyperplasia [27]. Moore *et al*. [17] demonstrated that treatment of castrated dogs with 17 β -estradiol results in a two-fold increase in the cytosolic androgen receptor content. Based on this observation they put forward the hypothesis that enhancement of the androgen receptor activity may be responsible for the synergism of estradiol and androgen in experimentally induced prostatic hyperplasia. Trachtenberg *et al*. [25] developed this concept and gave new insight into the mechanism of steroid-induced prostatic hyperplasia by measuring the nuclear and cytosolic androgen and estrogen receptor contents; in castrated dogs administration of estradiol plus 3 alpha-androstanediol synergistically increased the nuclear androgen receptor content to an extent that exceeded that found in castrated dogs treated with 3 alpha-androstanediol only.

It was reported that the active center of GSH-PO consists of selenocystein [9]. In addition, the disappearance of GSH-PO in selenium-deficient rat liver was immunochemically and immunocytochemically proved by us [26] and others [23]. We found that only a negligible amount of GSH-PO mRNA was exhibited in the case of selenium deficient rat liver, while the mRNA coding for beta-actin [29] was outstandingly increased with selenium deficiency. Therefore, we postulated that the transcription of GSH-PO mRNA was regulated by selenium and the *de novo* synthesis of GSH-PO would thus be regulated at transcription level by selenium [29]. In the prostate, testosterone is irreversibly converted to dihydrotestosterone (DHT) by an enzyme, 5 alpha-reductase. DHT, the major androgen of the prostate, has a higher affinity for androgen receptors than testosterone [11], and is required for the normal development and function of the gland. Based on our data and these facts, it is suggested that the transcription of prostatic GSH-PO mRNA was regulated by testosterone (DHT) or E_2, and *de novo* synthesis of GSH-PO would thus be regulated at transcription level by testosterone (DHT) or E_2.

GSH-PO is a selenium-dependent enzyme that exists as a homotetramer with each 22-kDa subunit containing a selenium atom incorporated within a catalytically active selenocysteine residure [4]. There are three other members of the selenium-dependent GSH-PO family, although cytosolic GSH-PO is the predominant form [6]. The gene encoding GSH-PO was mapped on chromosome 3q11-13 [8]. Because GSH-PO decomposes hydrogen peroxide and organic hydroperoxides produced during normal metabolism and after oxidative insults, GSH-PO prevents peroxide-induced DNA damage, lipid peroxidation, and protein degradation [10,26]. P53, after being activated by DNA-damaging reagents, has been shown either to induce G1 growth arrest or apoptosis [14]. The p53 target genes that mediate or associate with p53-induced apoptosis include Bax [13], Fas/APO 1 [10], as well as those involving generation of reactive oxygen species [20]. Recently, Tann *et al*. [24] have identified and characterized GSH-PO, an antioxidant enzyme, as also induced by p53. It appears paradoxical that p53, on one hand, induces the gene responsible for reactive oxygen species generation, which mediates apoptosis [20], and on the other hand, induces expression of a protective antioxidant enzyme, GSH-PO, which protects cells from oxidative damage and apoptosis [12,15]. It is known that p53-induced reactive oxygen species generation is a rather later event [20]. Thus, p53 may regulate cellular redox status in a time-dependent manner: it increases antioxidant synthesis at an early stage followed by an increase in reactive oxygen species generation.

It has been shown that rat prostatic glandular epithelium undergoes apoptosis within hours of castration and results in involution of the gland within 7 days [12]. In our previous report [18], castration induced apoptosis in the prostatic glandular epithelial cells and apoptosis was reduced by testosterone administration to the castrated rats. Furthermore, we found that the intensity of the staining for bcl-2, a proto-oncogene that blocks apoptosis in multiple contexts, in the prostatic glandular epithelial cells was greatly decreased after castration, and that it was clearly recovered by testosterone administration to the castrated rats [18].

The relationship between GSH-PO expression, apoptosis, and testosterone or estosterone plus estrogen stimulation of the ventral prostate requires further study.

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A NEW ERYTHROCYTE FRAGILITY TEST : A SIMPLE PROCEDURE UTILIZING STIRRING

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ABSTRACT. In this study, a new simple method to measure erythrocyte fragility with stirring of diluted blood (stirring method) was introduced and evaluated with anemic rats given β-acetylphenylhydrazine (APHZ) or clofibrate. APHZ at a dose of 40 mg/kg caused significant decreases in hemoglobin and hematocrit 24 hr after administration. However, the marked elevation of erythrocyte fragility was already detectable after 6 hr by our stirring method. At a dose of 10 mg/kg APHZ, although no significant changes in the erythrocytic parameters were observed throughout the experimental period (72 hr), the blood stirring method revealed a marked elevation of erythrocyte fragility 6 hr after administration. Similarly with clofibrate, no changes in erythrocytic parameters were noted following 100 mg/kg or 300 mg/kg administration, but the enhanced fragility was evident with the stirring method. Thus, using our approach, the erythrocyte fragility could be detected at an earlier stage and with greater sensitivity than by decreases in erythrocytic parameters. The results suggest that the stirring method will prove to be useful for detecting erythrocyte fragility in safety studies. － Key Words: Erythrocyte fragility, β-acetylphenylhydrazine, Clofibrate, Hemolysis test, Anemic animal model

CD(SD)IGS-2001: 61-65

INTRODUCTION

Evaluating hemolysis *in vitro* should be performed under physiological conditions resembling those *in vivo* as far as possible. In safety studies, when hemolytic anemia is suspected by changes in erythrocyte parameters and blood chemistry values, various methods such as the measurement of lipids in erythrocyte membranes, the Coil Planet Centrifuge method (Kitazima and Shibata, 1975) and the erythrocyte osmotic resistant test have been employed for definite diagnosis. These methods, however, suffer problems in toxicity studies because the operations are complicated, the equipment is expensive and the detection sensitivity is imperfect. Therefore, we have developed a novel approach to measure erythrocyte fragility with a stirrer, in which blood is diluted under isotonic conditions. The advantage of this method is that a stirrer, one of the general-purpose instruments, is used to measure erythrocyte fragility. In this paper, we introduce our new hemolysis test, and its utility was evaluated in anemic animals administered (β-acetylphenylhydrazine (APHZ)(Ishijima *et al*., 1995) or clofibrate (Sugawara *et al*., 1977).

MATERIALS AND METHODS

Animals

Male CD(SD)IGS rats (SPF) purchased from Charles River Japan, Inc. were used in the present study. Their ages at administration were 9 weeks old in "Experiment 1", 10 weeks old in "Experiment 2" and 8 weeks old in "Experiment 3".

The animals were housed in stainless steel bracket-type cages (Japan CLEA Inc., Tokyo, Japan, W260×D380×H180 mm) in an animal room under the following conditions: temperature, 20 ～ 26 ℃ ; relative humidity, 30 ~ 70%; 12 hr lighting period; and frequency of air changes, 10 times or more/hr. The animals were given pellet diet (CE-2, Japan CLEA Inc., Tokyo, Japan) and tap water *ad libitum*.

Fragility test

To measure erythrocyte fragility with a stirrer the following was performed:

(1) Ten milliliters of physiological saline (Otsuka Pharmaceutical Factory, Inc.) were added to 50 μL of blood and mixed gently.
(2) One milliliter of diluted blood was transferred into a plastic test tube with a round bottom.
(3) Immediately after transfer, 50 μL was taken, added to 10 mL of physiological saline, and the number of red blood cells counted with a Celltac MEK-5153 (NIHON KOUDEN Co., Ltd., Tokyo, Japan) to give V_0.
(4) After stirring diluted blood in plastic test tubes for 10, 30 and 60 sec, the operation in (3) was performed to give V_{10}, V_{30} or V_{60}. A stirrer (Tube touch mixer, Iuchi-seieido, Osaka, Japan), set the speed control dial at 5.5 (approximately 1300 rpm) , was used for stirring. Percentage of residue erythrocyte after stirring for 10, 30 or 60 sec was calculated by the following formula: $V_{10}/V_0 \times 100$, $V_{30}/V_0 \times 100$ or $V_{60}/V_0 \times 100$ (erythrocyte count after stirring for 10, 30 or 60 sec / erythrocyte count without stirring × 100).

All procedures, from blood collection through to measurement of the number of red blood cells, were performed within 2 hr.

Experiment 1: Administration of APHZ (40 mg/kg)

APHZ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) and administered subcutaneously once to rats at a dose of 40 mg/kg. A non-treated group was provided as a control. Blood was taken under ether anesthesia from the vena cava of 3 rats each at 6, 24, 48 and 72 hr after administration and introduced into EDTA-2K added vials. The number of red blood cells (RBC: flow cytometry method), hemoglobin (Cyan-free hemoglobin method) and hematocrit (calculated from the red blood cell counts and MCV) were measured with a Multi-Species hematology system analyzer Technicon H･1E™ (Bayer Co., NY, USA) and the erythrocyte fragility measured with the above stirrer method.

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Experiment 2: Administration of a low dose of APHZ (10 mg/kg)

APHZ was dissolved in physiological saline and administered subcutaneously once to rats at a dose of 10 mg/kg. As a control, a non-treated group was provided. The procedure for blood collection, the red blood cell counts, measurement of hemoglobin, hematocrit, and erythrocyte fragility with a stirrer were as stated in "Experiment 1".

Experiment 3: Administration of Clofibrate

Clofibrate (Wako Pure Chemical Industries, Ltd.) was dissolved in sterilized 5 w/v% gum arabic solution and administered orally to rats at doses of 100 and 300 mg/kg for 3 weeks. The control group received the vehicle alone. At the end of the administration period, animals were fasted for 4 hr and blood was sampled from the vena cava for hematological examination. In the former, RBC, hemoglobin, hematocrit, MCV, MCH and MCHC were measured with a Multi-Species hematology system analyzer Technicon H･1E(tm). Reticulocyte counts were made with the Brilliant cresyl blue stain. After taking blood, the livers were weighed and the relative weights were calculated based on body weights on the necropsy day. Liver tissue was fixed in 20 or 10% phosphate buffered formalin and paraffin blocks routinely prepared and stained with hemotoxyline and eosin for histopathological examination. A small piece of the left lateral lobe of the liver was taken from one representative animal in each group, prefixed in 2.5% glutaraldehyde and post-fixed in 1.0% osmium tetroxide, dehydrated in graded ethanol and embedded in epoxy resin. Thin sections were double-stained with uranium acetate and lead citrate, and examined under an electron microscope (JEM 1200EX, JEOL Co., Ltd., Tokyo, Japan).

Statistical analysis

Experiment 1 and 2 : The data for hematological parameters were assessed by F-test for homogeneity of variance. If homogeneous, the data were analyzed by Student's t-test, and if not homogenous, the data were analyzed by the Aspin-Welch test (Gad and Weil, 1982).

Experiment 3 : The data for hematological as well as the absolute and relative liver weights were assessed by Bartlett's method (Snedecor and Cochran, 1967) for homogeneity of variance. If homogeneous, the data were analyzed by one-way ANOVA (Snedecor and Cochran, 1967), and if not homogenous, the data were analyzed by the Kruskal Wallis test (Hollander and Wolfe, 1973). In significant cases, the Dunnett's test (Dunnett, 1955, 1964) for multiple group comparisons was performed.

RESULTS

Experiment 1

Hemoglobin and hematocrit were both decreased significantly from 24 hr after administration of APHZ (40 mg/kg) and the number of red blood cells decreased at 72 hr (Table 1). In the erythrocyte fragility test, the percentage of residual erythrocytes was reduced following APHZ administration, and significant decreases were usually found after 60 sec stirring (Table 2).

Experiment 2

There were no changes in values for red blood cells, hemoglobin and hematocrit at any time after administration of 10 mg/kg APHZ (Table 1). In the fragility test, however, significant decreases in the percentage of residual erythrocytes after 60 sec stirring were observed (Table 2).

Experiment 3

1. Hematological examination

In the groups given 100 or 300 mg/kg of clofibrate, there were no changes in the erythrocytic parameters (Table 3). In the fragility test, significant decreases in the percentage of residual erythrocytes after 30 and 60 sec stirring were observed (Table 3).

2. Necropsy

At necropsy, an increase in liver size and weight was observed in 3 out of 6 animals given 100 mg/kg and in all those administered 300 mg/kg (Table 4).

3. Histopathological examination

Hypertrophy of hepatocytes was apparent in all animals given 100 and 300 mg/kg with decrease in the amount of glycogen, which was confirmable by electron microscopic observation, in 4 out of 6 animals given 100 mg/kg and in all those administered 300 mg/kg.

4. Electron microscopic findings

The amounts of peroxisomes in hepatocytes were increased in a dose-dependent manner.

Table 1 Hematology data for male rats after subcutaneous administration of APHZ.

Item (unit)	Stage	Experiment 1		Experiment 2	
		Control	APHZ 40 mg/kg	Control	APHZ 10 mg/kg
RBC ($\times 10^6/\mu$ l)	6hr	6.83 ± 0.39	6.75 ± 0.35	7.93 ± 0.29	7.60 ± 0.38
	24hr	7.38 ± 0.18	6.78 ± 0.43	7.57 ± 0.10	7.16 ± 0.37
	48hr	7.61 ± 0.56	6.60 ± 0.29	7.25 ± 0.25	7.36 ± 0.01
	72hr	7.30 ± 0.24	6.08 ± 0.29**	7.51 ± 0.22	7.36 ± 0.24
Hemoglobin (g/dl)	6hr	13.9 ± 0.9	13.7 ± 0.2	15.8 ± 0.9	14.9 ± 0.4
	24hr	15.2 ± 0.1	12.7 ± 0.9*	15.4 ± 0.5	14.3 ± 0.6
	48hr	15.3 ± 0.6	12.6 ± 0.2**	14.6 ± 0.4	14.4 ± 0.4
	72hr	14.9 ± 0.2	12.1 ± 0.8**	14.9 ± 0.2	14.4 ± 0.6
Hematocrit (%)	6hr	39.2 ± 2.1	37.8 ± 0.3	43.9 ± 2.2	40.9 ± 1.3
	24hr	41.7 ± 0.4	34.5 ± 2.5*	41.9 ± 1.4	38.7 ± 1.6
	48hr	42.2 ± 2.0	34.6 ± 0.3*	40.7 ± 1.7	40.2 ± 0.9
	72hr	41.1 ± 0.7	34.1 ± 1.9**	41.7 ± 0.5	40.1 ± 1.7

Data are Mean±S.D.of 3 rats.
Significant difference from control(*:P< 0.05, **:P< 0.01)
Statistical method : t-test or Aspin-Welch test

Table 2 Percentage of residual erythrocytes after stirring for male rats treated subcutaneously with APHZ.

Item (unit)	Stage	Stirring time	Experiment 1		Experiment 2	
			Control	APHZ 40 mg/kg	Control	APHZ 10 mg/kg
Residual erythrocyte (%)	6hr	10 sec.	95.2 ± 1.9	88.7 ± 3.8	93.1 ± 2.0	91.9 ± 1.2
		30	89.2 ± 4.1	78.4 ± 6.0	87.9 ± 4.3	85.3 ± 1.8
		60	81.6 ± 3.2	70.0 ± 4.8*	83.3 ± 2.0	77.3 ± 1.2*
	24hr	10	94.6 ± 1.4	84.5 ± 4.1*	96.3 ± 1.4	92.6 ± 1.0*
		30	90.1 ± 0.5	76.9 ± 5.4	90.5 ± 0.7	86.4 ± 2.0*
		60	80.2 ± 1.1	66.1 ± 2.9**	83.2 ± 1.7	78.3 ± 2.5*
	48hr	10	95.7 ± 0.5	86.7 ± 5.7	93.8 ± 3.5	88.2 ± 1.4
		30	91.4 ± 1.0	77.0 ± 4.2*	90.1 ± 3.3	81.5 ± 2.3*
		60	86.7 ± 0.6	66.1 ± 4.1*	82.8 ± 3.9	72.4 ± 3.1*
	72hr	10	96.0 ± 1.9	85.0 ± 0.7**	94.6 ± 0.4	86.9 ± 3.2
		30	89.6 ± 2.8	75.4 ± 4.7*	88.4 ± 2.1	79.5 ± 3.1*
		60	82.8 ± 2.0	63.0 ± 6.2**	81.2 ± 0.6	69.0 ± 2.3**

Data are Mean±S.D. of 3 rats.
Significant difference from control(*:P< 0.05, **:P< 0.01)
Statistical method : t-test or Aspin-Welch test

Table 3 Hematology data and percentage of residual erythrocytes after stirring for male rats treated with clofibrate for 3 weeks

Item (unit)	Stirring time	Control	Clofibrate 100 mg/kg	Clofibrate 300 mg/kg
RBC ($\times 10^6$/μl)		7.99 ± 0.2	7.98 ± 0.34	7.84 ± 0.24
Hemoglobin (g/dl)		15.9 ± 0.4	15.7 ± 0.5	15.4 ± 0.4
Hematocrit (%)		45.3 ± 1.4	45.0 ± 1.2	44.6 ± 1.2
MCV (fl)		56.8 ± 2.4	56.5 ± 1.7	56.9 ± 1.3
MCH (pg)		20.0 ± 0.6	19.7 ± 0.5	19.6 ± 0.5
MCHC (g/dl)		35.6 ± 0.5	35.0 ± 0.4	34.6 ± 0.1
Reticulocyte (%)		1.92 ± 0.48	1.75 ± 0.42	1.95 ± 0.51
Reticulocyte ($\times 10^3$/μl)		153 ± 38	139 ± 33	152 ± 38
Residual erythrocyte (%)	10 sec.	95.5 ± 1.4	91.8 ± 4.3	92.7 ± 1.8*
	30	91.0 ± 2.1	85.3 ± 3.8*	83.8 ± 1.2**
	60	85.3 ± 3.0	76.5 ± 6.1**	76.3 ± 3.7**

Data are Mean±S.D.for 6 rats.
Significant difference from control(*:P< 0.05, **:P< 0.01)
Statistical method : Dunnett or Kruskal-Wallis

Table 4 Liver weight of male rats treated with clofibrate for 3 weeks

Item (unit)	Control	Clofibrate 100 mg/kg	Clofibrate 300 mg/kg
Absolute (g)	13.28 ± 0.70	16.07 ± 1.58	21.65 ± 3.21**
Relative (g/100g BW)	3.39 ± 0.19	4.22 ± 0.30**	5.79 ± 0.46**

Data are Mean±S.D.for 6 rats.
Significant difference from control(** : P<0.01)
Statistical method : Dunnett
BW : Body Weight

DISCUSSION

It is well known that different kinds of drugs can act on hemoglobin, erythrocyte lipids and membrane proteins as oxidizing agents, resulting in the production of damaged red blood cells which are trapped and destroyed in organs of the reticuloendothelial system such as the spleen. The number of red blood cells containing Heinz bodies is increased in the hemolytic anemia rat model with APHZ administration (Ishijima *et al*., 1995), and those with unstable hemoglobin exert various effects on red cell membranes (Chiu and Lubin, 1989, Hebbel and Eaton, 1989, Winterbourn, 1990). It has been reported that one of the causes of slight hemolytic anemia following clofibrate administration (Sugawara *et al*., 1977) is elevation of fragility of erythrocytes due to lipid peroxidation of their membranes (Okazaki *et al*., 1998). In the present study, the usefulness of a stirring approach was demonstrated using both APHZ and clofibrate models with male rats.

In the APHZ case, erythrocyte fragility was thus found both earlier and with greater sensitivity than as assessed in erythrocytic parameters.

Similarly, while no clear hematological changes suggestive of anemia were observed after clofibrate administration, erythrocyte fragility was apparent after 30 and 60 sec stirring. Increase in liver weight was also observed at necropsy with hypertrophy of hepatocytes on histopathological examination. This appeared due to increased peroxisomes in hepatocytes observed dose-dependently under the electron microscope in line with earlier findings (Hess *et al*., 1965).

In conclusion, erythrocyte fragility can be detected at an earlier time than decrease in the erythrocytic parameters and at doses below the threshold for the latter. The method, with its simple operation and widely available equipment, showed therefore a wide application in safety assessment.

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Evaluation of the general suitability of the rat for the micronucleus assay: the effect of cyclophosphamide in 14 strains

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ABSTRACT. To evaluate the general suitability of the rat for the micronucleus assay, we conducted the assay in males of 14 different strains, 13 inbred (ACI, BN, BUF, COP, DRH, F344, IS, LEW, RCS, SHR, WAG, WKYO, WTC) and 1 outbred (SD), using cyclophosphamide as the test chemical. Cyclophosphamide at 0 (vehicle), 5, 10, or 20 mg/kg/day was administered orally twice, 24-h apart, to 5 rats per dosage group. Bone marrow and peripheral blood were collected 24 h after the second treatment.

All 14 strains showed a positive response to cyclophosphamide, with slight differences in sensitivity. We concluded that the rat is suitable for the micronucleus assay regardless of strain. — Key words: micronucleus assay, rat, strain differences, cyclophosphamide, CSGMT

CD(SD)IGS-2001: 66-73

INTRODUCTION

The mouse has conventionally been used for the micronucleus assay, with bone marrow the usual target organ. The rat, on the other hand, is usually used for general toxicologic and toxicokinetic studies. If micronucleus induction could be evaluated in the same animals that are used for nongenetic studies, general and genetic toxicological information could be gathered concomitantly. The Collaborative Study Group for the Micronucleus Test (CSGMT) is a working group of the Mammalian Mutagenicity Study Group (MMS), which is a subgroup of the Environmental Mutagen Society of Japan (JEMS). CSGMT previously evaluated several factors that could affect the results of the micronucleus assay in mice, i.e., sex [1], strain [2], administration route [3], number of treatments [4], peripheral blood (with acridine orange supravital staining) vs bone marrow sampling [5, 6], and aging [7-10], but similar studies in rats are limited [11-14]. In the present study, we investigated the effect of strain differences on the induction of micronuclei in rats by cyclophosphamide.

MATERIALS AND METHODS

2.1. Chemicals

Cyclophosphamide (CP, CASRN 50-18-0, Lot No. 9014) obtained from Shionogi & Co., Ltd. (Osaka, Japan), was dissolved in distilled water just before use.

2.2. Animals

Male rats of thirteen inbred strains and one closed colony strain were used (Table 1, Fig.1). The BN, BUF, COP, DRH, LEW, and SD strains were purchased. The ACI, F344, IS, RCS, SHR, WAG, WKYO, and WTC strains were bred at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan, where the micronucleus assays were performed. The rats were at least 7 weeks old at the time of treatment, and we used 5 rats of each strain for each dosage group. Rats were given commercial pellets and tap water ad libitum throughout the acclimation and experimental periods and were subjected to a 12 hour light/dark cycle.

2.3. Micronucleus Assay Protocols

To perform the micronucleus assay using bone marrow and peripheral blood simultaneously in the same animal, double-dosing regimen was selected [4,6,15].

CP dissolved in distilled water at 0 (vehicle), 5, 10, or 20 mg/kg/day was administered orally twice, 24-h apart, to 5 male rats per dosage group. Peripheral blood and bone marrow were collected 24h after the last treatment. Acridine orange staining [16] was used to identify micronuclei. Both immature and mature erythrocytes in bone marrow and peripheral blood were analyzed. Micronucleated polychromatic erythrocyte (MNPCE), micronucleated normochromatic erythrocyte (MNNCE) and micronucleated reticulocyte (MNRET) frequencies were based on the observation of 2000 polychromatic erythrocytes (PCEs), 2000 normochromatic erythrocytes (NCEs) or 2000 reticulocytes (RETs), respectively. To assess cytotoxicity, 300 bone marrow erythrocytes and 2000 peripheral blood reticulocytes were analyzed.

2.4. Statistical Analysis

To compare the responses of each dosage group with those of the control group, we used the Kastenbaum and Bowman test [17] for the frequency of MNPCEs, MNRETs, and MNNCEs and the t-test for the frequency of PCEs and RETs.

To investigate the effect of strain differences, we analyzed MNPCE values with stability and sensitivity indexes. To evaluate stability, we used MNPCE variance for each animal, pooled for each dose; we applied the square root transformation for each value to stabilize the variance. We analyzed the equality of these variances across strain using the Bartlett homogeneity of

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Note: This paper is reprinted from original paper (Mutation Research 495, 127-134, 2001)

variance tests [18]. To evaluate sensitivity, we used the slope of the simple regression line for the incidence of MNPCEs against dose. We analyzed the equality of the slopes across strain using the chi-square test with 13 degrees of freedom.

RESULTS

The results of the bone marrow and peripheral blood micronucleus assays are shown in Table 2, Fig.2, and Fig.3.

In the bone marrow micronucleus assay, the MNPCE frequency was significantly increased in the groups treated with 5 mg/kg/day or more CP in all 14 strains. The MNNCE frequency was significantly increased in the group treated with 20 mg/kg/day in 6 strains (COP, RCS, SHR, WAG, WTC, and SD). There were no significant increases in the remaining 8 strains. The PCE frequency was significantly decreased in the group treated with 5 mg/kg/day CP in 6 of 14 rat strains and in the 20 mg/kg/day group in 13 of 14 rat strains.

In the peripheral blood micronucleus assay, the MNRET frequency in the groups treated with 5 mg/kg/day or more CP was significantly increased in 12 strains. In the ACI and COP strains, the MNRET frequency was significantly increased in the 10 mg/kg/day and 20 mg/kg/day groups, but not in the 5 mg/kg/day group. There were no significant increases in the MNNCE frequency in any dosage groups in any strain. The RET frequency was significantly decreased in the group treated with 5 mg/kg/day CP in 6 strains and in the 20 mg/kg/day group in 13 strains.

Analysis for strain differences in MNPCE frequency revealed no difference in stability ($p=0.244$), but a significant difference in sensitivity ($p<0.001$). The DRH strain was the most sensitive to the drug (slope: 0.00255), and BN was the least sensitive (slope: 0.00058). For all 14 strains, the slope of dose with response was statistically significant ($p<0.001$).

DISCUSSION

CP at 5 mg/kg/day or more induced a significant increase in MNPCEfrequency in all 14 rat strains. CP at 5 mg/kg/day did not induce a significant increase in MNNCE frequency in any strain. These results suggest that the micronucleus-inducing activity of compounds can be evaluated in bone marrow regardless of strain if MNPCEs, but not MNNCEs, are scored. Furthermore, among the strains investigated in this study, BN, COP, and DRH are resistant to chemical carcinogenesis via a protective mechanism may act at the post-initiation stage [19,20,21]. In two of those strains, BN and COP, 20 mg/kg/day CP induced a lower frequency of MNPCEs than it did in other strains. Thus, the results of this study suggest that there may be a mechanism that inhibits the chemical induction of DNA damage at the initiation stage. 20 mg/kg/day CP induced the highest MNPCE frequency in the DRH strain, suggesting a low level of protection against chemical induction of DNA damage. That is in accord with the report by Denda et al. [21] that suggests that the carcinogenesis-inhibiting mechanism of the DRH strain acts at the post-initiation stage.

The results of the peripheral blood micronucleus assay were similar. The micronucleus-inducing activity of compounds could be evaluated in rats regardless of the strain by evaluation of MNRET frequency, but not MNNCE frequency.

The results of the analysis for strain differences on the frequency of MNPCEs suggest that there was little difference in stability, but between-strain differences in sensitivity were evident. It is well known that CYP2C metabolizes CP and the metabolite itself induces genotoxicity [22]. The sensitivity of micronucleus assay for CP may depend on the metabolic activity of CYP2C, but it needs further evaluation. All strains were adequately sensitive, however, and in practical terms the differences are slight because all the slopes used in the index of evaluation were highly significant.

In summary, these results suggested that the chemical induction of MNPCEs in bone marrow or of MNRETs in peripheral blood could be evaluated in the rat micronucleus assay regardless of strain, with slight between-strain differences in sensitivity.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Tsutikawa Memorial Fund for Study in Mammalian Mutagenicity, which partially sponsored this study.

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Table 1. Rat strains used in micronucleus assays.

Strains (abbreviations)	Chacteristics[a]	Inbred or outbred	Source	Age (weeks)
ACI/Nkyo (ACI)	as a reference strain	inbred	Kyoto University	8-9
BN/Crj (BN)	resistant to carcinogen	inbred	Charles River Japan	8
BUF/NacJcl (BUF)	sensitive to carcinogen	inbred	CLEA Japan	9-10
COP/CrCrlBR (COP)	resistant to carcinogen	inbred	Charles River Laboratories	8
DRH/Sea (DRH)	resistant to carcinogen	inbred	Seac Yoshitomi	8
F344/Nkyo (F344)	as a reference strain	inbred	Kyoto University	8-12
IS/Kyo (IS)	scoliosis	inbred	Kyoto University	10-12
LEW/Crj (LEW)	as a reference strain	inbred	Charles River Japan	8
RCS	retinal dystrophy	inbred	Kyoto University	10
Crj:CD(SD)IGS (SD)	as a reference strain	outbred	Charles River Japan	8
SHR/Kyo (SHR)	hypertention	inbred	Kyoto University	7-8
WAG/RijKyo (WAG)	absence-like seizure	inbred	Kyoto University	7-11
WKYO	as a reference strain	inbred	Kyoto University	7
WTC	as a reference strain	inbred	Kyoto University	7-10

[a]Data from database of The Jackson Laboratory (Inbred strains of mice and rats), NIH Animal Genetic Resource (Strain and Stock Listings), and Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Rat Models for Human Diseases, List of Mouse and Rat Strains), 11 April 2001.

Table 2. Results of the micronucleus assay of rats treated with cyclophosphamide.

Strains	Dose	Number	Bone marrow			Peripheral blood		
	(mg/kg)	of rats	MNPCE (%)	MNNCE (%)	PCE (%)	MNRET (%)	MNNCE (%)	RET (%)
ACI	0	5	0.08±0.06	0.02±0.03	45.16±5.47	0.09±0.07	0.01±0.02	2.42±0.57
	5	5	0.21±0.11*	0.03±0.05	42.78±6.04	0.13±0.03	0.02±0.03	1.60±0.29*
	10	5	0.63±0.19**	0.02±0.03	41.48±4.92	0.42±0.10**	0.02±0.03	1.80±0.61
	20	5	2.46±0.71**	0.04±0.07	11.42±2.97**	0.67±0.24**	0.02±0.03	0.40±0.14**
BN	0	5	0.04±0.04	0.01±0.02	63.76±5.20	0.04±0.04	0.01±0.02	1.68±0.47
	5	5	0.35±0.09**	0.01±0.02	49.14±9.78*	0.20±0.06**	0.02±0.03	1.04±0.15*
	10	5	1.00±0.26**	0.06±0.07	27.06±5.52**	0.46±0.27**	0.04±0.04	0.70±0.33**
	20	5	1.17±0.17**	0.06±0.04	19.88±7.62**	0.68±0.16**	0.02±0.03	0.48±0.16**
BUF	0	5	0.14±0.13	0.01±0.02	72.98±2.30	0.05±0.09	0.00±0.00	2.20±0.50
	5	5	0.65±0.20**	0.03±0.03	71.70±6.88	0.15±0.06*	0.00±0.00	2.20±0.29
	10	5	1.14±0.48**	0.03±0.05	56.28±9.57	0.30±0.12**	0.02±0.03	1.56±0.38
	20	5	2.41±0.36**	0.06±0.04	36.76±3.09**	0.54±0.11**	0.01±0.02	1.16±0.44**
COP	0	5	0.04±0.02	0.00±0.00	78.62±4.52	0.03±0.03	0.00±0.00	3.10±0.41
	5	5	0.41±0.08**	0.02±0.03	66.38±4.49**	0.14±0.06	0.01±0.02	1.82±0.15
	10	5	1.28±0.12**	0.04±0.04	55.24±6.35**	0.59±0.24**	0.00±0.00	1.20±0.17**
	20	5	1.41±0.51**	0.05±0.05*	30.50±2.21**	0.54±0.13**	0.02±0.03	0.60±0.07**
DRH	0	5	0.09±0.07	0.02±0.03	84.06±6.76	0.10±0.06	0.01±0.02	5.22±0.74
	5	5	0.62±0.18**	0.02±0.03	67.96±2.55**	0.32±0.10*	0.01±0.02	3.48±0.37**
	10	5	2.28±0.76**	0.06±0.02	59.20±7.38**	0.58±0.15**	0.01±0.02	2.92±0.66**
	20	5	4.99±1.37**	0.07±0.03	33.94±7.30**	0.59±0.16**	0.01±0.02	1.44±0.32**
F344	0	5	0.04±0.04	0.02±0.03	64.86±9.24	0.04±0.04	0.01±0.02	2.52±0.44
	5	5	0.87±0.25**	0.02±0.03	36.62±3.95**	0.19±0.13**	0.02±0.03	1.88±0.87
	10	5	1.40±0.29**	0.07±0.05	39.00±13.25**	0.23±0.10**	0.00±0.00	1.50±0.53*
	20	5	2.72±0.59**	0.08±0.05	27.34±4.67**	0.33±0.10**	0.01±0.02	0.88±0.29**
IS	0	5	0.08±0.08	0.02±0.03	36.74±10.79	0.08±0.07	0.01±0.02	3.04±0.36
	5	5	0.26±0.06**	0.00±0.00	65.28±7.41*	0.19±0.09*	0.02±0.03	1.82±0.24**
	10	5	0.69±0.16**	0.05±0.04	37.56±3.93	0.45±0.19**	0.02±0.03	0.94±0.11**
	20	5	3.34±0.48**	0.07±0.03	18.44±0.94*	0.98±0.28**	0.01±0.02	0.84±0.27**
LEW	0	5	0.05±0.05	0.01±0.02	60.06±5.81	0.04±0.02	0.01±0.02	2.16±0.17
	5	5	0.71±0.17**	0.04±0.02	36.04±10.12**	0.32±0.14**	0.01±0.02	1.50±0.51**
	10	5	1.90±0.76**	0.03±0.03	18.46±4.78**	0.75±0.19**	0.01±0.02	0.78±0.24**
	20	5	2.14±0.49**	0.05±0.04	11.90±2.75**	0.67±0.26**	0.03±0.05	0.44±0.17**

Data shown in the table are mean±SD. * P<0.05, ** P<0.01, compared to the results of vehicle control group in each strain.
PCE: polychromatic erythrocytes, RET: reticulocytes, MNPCE: micronucleated PCE, MNRET: micronucleated RET, MNNCE: micronucleated normochromatic erythrocytes.

Table 2. (continued)

Strains	Dose	Number	Bone marrow			Peripheral blood		
	(mg/kg)	of rats	MNPCE (%)	MNNCE (%)	PCE (%)	MNRET (%)	MNNCE (%)	RET (%)
RCS	0	5	0.06±0.04	0.02±0.03	67.40±6.04	0.05±0.05	0.01±0.02	1.80±0.37
	5	5	0.43±0.18**	0.02±0.03	44.06±7.93**	0.15±0.05*	0.01±0.02	1.26±0.23
	10	5	0.99±0.22**	0.09±0.08*	31.54±2.52**	0.36±0.14**	0.02±0.03	0.72±0.18*
	20	5	2.11±0.25**	0.10±0.07*	14.12±3.50**	0.57±0.30**	0.02±0.03	0.28±0.04**
SD	0	5	0.11±0.06	0.02±0.03	71.80±5.23	0.12±0.03	0.02±0.03	4.14±0.46
	5	5	0.70±0.23**	0.01±0.02	69.32±10.99	0.39±0.11**	0.01±0.02	2.86±0.50**
	10	5	1.72±0.44**	0.04±0.02	66.86±1.75	0.67±0.15**	0.03±0.05	2.78±0.50**
	20	5	3.39±0.80**	0.10±0.07*	35.20±9.02**	1.20±0.49**	0.02±0.03	0.80±0.21**
SHR	0	5	0.08±0.08	0.01±0.02	59.64±16.25	0.03±0.03	0.02±0.05	2.58±0.79
	5	5	0.35±0.24**	0.01±0.02	72.32±6.07	0.18±0.08**	0.01±0.02	3.50±0.40
	10	5	0.92±0.38**	0.01±0.02	68.86±5.43	0.48±0.27**	0.00±0.00	3.14±0.61
	20	5	2.77±0.59**	0.08±0.08*	36.38±7.51**	0.63±0.26**	0.01±0.02	1.18±0.13
WAG	0	5	0.08±0.03	0.01±0.02	60.08±5.59	0.05±0.05	0.01±0.02	2.20±0.16
	5	5	0.51±0.09**	0.03±0.03	62.80±5.99	0.33±0.13**	0.01±0.02	3.96±0.53**
	10	5	2.18±0.55**	0.11±0.04**	33.10±9.92**	0.48±0.23**	0.02±0.03	1.52±0.57
	20	5	2.52±0.58**	0.08±0.06*	26.26±11.24**	0.64±0.31**	0.01±0.02	0.96±0.40**
WKYO	0	5	0.03±0.03	0.01±0.02	48.06±13.16	0.05±0.05	0.01±0.02	1.70±0.40
	5	5	0.61±0.19**	0.02±0.03	80.56±6.05*	0.29±0.06**	0.01±0.02	1.38±0.15
	10	5	1.26±0.34**	0.01±0.02	76.88±1.92	0.64±0.26**	0.01±0.02	0.58±0.15**
	20	5	1.82±0.23**	0.01±0.02	8.22±1.88	0.85±0.28**	0.02±0.03	0.50±0.12**
WTC	0	5	0.07±0.06	0.01±0.02	73.46±10.73	0.08±0.03	0.01±0.02	4.90±2.30
	5	5	0.87±0.20**	0.03±0.05	74.90±5.99	0.36±0.17**	0.01±0.02	5.06±0.61
	10	5	2.16±0.61**	0.06±0.04	36.40±6.47	0.45±0.13**	0.02±0.03	1.70±0.34
	20	5	3.12±0.22**	0.13±0.06**	15.74±1.63**	1.11±0.55**	0.02±0.03	0.48±0.15**

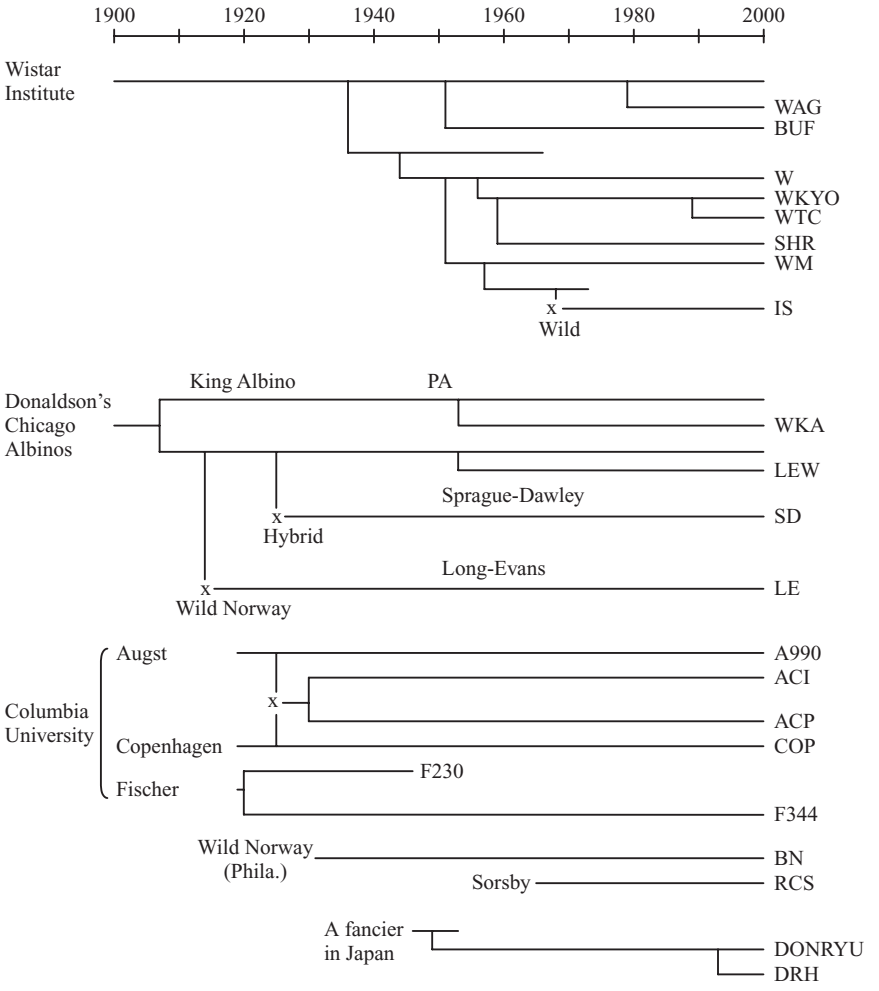

Fig. 1. Genealogy of major rat stock and strains used in this study. Data shown in Fig.1 was collected from database of The Jackson Laboratory (Inbred strains of mice and rats), NIH Animal Genetic Resource (Strain and Stock Listings), and Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Rat Models for Human Diseases, List of Mouse and Rat Strains), 11 April 2001.

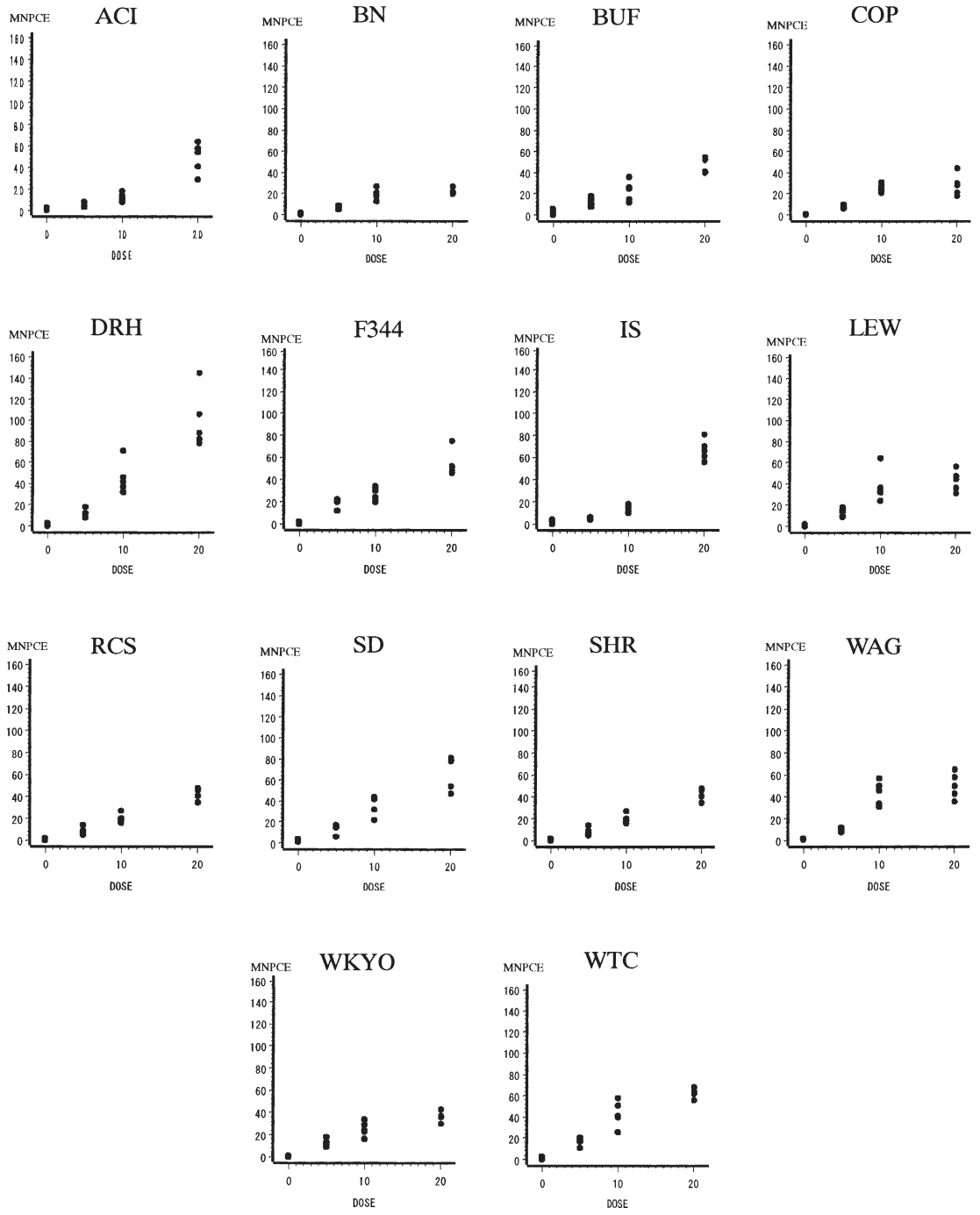

Fig. 2. MNPCE frequencies in bone marrow of male rats following oral administration of cyclophosphamide once a day for 2 days. Data points represent the MNPCE frequency for each rat.

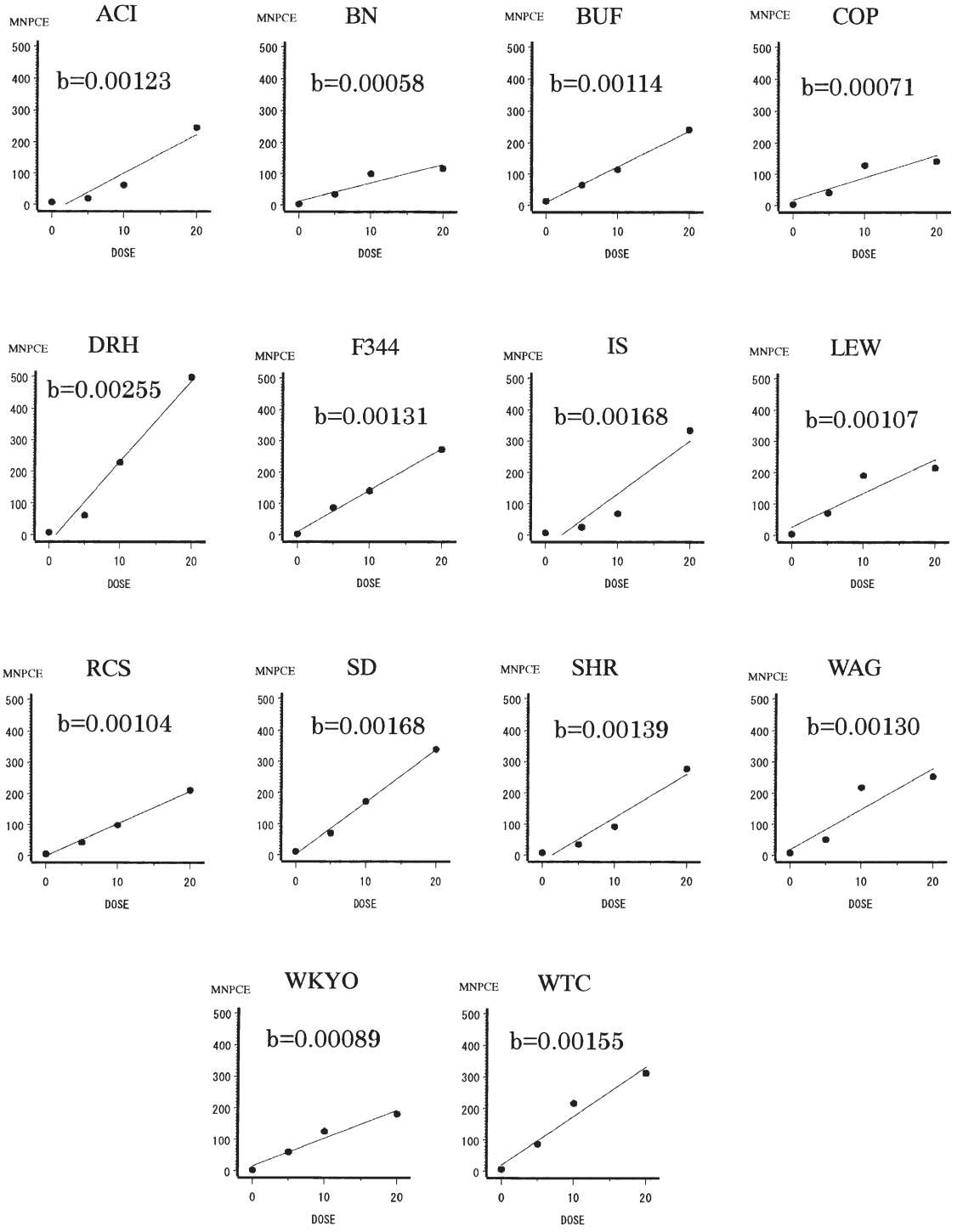

Fig. 3 The sensitivity of 16 rat strains to cyclophosphamide in the bone marrow micronucleus assay. Data points represent the total number of MNPCEs for each dosage group. The solid line represents regression; values (b) are estimates of the slope.

Spontaneous Mucin-secreting Adenocarcinoma of the Ileum in a Male Crj:CD (SD) IGS Rat

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ABSTRACT. A small intestinal mucin-secreting adenocarcinoma was found in a 78-week-old male Crj:CD (SD) IGS rat. The neoplasm was present in a portion of the ileum as a white soft mass. Histologically, it consisted of pleomorphic mucin-producing glands, multiple cysts of various sizes, and a well-developed dense fibrous tissue stroma. Chronic inflammatory reaction and osseous metaplasia were observed in the stroma. Ultrastructurally, the microvilli of the epithelium at the luminal surface contained core filaments, and often appeared more stubby and sparse than usual.
— Key words: Ileum, Mucin-secreting adenocarcinoma, Osseous metaplasia, Rat, Spontaneous

CD(SD)IGS-2001: 74-76

Although many spontaneous tumors have been reported in rats, spontaneous tumors of the small intestine in animals and particularly in rats are extremely rare[1,2]. We recently encountered a spontaneous small intestinal tumor in a Crj:CD (SD) IGS rat. This article describes the features of the histological and ultrastructural findings of that tumor.

A male Crj:CD (SD) IGS rat, which was supplied to a non-treated life span study, was sacrificed under diethyl ether anesthesia at 78 weeks of age. The small intestine tumor mass was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 μm-thick sections, and stained routinely with hematoxylin-eosin (HE), periodic acid-Schiff's reaction (PAS), alcian blue (pH 2.5 and pH 1.0), von Kossa, proliferating cell nuclear antigen (PCNA) (DAKO, Japan), and CM5 polyclonal rabbit anti-human p53 antibody against wild and mutant forms of p53 (Novacastra, UK), which was known useful for immunohistochemical assessment of rat *p53* mutation[3]. For ultrastructural examination, the formalin-fixed tumor tissue was postfixed in 1% solution of osmium tetroxide, dehydrated in ethanol, and embedded in Spurr. Ultrathin sections were stained with $KMnO_4$ and lead citrate.

Clinically, the body weight began to decrease from 74 weeks of age. At that time, the body weight was 892g. A body weight loss of 122g was recorded at 78 weeks of age, and the rat was autopsied in a moribund state. At necropsy, a tumor mass was present in a portion of the ileum as a soft yellowish white mass, 30×25×20 mm in size. The border between the ileum and the tumor mass was not obscured. A pituitary gland tumor, a hepatocellular adenoma, and myelogenic leukemia were also observed.

Histologically, the tumor consisted of pleomorphic irregular mucin-producing glands and a well-developed dense fibrous tissue stroma (Fig. 1). The lumina of these glands, cysts, and crypts were filled with mucin and cellular debris. Mucin was stained positively with alcian blue (pH 2.5 and pH 1.0) and PAS. These findings were similar to mucin, which was contained in the normal ileum. The glandiform structures were lined with cuboidal, cylindrical, or flattened epithelium and goblet cells arranged in one or several rows (Fig. 2). A few Paneth-like cells were located at the base of the glands. These epithelia exhibited indistinct cell borders and eosinophilic cytoplasm. The cell nuclei were pleomorphic, and differed in chromatin content, size, shape, and position with respect to the longitudinal axis of the cell. There were many mitotic figures and most of the cells were stained positively with PCNA antibody (Fig. 3). The tumor cells were negative for p53 antibody. Chronic inflammatory response and osseous metaplasia were observed in a stroma. The osseous tissues contained some osteoclast-like and osteoblast-like cells and were stained positively with von Kossa. Although the neoplasms invaded the serosa, no metastatic lesions were observed in any other organs. Ultrastructurally, the microvilli of the epithelium at the luminal surface contained core filaments, and often appeared more stubby and sparse than usual. A fine fibrillar network was sometimes seen under the luminal surface. Most tumor cells were comparatively rich in rough endoplasmic reticulum. Electron-lucent materials, which might have been secreting granules, were observed in some tumor cells. Desmosomes were seen at the cytoplasmic boundaries of adjoining cells (Fig. 4).

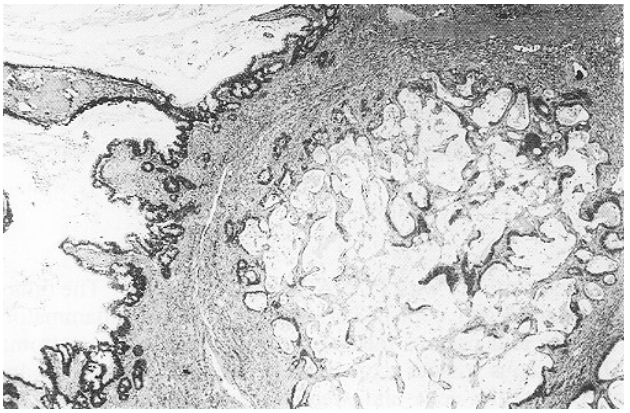

Figure 1. The tumor consists of pleomorphic irregular mucin-producing glands and a well-developed dense fibrous tissue stroma. HE. ×108.

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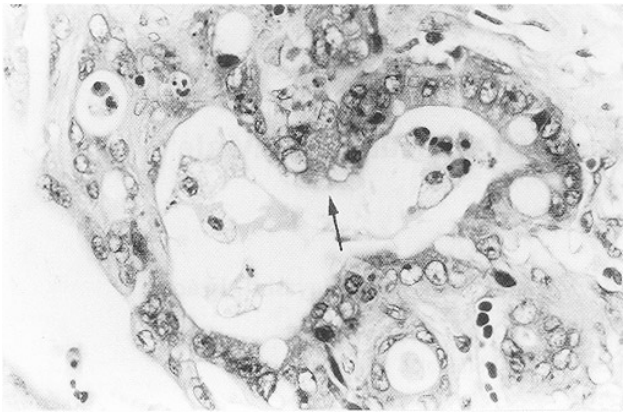

Figure 2. The glandiform structures are lined with cuboidal or flattened epithelium. Paneth-like cells are located at the base of the glands (arrow). HE. ×810.

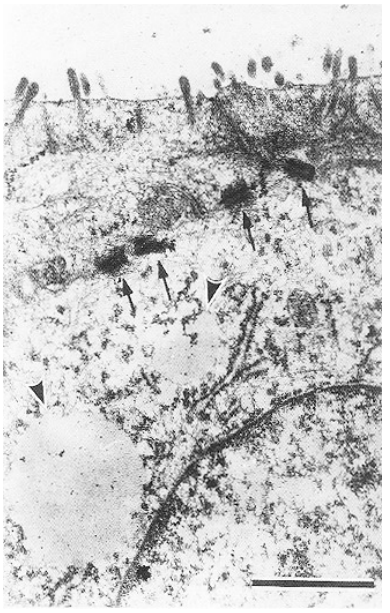

Figure 4. Tumor cells have microvilli, electron-lucent materials (arrow heads), and desmosomes (arrows). Bar=1μm. ×18000.

The morphological structures in the present case were similar to those of reported intestinal mucin-producing carcinomas with ossification and calcification as secondary alterations in rats[4,5] and humans[6]. In the World Health Organization (WHO) classification of intestinal tumors in rats[2], mucin-producing carcinomas are divided into mucin-secreting adenocarcinoma, mucinous carcinoma, and signet-ring carcinoma. Because the two latter types show no tendency to form glandular structures, we diagnosed the present case as a mucin-secreting adenocarcinoma. Spontaneous neoplasms of the intestine occur more commonly in the large intestine than in the small intestine, and are seen more often in males than in females in rats[7]. Although our case involved a male rat, this animal was comparatively younger than the rats in other reports and this might be the first reported case of this tumor in Crj-CD (SD) IGS rats.

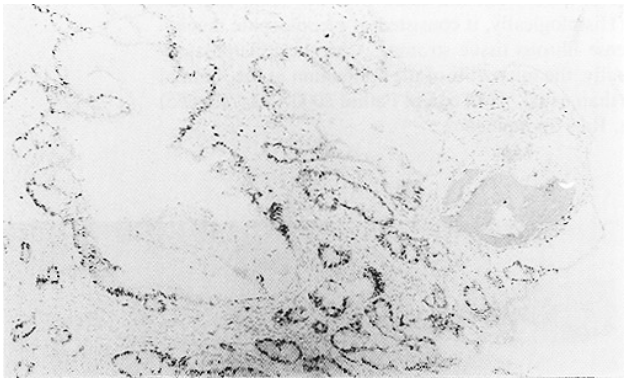

Figure 3. Some tumor cells are stained positively with PCNA. ×135.

Meckel's diverticulum is rarely encountered as persistence of the fetal yolk sac in ileum. It was known that some tumors might originate there[8]. The present case was found in the end part of ileum, where Meckel's diverticulum is not located[9,10]. Therefore, we considered that this tumor did not originate from Meckel's diverticulum.

The *p53* tumor suppressor gene is the most common target in human cancers, its point mutations or short deletions are detected frequently in human colon tumors, and may be important for tumor development[11]. On the other hand, it was reported that the incidence of the *p53* point mutation in chemical-induced adenocarcinoma of the small intestine in rats was only 31%[12]. Because p53 protein expression was not observed in the present case, it was suggested that this tumor might have occurred through a pathway which is independent of the *p53* mutation. However, because immunohistochemistry is less sensitive in detecting *p53* mutations than DNA sequencing[11], DNA sequencing would be required to confirm whether a *p53* mutation was associated with this tumor.

ACKNOWLEDGEMENTS

The authors thank Mr. Katsuhiro Yoshizawa (Fujisawa Pharmaceutical Co., Ltd.) for his kind help in immunohistochemistry and Ms. Yoko Fujieda, Mr. Atsushi Funakoshi, Ms. Rie Kubota, Ms. Kaori Maejima, and Mr. Mitsuo Kawashima for their excellent technical assistance.

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Spontaneous Pinealoma in a Male Crj:CD (SD) IGS Rat

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ABSTRACT. A pinealoma (benign) was found in a 61-week-old male Crj:CD (SD) IGS rat. The neoplasm was located between the cerebral hemispheres and the cerebellum. Histologically, the tumor cells consisted of two cell types : large, pale-staining cells and small dark-staining cells. A fibrovascular stroma divided the tumor cells into incomplete lobules or nest structures. Relatively numerous mitoses were noted in the tumor cells. Ultrastructurally, the tumor cells contained dense-cored vesicles, approximately 120 nm in diameter. — Key word: Crj:CD (SD) IGS, pinealoma (benign), spontaneous.

CD(SD)IGS-2001: 77-80

Although many spontaneous tumors have been reported in rats [2], primary tumors in the pineal gland are apparently very rare [13]. Their incidence has been reported to be 0.01 - 0.8 % [3-5, 6, 9, 11, 13]. The Crj:CD (SD) IGS strain was established by Charles River Inc. as a worldwide standardized Sprague-Dawley strain with minimal genetic divergence. These rats have been available since 1996. We recently encountered a spontaneous pineal gland tumor in a Crj:CD (SD) IGS rat. This article describes the features of the histological and ultrastructural findings of that tumor.

A male Crj:CD (SD) IGS rat, which was supplied to a non-treated life span study, was killed at 61 weeks of age. The pineal gland tumor mass was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 μm thick sections and stained routinely with hematoxylin-eosin, reticulin silver stain proliferating cell nuclear antigen (PCNA) (DAKO). For ultrastructural examination, the formalin-fixed tumor tissue was postfixed in 1% solution of osmium tetroxide, then dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate.

Clinically, the bodyweight began to decrease from week 54. At that time, the body weight was 730 g. A bodyweight loss of 115 g was recorded at 61 weeks of age and the rat was killed in a moribund state.

At necropsy, the pineal neoplasm was observed as a hemorrhagic mass, 3 mm in diameter. It was located between the cerebral hemispheres and the cerebellum (Fig. 1). The tumor mass was well-demarcated from adjacent normal brain tissue but slight compression was present. A pituitary gland tumor measuring 8 mm in diameter was also observed.

Histologically, the tumor cells were surrounded by a connective tissue stroma and demarcated from the adjacent brain tissues (Fig. 2). In the marginal areas, there were small amounts of normal pineal tissue. The tumor was divided by a fibrovascular

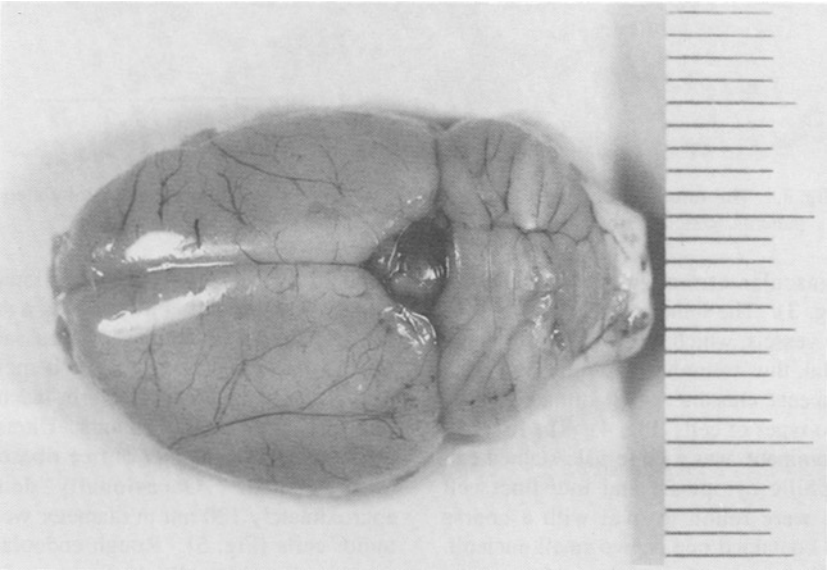

Figure 1. The pineal gland tumor is situated dorsally between the cerebral hemispheres and the cerebellum as a hemorrhagic mass.

Note: This paper is reprinted from original paper (J. Vet. Med. Sci., 61(1), 41-44, 1999)

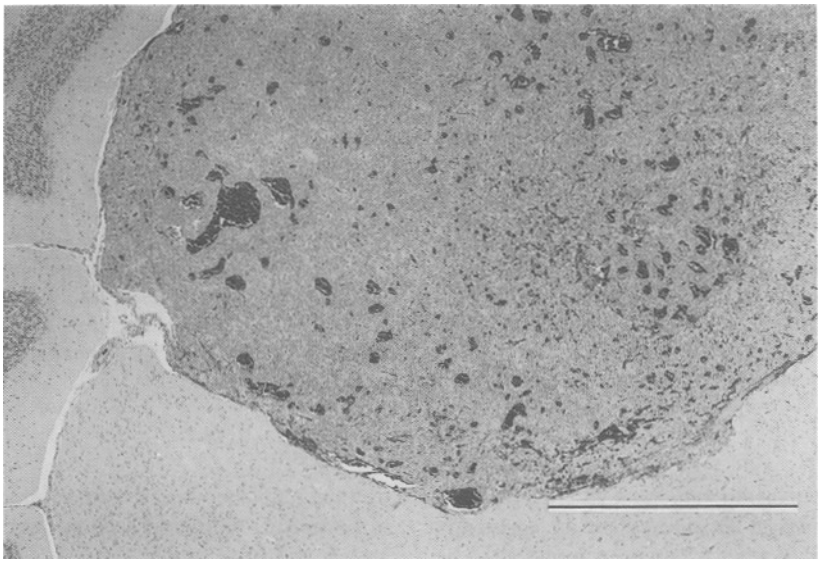

Figure 2. The tumor mass is surrounded by a connective stroma. HE. Bar=1mm.

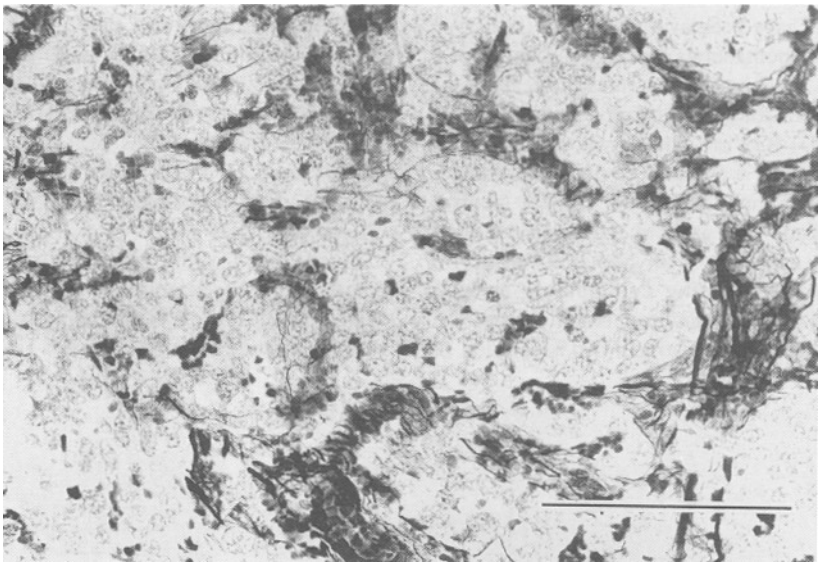

Figure 3. The tumor is divided by a fibrovascular stroma into incompletely lobulated patterns. Reticulin silver stain Bar=100 μm.

stroma into incompletely lobulated patterns (Fig. 3). The tumor cells were arranged along expanded blood vessels, which were filled with blood or eosinophilic material, thus resembling normal endocrine tissue. They formed sheets, clusters or nest structures. The tumor consisted of two types of cells (Fig. 4). The first cell type, which was predominant, was a large pale-stained cell with slightly eosinophilic cytoplasm and indistinct cell borders. The nuclei were round to oval with a coarse chromatin pattern and contained one or two small nucleoli. The cells showed excessive mitotic activity. Most of the cells were stained positively with PCNA antibody. Gigantic or multinuclear cells were occasionally scattered in the tumor. The second cell type was a small cell with dark-staining irregularly-shaped nucleus and scant cytoplasm. There were a few small foci of hemorrhage and necrosis, infiltration of a few hemosiderin-laden cells, and scattered single cell necrosis in the tumor. Ultrastructurally, the large cells contained a number of free ribosomes, polysomes and mitochondria. Occasionally dense-cored vesicles approximately 120 nm in diameter were observed in some tumor cells (Fig. 5). Rough endoplasmic reticulum was developed, and lamellar bodies were formed in some cells. Some junctional complexes, such as desmosomes were evident between the cells. Although

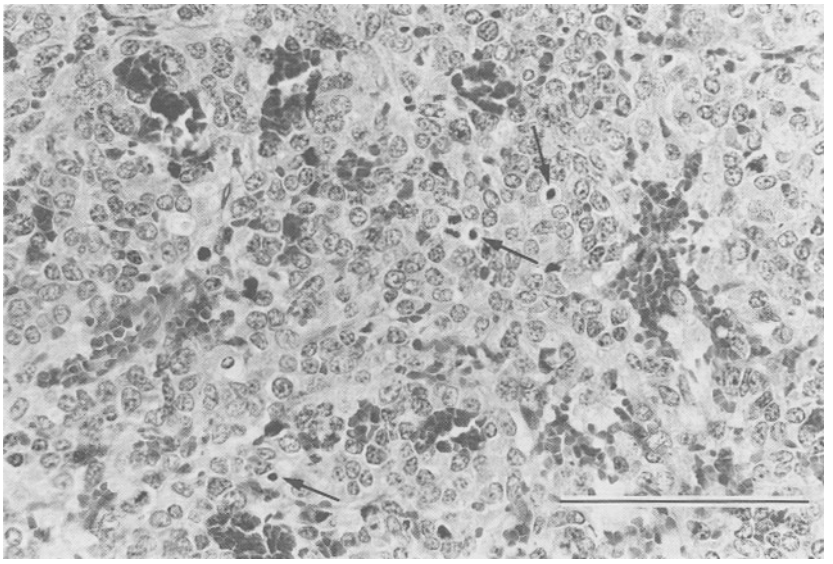

Figure 4. The tumor consists of a number of large pale-stained cells and a few dark cells (arrows). HE. Bar=100 μm.

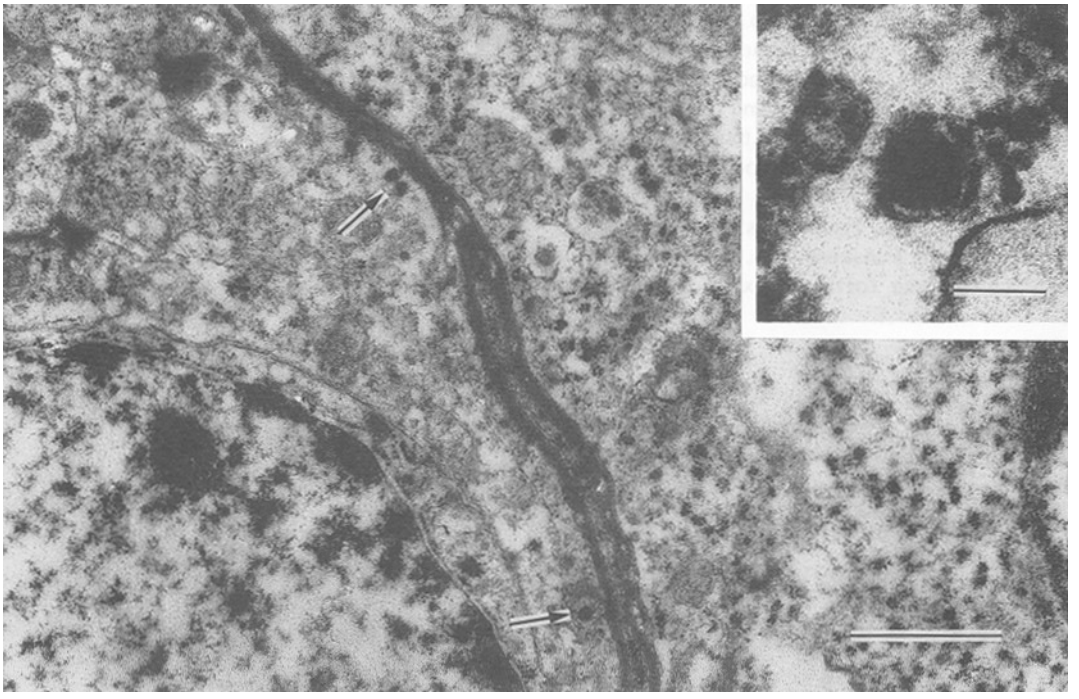

Figure 5. Electron micrograph. Tumor cells contain free ribosomes, polysomes, and dense-cored vesicles (arrows). Bar=1 μ m. Inset: Higher magnification of dense-cored vesicles. Bar=100nm.

the small cells contained a number of free ribosomes and polysomes, other organelles were poorly developed. The morphology of these cells was largely similar to that of the large cells.

The morphological structures of the present case were similar to those of reported pineal gland tumors [3, 8, 10]. While the incidence of pineal gland tumors in rats is apparently very low, it is known to be more common in Sprague-Dawley rats than other strains such as the Fischer and Osborn-Mendel strain [10]. Pineal gland tumors are more likely to occur in older (2-year-old or more) animals than in younger rats [8]. Although our case involved a Sprague-Dawley rat, this animal was comparatively younger than the rats in other reports, and this might

be the first reported case of this tumor in Crj-CD (SD) IGS rats.

The presence of two cell types [3, 6, 10, 13] and a few dense-cord vesicles in tumor cells similar to the neurosecretory granules [14] strongly suggested that the present tumor was derived from pineal parenchymal cells. The malignant pineal gland tumor is characterized by cellular pleomorphism, high mitotic activity, necrosis, and, most important, invasive growth into adjacent tissue [1, 8, 12]. However, it has been reported that high mitotic activity is common in benign pineal gland tumors in rats [3 ,9-11, 13]. Based on present findings, this tumor was diagnosed as benign. The formation of a rosette or a pseudo-rosette pattern and smooth muscle fibers in the such stroma have also described in the pineal gland tumors in rats [1, 6, 9, 12], but there were no findings in the present case.

In World Health Organization (WHO) classification of central nervous system neoplasms, human pineal parenchymal tumors are divided into pineocytoma, pineoblastoma and mixed pineocytoma-pineoblastoma [7]. In the rat, they are divided into pinealoma (benign) and pinealoma (malignant) by the International Agency for Research on Cancer (IARC) [12]. Koestner and Solleveld recommended that the classification of pineal parenchymal tumors of the rat should be adapted to the WHO criteria proposed for human pineal gland tumors, although they considered the term pineocytoma a synonym for the term pinealoma [8]. The terms for pineal gland tumors currently do not agree however, we diagnosed this tumor as pinealoma (benign) according to IARC classification.

ACKNOWLEDGEMENTS

The authors thank Ms. Misako Yamaguchi, Mr. Atsushi Funakoshi, Ms. Yoko Fujieda and Mr. Mitsuo Kawashima for their excellent technical assistance.

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CHAPTER 3

Reproduction Toxicology Related To

A Study of the Effects of Restraint Duration for Nose-Only Inhalation Exposure on Embryo/Fetal Development in Rats

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ABSTRACT. The current study, following the ICH 4.1.3 design, assessed the effects of various nose-only (N-O) inhalation exposure/restraint durations on pregnancy, maternal body weight, food consumption, clinical condition, and embryo/fetal development in Crl:CD®(SD)IGS BR rats. Animals were incrementally acclimatized to the restraint tubes for at least four days prior to mating. The maximum acclimatization time for each animal matched its designated exposure duration. Following acclimatization, 25 mated female rats per group were exposed to filtered air using N-O inhalation techniques for periods of 1, 2, or 6 hours on gestation days (GD) 6 through 17. Comparative controls were exposed to filtered air for 6 hours using a whole-body inhalation technique. Acclimatization to N-O restraint had no effects on the number of corpora lutea, nidation (implantation) or the pregnancy rate. Daily 1-, 2- or 6-hour N-O exposure/restraint from GD 6-17 had no effects on clinical condition or food consumption. Slight, but statistically significant, reduced mean body weight gains or body weight losses were observed early in the gestation period (GD 6-9) for all N-O exposure/restraint groups. The reductions in body weight gain were sufficiently minor as to create no statistically significant difference in mean body weight for any interval in the study. Considering the entire exposure period (GD 6-18), mean body weight gains in the 1-, 2-, and 6-hour N-O exposure/restraint groups were 9, 9, and 12 g lower, respectively, than the mean gain observed on the whole-body group. However, body weight gain throughout gestation (GD 0-20) was similar across all N-O exposure/restraint groups. Mean net body weight, net body weight gain (mean GD 20 weight minus initial weight and gravid uterine weight), gravid uterine weight, and fetal viability and weights were also comparable across groups. Spontaneous malformations were limited to one fetus in the whole-body group and one fetus in the 1-hour N-O group. Developmental variations were not increased in the N-O exposure/restraint groups when compared to the whole-body group or the WIL Historical Control database. The no-observed-effect level (NOEL) for developmental toxicity for N-O inhalation restraint duration was 6 hours under the conditions of this study. Therefore, the N-O inhalation route of exposure is suitable for guideline-driven prenatal (embryo/fetal) developmental toxicity studies. — Key Words: Restraint, Nose-Only Inhalation, and Embryo/Fetal Development

CD(SD)IGS-2001: 81-91

INTRODUCTION

Worldwide regulatory guidelines *e.g*., International Conference on Harmonisation (ICH), Organisation of Economic Cooperation and Development (OECD) and U.S. Environmental Protection Agency, either state that the route of exposure in studies of reproduction and developmental toxicity should mimic the route of clinical administration (ICH) or provide specific guidelines for inhalation exposure (OPPTS 870.3600). For pharmaceutical development, essentially only inhalants, whole-body exposure may not adequately mimic the clinical route of exposure. It may also be inappropriate for some agricultural compounds and other chemical industry products to expose animals to the test material by whole-body inhalation techniques. For example, animals exposed to solvents by whole-body inhalation exposure may absorb quantities of the test material dermally and chemicals that must be administered as an aerosol may be ingested during preening after and during exposure along with dermal absorption [1]. Therefore, in order to more adequately mimic the clinical route of exposure (pharmaceuticals) or limit secondary routes of exposure (aerosol, solvents), the use of nose-only (N-O) inhalation exposure may be considered a suitable alternative. However, because maternal stress (primarily mice [2-7]) and hyperthermia (rats and mice [8-14]) have been shown to result in developmental toxicity (*i.e*., prenatal mortality, malformations) the purpose of this study was to evaluate the effects of the duration of restraint for N-O inhalation exposure on embryo/fetal development, maternal body weight and clinical condition in Sprague-Dawley Crl:CD®(SD)IGS BR rats.

MATERIALS and METHODS

Test Species and Husbandry

One hundred forty female Crl:CD®(SD)IGS BR rats were obtained from Charles River Laboratories, Inc. (Kingston, NY, USA). The animals were individually housed in clean suspended wire-mesh cages in an environmentally controlled room during acclimatization. Prior to mating, the animals were randomly assigned to three acclimatization regimes that represented the durations of N-O restraint commonly used on inhalation toxicity studies in rats. An incremental acclimatization approach was used over four days up to final durations of one, two and six hours. After four days of acclimatization, the females were paired in a suspended wire-mesh cage (1 male:1 female) with resident males of the same strain and from the same supplier. Mating was confirmed by the presence of a vaginal plug or the presence of sperm in a vaginal smear, and the day of positive confirmation was designated as day 0 of gestation. Animals from the 1-, 2- and 6-hour acclimatization regimes were assigned to exposure groups with the same respective durations. Mated females from the three acclimatization regimes were also assigned to the whole-body control group.

In the animal room, controls were set to maintain temperature at 72°±4°F and relative humidity between 30-70%. Air handling units were set to provide at least 10 fresh air changes per hour. Fluorescent lighting controlled by light timers provided illumination for a 12-hour light/dark photoperiod (6:00 a.m. - 6:00 p.m.). Temperature and relative humidity were recorded once daily. Reverse osmosis-purified water and PMI Nutrition International, Inc. Certified Rodent LabDiet® 5002 were available *ad libitum* (except during inhalation exposures).

Exposure Methods

Using either the N-O or whole-body system, the animals were exposed to HEPA- and charcoal-filtered air for the requisite durations. Exposure atmosphere temperature, humidity, flow rate and pressure were monitored continuously throughout the exposures via National Instruments LabVIEW® software.

Figure 1. The Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (12-port). Photo courtesy of Rudolph Jaeger, Ph.D., D.A.B.T., CH Technologies, Inc., Westwood, NJ.

Figure 2. Polycarbonate exposure tube with slotted polystyrene backstop. Photo courtesy of Rudolph Jaeger, Ph.D., D.A.B.T., CH Technologies, Inc., Westwood, NJ.

Nose-Only Exposures: Exposures were conducted utilizing the Jaeger-NYU Directed-Flow Nose-Only Inhalation Exposure System (Figure 1). The systems used polycarbonate exposure/restraint tubes fitted with slotted polystyrene backstops (Figure 2). The slotted-backstop feature of these exposure/restraint tubes allows for better thermoregulation in the rats than the older previously employed solid-backstop designs. The size of exposure/restraint tube was increased on gestation day (GD) 10 to accommodate maternal body weight gain. Air flow rate through the system was set to provide adequate volumes for the animals. Controls for daily average chamber temperature and relative humidity were set to maintain 22±2°C and 30-70%, respectively. The supply air was conditioned as necessary to maintain temperature and relative humidity within these ranges. Nose-only chamber exhaust air flow was monitored continuously and recorded at approximately 30-minute intervals during each exposure. Chamber temperature and relative humidity were measured and recorded at approximately 35-minute intervals during each exposure. Oxygen content of the exposure atmospheres was at least 19% and was confirmed once during the study.

All animals were housed in standard animal rooms during non-exposure hours. For each day's exposure, the animals were placed in restraint tubes, transported to the exposure room, exposed for the requisite duration and returned to their home cages. To allow the restrained animals to acclimate, they were housed in holding tubes for at least 20 minutes, but no more than 40 minutes, prior to initiation of exposure. The animals were rotated around the available exposure ports on a daily basis.

Whole-Body Exposures: Control group animals were exposed using whole-body techniques in a 1 m^3 stainless-steel and glass exposure Hazleton (H-1000) chamber. The chamber was operated under dynamic conditions with at least 12 air changes per hour and a slight negative pressure (ca. 0.5 in. of water). Oxygen content of the exposure atmosphere was at least 19% and was confirmed once during the study. Controls for daily average chamber temperature and relative humidity were set to maintain daily averages between 20-26°C, and 30-70%, respectively. The supply air source was the same as that used for the N-O exposures and the air was conditioned as necessary to maintain temperature and relative humidity within the stated ranges.

The animals were housed in standard animal rooms during non-exposure hours. For each day's exposure, the animals were transferred to exposure caging, transported to the exposure room, exposed for the requisite duration, and returned to their home cages. Animal cage batteries were rotated through the various possible chamber locations on a daily basis.

Experimental Design

The mated females (25 animals/group) were exposed to filtered air for the appropriate daily durations during GD 6 through 17 using N-O or whole-body inhalation exposure techniques. Clinical findings were recorded daily throughout gestation. Body weights and food consumption were measured on GD 0, 6-18 (daily) and 20. On GD 20, the animals were euthanized, laparohysterectomies performed and uterine parameters recorded. Uteri that appeared nongravid by macroscopic examination were opened and

placed in 10% ammonium sulfide for detection of early implantation loss [15].

Fetuses were weighed, sexed and subjected to a fresh external and visceral [16] examination. One-half of the heads of fetuses in each litter were examined by a mid-coronal slice and the remaining half of the heads were removed, placed in Bouin's solution and subsequently examined using the Wilson [17] sectioning technique. All carcasses were processed and stained with Alizarin Red S [18] for subsequent skeletal examinations for malformations and variations.

Statistical Analyses

All analyses were two-tailed for significance levels of 5% and 1%. All statistical tests were performed by a computer with appropriate programming as referenced below. The litter, rather than the fetus, was considered as the experimental unit. Statistical comparisons of the N-O exposure/restraint groups were made to the whole-body exposure group.

Continuous data variables were subjected to a parametric ANOVA [19] to determine intergroup difference. If the result of the ANOVA were significant ($p < 0.05$), Dunnett's test [20] was applied to the data. The mean litter proportions of prenatal data (% per litter of viable and nonviable fetuses, early and late resorptions, total resorptions, pre- and post-implantation loss and the fetal sex distribution) were subjected to the Kruskal-Wallis nonparametric ANOVA [21] to determine intergroup difference. If the result of the ANOVA were significant ($p < 0.05$), the Mann-Whitney U-Test [21] was applied to the data.

The mean litter proportion (% per litter) of total fetal malformations and developmental variations (external, visceral, skeletal and combined) and of each particular external, visceral and skeletal malformation or variation were tabulated. The mean litter proportions of fetal malformations and developmental variations were subjected to the Kruskal-Wallis nonparametric ANOVA followed by the Mann-Whitney U-Test, where appropriate.

RESULTS

Maternal:

Acclimatization to the N-O exposure/restraint tubes had no effects on ovulation, nidation or the pregnancy rate (Table 1). No clinical findings related to the N-O exposure/restraint were observed (data not shown) nor was food consumption affected by the N-O exposure/restraint (Figure 3). A slight, statistically significant mean body weight loss induced in all N-O groups on the first day (GD 6) of restraint (Figure 4) contributed to a statistically significant decrease in body weight gain over the GD 6-9 interval (Figure 5). While these initial reductions were sufficiently minor as to create no statistically significant difference in mean body weight throughout the study (Figure 6), a statistically significant reduction in the GD 6-18 (exposure period) body weight gain was noted in all N-O exposure/restraint groups. The exposure period reductions and losses in mean body weight gain were considered evidence of stress (″maternal toxicity″). However, when the entire gestation period (GD 0-20) was considered, maternal mean body weight gain for the 1-, 2- and 6-hour N-O exposure groups was decreased only 4, 1 and 8 g, respectively suggesting that the stress produced by restraint during the exposure period was minimal. Lastly, net mean body weight (GD 20 body weight minus gravid uterine weight), net mean

Table 1. The effect of N-O exposure/restraint on maternal and intrauterine endpoints (resorptions, litter sizes, corpora lutea *etc*.)

Parameter	Whole-Body	1-Hour Nose Only	2-Hour Nose Only	6-Hour Nose Only
Mean no. corpora lutea	17.7±2.5	18.2±2.8	18.3±2.2	17.6±3.0
Percent (%) Pregnant	100(25/25)	100(25/25)	96(24/25)	100(25/25)
Mean no. implantation sites	16.2±2.4	15.9±2.4	16.7±1.7	16.0±1.8
Mean pre-implantation loss (% per litter)	1.5±1.7 (8.2±9.1)	2.3±2.3 (12.0±11.2)	1.7±2.0 (8.4±9.6)	1.6±2.2 (8.2±9.3)
Mean no. viable fetuses (%)	15.4±3.0 (94.3)	15.1±2.5 (94.9)	15.9±1.7 (95.4)	15.2±1.9 (94.8)
Mean early resorptions (% per litter)	0.8±1.4 (5.7±10.5)	0.8±0.8 (4.9±5.8)	0.8±0.8 (4.7±4.7)	0.8±1.1 (4.9±6.8)
Mean late resorptions (% per litter)	0.0±0.0 (0.0±0.0)	0.0±0.2 (0.3±1.3)	0.0±0.0 (0.0±0.0)	0.0±0.2 (0.3±1.3)
Mean Post-implantation loss (% per litter)	0.8±1.4 (5.7±10.5)	0.8±0.9 (5.1±6.0)	0.8±0.8 (4.7±4.7)	0.8±1.1 (5.2±6.8)
Percent males (females)	50.7(49.3)	51.7(48.3)	51.0(49.0)	47.9(52.1)
Mean combined (male + female) fetal weight (g)	3.7±0.23	3.7±0.27	3.7±0.24	3.6±0.28

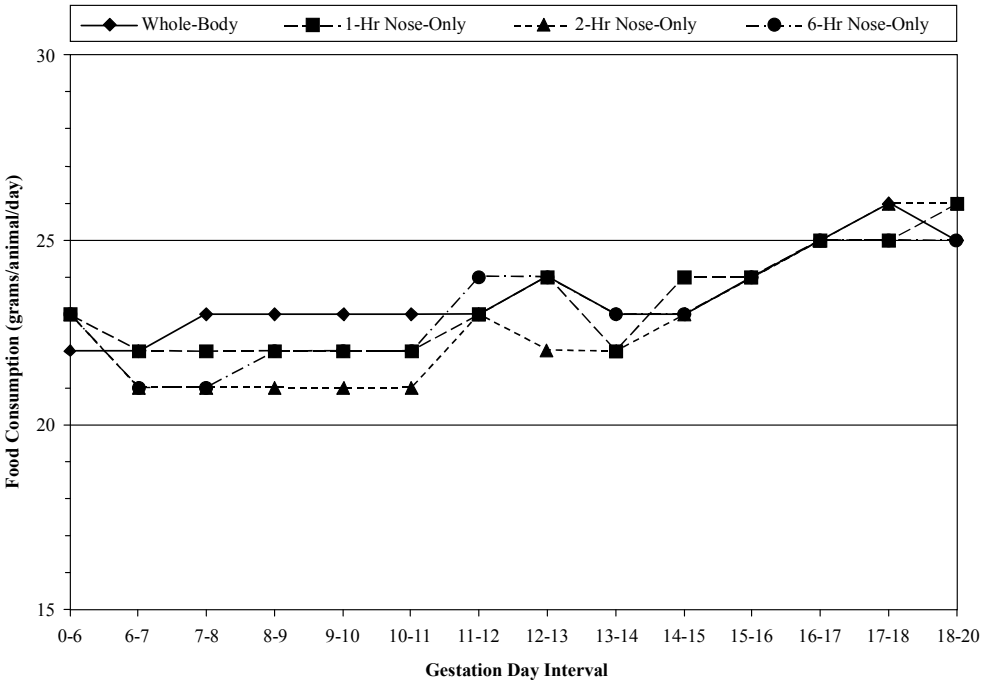

Figure 3. Mean food consumption during gestation of dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods.

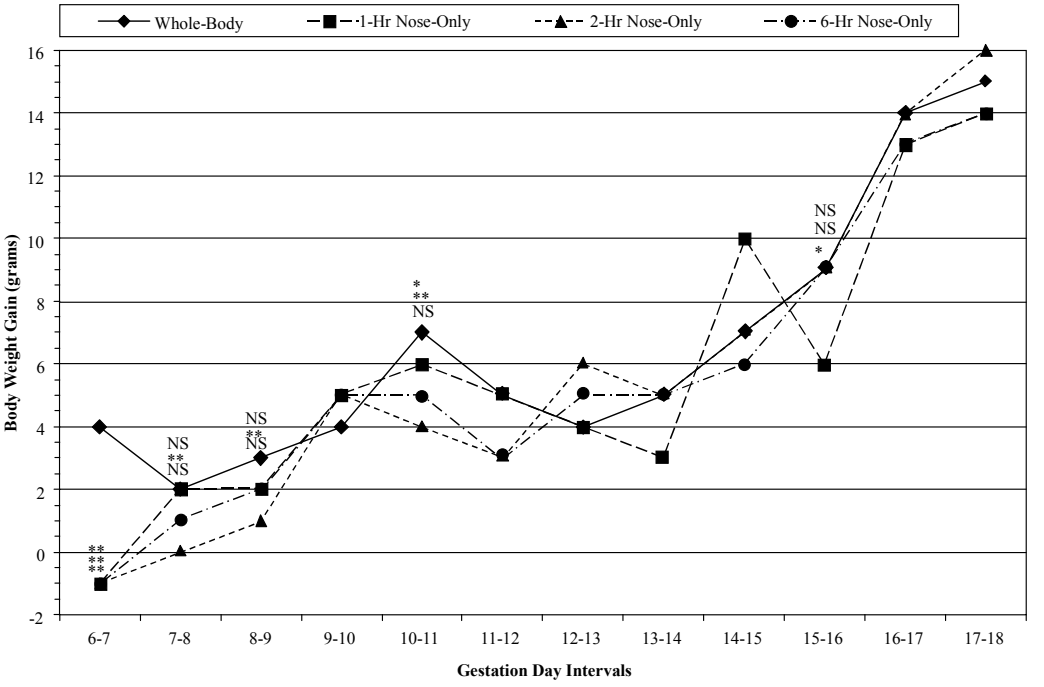

Figure 4. Mean daily body weight gain during gestation of dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods. Statistical significance between the control and N-O groups is indicated by * ($p<0.05$) and ** ($p<0.01$). The row of */** indicates which group(s) is different from the whole-body exposure (control) group with bottom, middle and top rows corresponding to 1-, 2- and 6-hourr exposure/restraint duration, respectively.

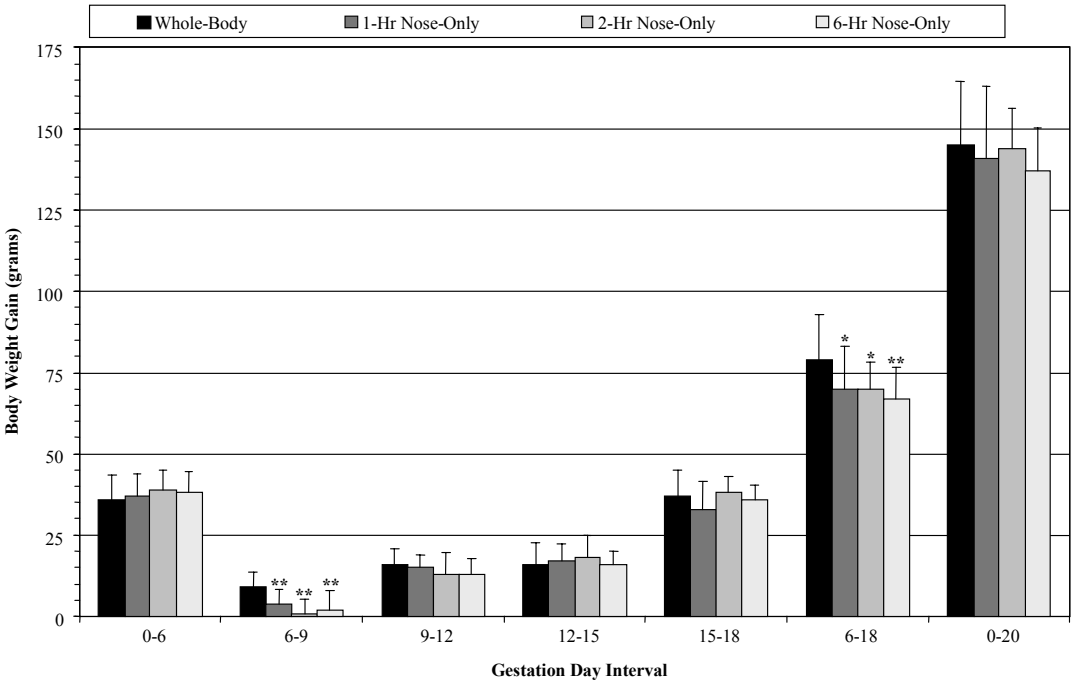

Figure 5. Mean±standard deviation interval body weight gain during gestation of dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods. Statistical significance between the whole-body exposure (control) and N-O groups is indicated by * ($p<0.05$) and ** ($p<0.01$).

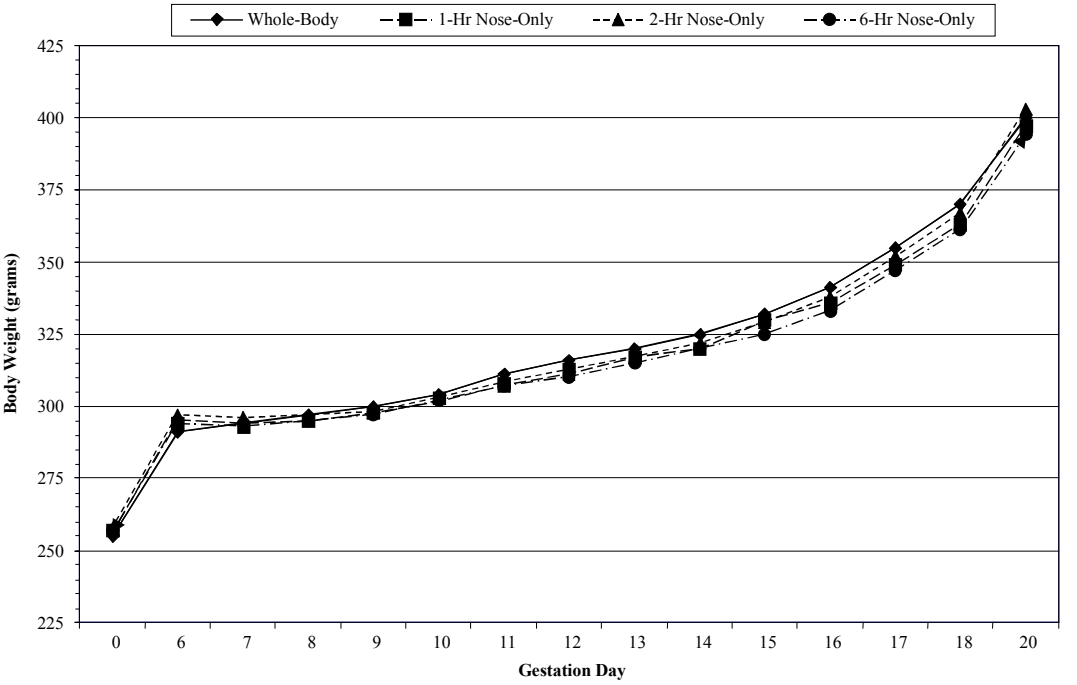

Figure 6. Mean body weight during gestation of dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods.

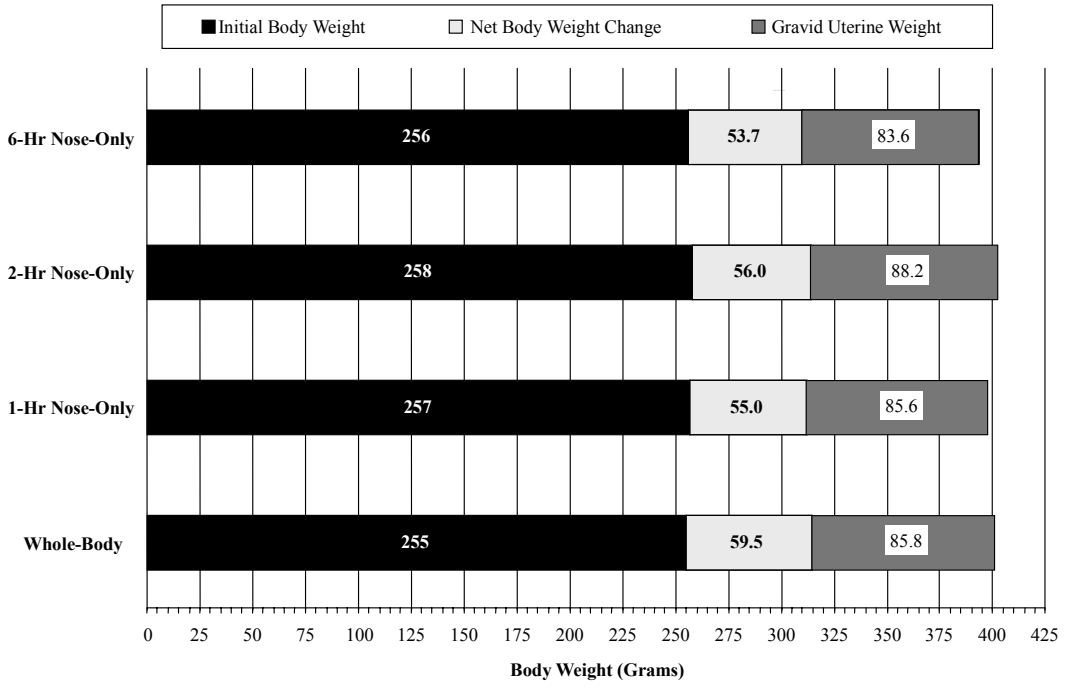

Figure 7. Mean terminal body weight, net body weight change and gravid uterine weight of dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods.

body weight gain and mean gravid uterine weight were unaffected by any restraint duration (Figure 7) indicating that N-O exposure/restraint had only a slight effect on maternal body weight gain.

Embryo/Fetal:

Fetal viability, the degree of embryonic resorptions and mean fetal weights were unaffected by N-O exposure/restraint duration (Table 1; Figure 8). No increase in the rate of malformations or variations were observed in the N-O exposure/restraint groups compared to the whole-body group (Tables 2-5) or the WIL Historical Control database.

DISCUSSION

Previously, maternal restraint stress *per se* (primarily via restraining tubes or taping in a prone position for 1.5-12 hours/day) has been shown to elicit fetal skeletal and external malformations/variations in mice. Generally, maternal restraint produces supernumerary ribs [2-4] and cleft palate [5-7] in mice. In rats, maternal restraint stress has been shown to affect a variety of fetal or pup endpoints such as baseline, stressed or drug-induced hormonal milieus [22-25], brain opioid receptors [26], behavior [27-29], male sexual behavior [30-34] and neuronal development [35-36]. However maternal restraint apparently does not elicit comparable fetal effects [3] but may [37] or may not [35,38] increase prenatal mortality. The present study using 1-, 2- and 6-hour N-O exposure/restraint paradigms produced no malformations or variations nor increased fetal mortality indicating that, unlike the mouse, maternal restraint during gestation is not teratogenic. Also, four days of premating acclimatization to the restraint procedure had no effect on fertility or fecundity as denoted by numbers of corpora lutea and implantation sites. Based on the mean daily chamber temperatures (Table 6), hyperthermia-inducing conditions (ambient temperatures >40°C [8-10] that raise the core body temperature >2.0°C producing malformations [11-14]) were presumed not to have been produced (core body temperatures were not measured). Therefore, it was not surprising that malformations, variations and embryo/fetal mortality similar to the effects produced by hyperthermia [8-12,39] were not evident in any of the groups.

In conclusion, when employing the proper equipment and an appropriate acclimatization schedule, restraint during N-O inhalation exposure, while slightly decreasing maternal body weight gain during the treatment period, does not increase the background incidence of malformations, variations and embryo/fetal mortality nor decrease fertility and fecundity. Therefore, the no-observed-effect level (NOEL) for developmental toxicity for N-O inhalation restraint duration was 6 hours under the conditions of this study and the N-O inhalation route of exposure is suitable for prenatal (embryo/fetal) developmental toxicity studies.

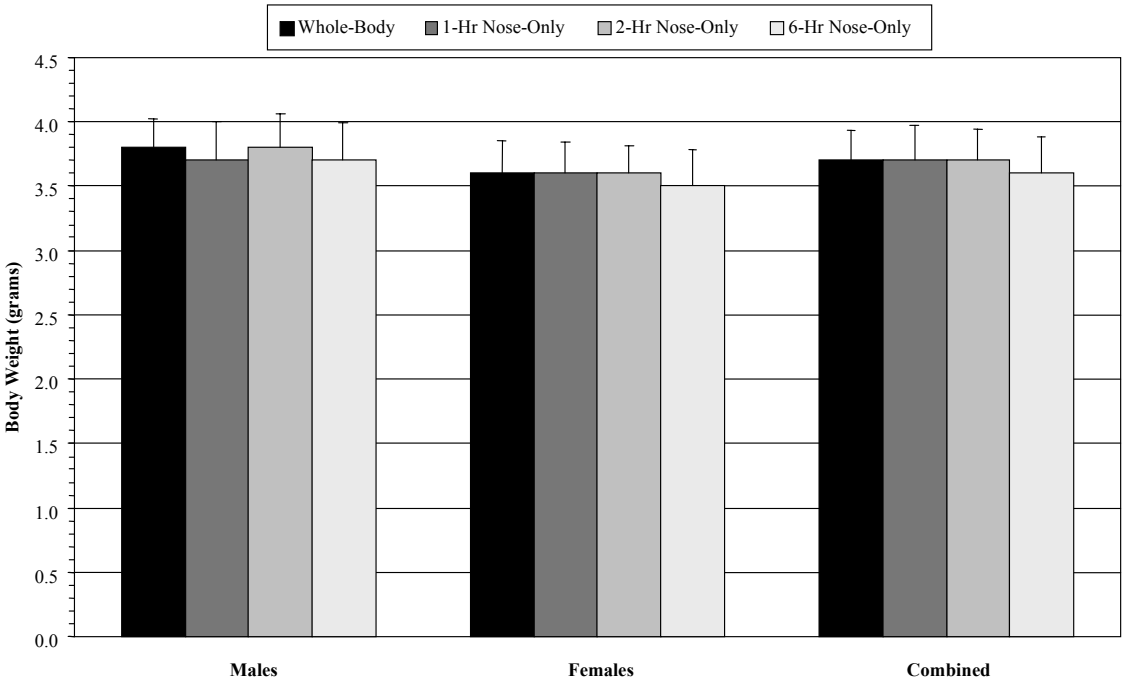

Figure 8. Mean±standard deviation body weight of fetuses from dams exposed to HEPA- and charcoal-filtered air by whole-body (control) exposure or by 1-, 2- or 6-hour N-O exposure methods.

Table 2. The effect of N-O exposure/restraint on fetal malformation incidence [no. of fetuses (litters) affected]

Parameter	Whole-Body	1-Hour Nose Only	2-Hour Nose Only	6-Hour Nose Only
No. Examined Externally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
Thoracogastroschisis	0	1 (1)	0	0
Amelia	0	1 (1)	0	0
Curly Tail	0	1 (1)	0	0
Micropthalmia and/or anophthalmia	1 (1)	1 (1)	0	0
Meningoencephalocele	0	1 (1)	0	0
Cleft Palate	1 (1)	0	0	0
Mandibular Microagnathia	1 (1)	0	0	0
No. Examined Viscerally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
Hydrocephaly	0	0	1 (1)	0
No. Examined Skeletally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
No. with findings	0	0	0	0

Table 3. The effect of N-O exposure/restraint on fetal variation incidence [no. of fetuses (litters) affected]

Parameter	Whole-Body	1-Hour Nose Only	2-Hour Nose Only	6-Hour Nose Only
No. Examined Externally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
No. with findings	0	0	0	0
No. Examined Viscerally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
Hemorrhagic ring around the iris	0	0	0	1 (1)
Spleen small in size	0	1 (1)	0	0
No. Examined Skeletally	**384 (25)**	**377 (25)**	**381 (24)**	**378 (25)**
Sternebra(e) #5 and/or #6 unossified	39 (13)	30 (10)	29 (7)	31 (11)
Cervical centrum #1 ossified	54 (20)	58 (17)	59 (22)	65 (15)
14^{th} rudimentary rib(s)	18 (10)	20 (10)	14 (9)	17 (10)
Reduced ossification of the 13^{th} rib(s)	4 (4)	7 (3)	2 (2)	4 (1)
Bent rib(s)	0	1 (1)	0	0
27 presacral vertebrae	1 (1)	1 (1)	0	0
7^{th} cervical rib(s)	2 (2)	1 (1)	5 (2)	0
Hyoid unossified	8 (4)	5 (3)	6 (3)	1 (1)
Sternebra(e) #1, #2, #3 and/or #4 unossified	0	1 (1)	0	1 (1)
Entire sternum unossified	1 (1)	0	0	0
Pubis unossified	2 (2)	0	0	0
Ilium unossified	1 (1)	0	0	0
Ischium unossified	1 (1)	0	0	0
Sternebra(e) malaligned (slight or moderate)	1 (1)	0	0	0
7^{th} sternebra	0	0	2 (1)	0
14^{th} full rib(s)	0	1 (1)	0	0

Table 4. The effect of N-O exposure/restraint on fetal malformations (% per litter)

Parameter	Whole-Body	1-Hour Nose Only	2-Hour Nose Only	6-Hour Nose Only
No. Examined Externally	**25**	**25**	**24**	**25**
Thoracogastroschisis	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
Amelia	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
Curly Tail	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
Micropththalmia and/or anophthalmia	0.2±1.2	0.3±1.25	0.0±0.0	0.0±0.0
Meningoencephalocele	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
Cleft Palate	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
Mandibular Microagnathia	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
No. Examined Viscerally	**25**	**25**	**24**	**25**
Hydrocephaly	0.0±0.0	0.0±0.0	0.3±1.28	0.0±0.0
No. Examined Skeletally	**25**	**25**	**24**	**25**
No. with findings	0	0	0	0
Total Malformations				
External	0.2±1.18	0.3±1.25	0.0±0.0	0.0±0.0
Visceral	0.0±0.0	0.0±0.0	0.3±1.28	0.0±0.0
Skeletal	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Combined	0.2±1.18	0.3±1.25	0.3±1.28	0.0±0.0

Table 5. The effect of N-O exposure/restraint on fetal variations (% per litter)

Parameter	Whole-Body	1-Hour Nose Only	2-Hour Nose Only	6-Hour Nose Only
No. Examined Externally	**25**	**25**	**24**	**24**
No. with findings	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
No. Examined Viscerally	**25**	**25**	**24**	**24**
Hemorrhagic ring around the iris	0.0±0.0	0.0±0.0	0.0±0.0	0.2±1.05
Spleen small in size	0.0±0.0	0.2±1.18	0.0±0.0	0.0±0.0
No. Examined Skeletally	**25**	**25**	**24**	**24**
Sternebra(e) #5 and/or #6 unossified	9.6±12.19	7.7±15.45	7.6±15.60	8.3±12.12
Cervical centrum #1 ossified	14.1±12.36	14.9±16.33	15.9±15.50	16.8±22.90
14^{th} rudimentary rib(s)	4.8±9.21	4.9±7.71	3.8±5.85	5.0±8.58
Reduced ossification of the 13^{th} rib(s)	1.1±2.66	1.7±4.75	0.5±1.76	1.2±6.15
Bent rib(s)	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
27 presacral vertebrae	0.3±1.25	0.3±1.25	0.0±0.0	0.0±0.0
7^{th} cervical rib(s)	0.6±2.21	0.2±1.05	1.3±5.21	0.0±0.0
Hyoid unossified	2.0±4.8	1.4±4.09	1.5±4.59	0.3±1.25
Sternebra(e) #1, #2, #3 and/or #4 unossified	0.0±0.0	0.3±1.54	0.0±0.0	0.2±1.18
Entire sternum unossified	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
Pubis unossified	0.6±2.12	0.0±0.0	0.0±0.0	0.0±0.0
Ilium unossified	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
Ischium unossified	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
Sternebra(e) malaligned (slight or moderate)	0.2±1.18	0.0±0.0	0.0±0.0	0.0±0.0
7^{th} sternebra	0.0±0.0	0.0±0.0	0.5±2.27	0.0±0.0
14^{th} full rib(s)	0.0±0.0	0.3±1.25	0.0±0.0	0.0±0.0
Total Variations				
External	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Visceral	0.0±0.0	0.2±1.18	0.0±0.0	0.2±1.05
Skeletal	28.7±17.91	28.9±22.40	27.3±19.83	28.9±22.81
Combined	28.7±17.91	28.9±22.40	27.3±19.83	29.2±22.81

Table 6. Descriptive statistics of mean daily N-O and whole-body exposure chambers.

Parameter	Whole-Body	Nose-Only
Daily Chamber Temperature	23.1	21.1
Standard Deviation	0.38	0.29
Coefficient of Variance	6.3	5.4
Minimum Temperature	21	20
Maximum Temperature	27	23

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Uterotrophic Assay in Immature Crj:CD(SD)IGS Rats- Effects of Aging and Weaning -

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ABSTRACT. Effects of aging and weaning on the uterotrophic assay were examined using immature Crj:CD(SD)IGS rats. Groups of 10 pups were exposed to 17-ethinyl estradiol (EE) of different concentrations ranging from 0.02 to 200 μg/kg body weight/day by oral gavage for 3 consecutive days. The uterotrophic response as indicated uterus weight to the EE administration did not change by aging but slightly increased by weaning. The body weight gain of both exposed and control pups significantly decreased by earlier weaning at the ages of 18 or 19 days as compared to later weaning at the ages of 20, 21 or 22 days. These results suggested that the uterotrophic assay using immature Crj:CD(SD)IGS rats should be commenced at the ages of 20-22 days under a weaning condition. — Key words: CD(SD)IGS Rat, Uterotrophic Assay, Immature Rats, Weaning, Age

CD(SD)IGS-2001: 92-97

INTRODUCTION

The rodent uterotrophic assay is one of the methods to assess effects of environmental chemicals on estrogenic activity. This assay was developed in the 1930s [1, 2] and has been reviewed recently [5]. Any detectable effect can not be observed for mature animals in the uterotrophic assay, because their hormonal system is homeostatically maintained and resistant even to small quantity of environmental chemicals. The rodent uterotrophic assay can afford to detect any subtle effect by using the animals bearing the immature hormonal function or the ovariectomized animals. Dosing of chemicals and necropsy must be performed within a limited short period spanning between the age of 18 days at a weaning time and the age of 25 days at a maturing time of ovary function [5]. In order to find out an appropriate age and weaning time for the effective uterotrophic assay, we undertook to examine the aging and weaning effects of 17-ethinyl estradiol (EE) administrations on growth rates and uterus and ovary weights in Crj:CD(SD)IGS rat pups. The present studies were financially supported by the Japan Environment Agency in 1999.

MATERIALS AND METHODS

Nine-week-old female and 10-week-old male Crj:CD(SD)IGS rats (F_0 animals) were purchased from Charles River Japan (Atsugi, Japan). F_1 female pups (the day of birth counted as day 0 of lactation) born in the Japan Bioassay Research Center animal facility were used. On day 4 of lactation, the number of pups per litter were adjusted to 8 animals (including both male and/or female pups). They were housed in aluminum or stainless steel cages with paper pulp tip as nesting material (ALPHA-dri., Shepherd Specialty Paper, INC., Kalamazoo, MI, USA) throughout the experimental period. After weaning the number of F_1 rats were adjusted to 5 female pups per cage. Room temperature and relative humidity were maintained at 22±2℃, 50±15%, respectively, with a 12-hr light/ dark cycle (08:00-20:00/ 20:00-08:00). Tap water and commercial pellet diet (CRF-1, γ-irradiated with 30 KGy, Oriental Yeast, Tokyo, Japan) were given *ad libitum*.

17-ethinyl estradiol (EE) (Sigma-Aldrich, St. Louis, USA) was administered daily (13:00-15:00) to the animals at dose levels of 0, 0.02, 0.2, 2, 20 and 200 μg/kg body weight/day by gavage for 3 consecutive days. The volume of dose was fixed at 5 mL/kg body weight. The dosing solutions were prepared by mixing the chemical with corn oil (Wako Pure Chemical Industries Ltd., Osaka, Japan). The control animals received an equivalent volume of the vehicle.

The effects of aging and weaning on the uterotrophic response to EE were examined by a total of ten studies including 5 studies each under a weaning or non-weaning condition. The animals used for the studies of the weaning condition were separated from their dams in the morning of the first day of dosing, and those used for the non-weaning condition were housed together with their dams until the necropsy. Age was 18, 19, 20, 21 and 22-day-old at the start of dosing under each weaning or non-weaning condition.

Each animal was weighed daily just before administration and necropsy. The animals were killed by exsanguination under diethylether anesthesia after approximately 24 hours of the last administration, and the uteri and ovaries were removed and weighed. The uterus weight was measured with fluid as wet weight and without fluid as blotted weight.

Statistical analyses were performed on all data including body and organ weights. Data were represented as mean±SD, and pre-tested for variance with Bartlett's test. When variance was homogeneous, a one-way analysis of variance (ANOVA) was carried out, and when any significant difference was detected, Dunnett multiplex comparison test was applied. When, however, variance was heterogeneous, Kruskal-Wallis's rank sum test was carried out, when any significant difference was detected, Dunnett's rank sum test was applied. Statistical significance was concluded with a probability of 5% for Bartlett's variance test and 5% and 1% for the final test.

RESULTS AND DISCUSSION

Under the non-weaning condition, body weight gain significantly decreased in 20 and 200 μg/kg groups when dosing started at the age of 20 days, and 200 μg/kg group when dosing started at the age of 22 days (Table 1). Under the weaning condition, body weight gain significantly decreased in 200 μg/kg groups when dosing started at the ages of 18, 19 and 20 days, and 20 μg/kg group when dosing started at the age of 21 days. It was note-

worthy that body weights of both exposed and control pups decreased next day of the weaning when dosing started at the ages of 18 and19 days (Table 2).

Under the non-weaning condition, the daily administrations of EE at the concentrations of 2 - 200 μg/kg induced a significant increase in both wet and blotted weights of the uterus. The same trend of the EE effect was also observed under the weaning condition except for a marginal but significant increase ($p<0.01$ or 0.05) in both wet and blotted uterus weights for the 0.2 μg/kg group of both 18 and 22 days old weaned animals. This revealed that the uterotrophic response to EE was more sensitive under weaning condition than under the non-weaning condition. It is noteworthy that the mean uterus weight of control group in the non-weaned rats was significantly higher than those in the weaned rats (Table 3). This result can be interpreted as that the urine and/or feces containing EE and its metabolites were ingested by the pups. In our studies, the non-weaned pups exposed to EE of different levels were housed together with a dam in a cage throughout the study period. A factor of weaning might be very important to assess effects of estrogenic chemicals in the uterotrophic assay using immature rats.

Weaning and aging effects of the EE administration on the ovary weight were not recognized (Table 4). This result can be taken to indicate that the ovary function was not matured at these ages.

As to the age of immature animals at the first dosing day of chemicals in the uterotrophic assay, the OECD guidance and protocol recommended in 1999 that the rat pups be between 18 to 22 days old (day of birth counted as day 1) at the age of the first dosing [3], and the revised OECD guidance in 2000 described that the first dosing start at the age of 18, 19 or 20 days (day of birth counted as day 0) [4]. In our studies, body weights of both exposed and control pups decreased on next day of the weaning when dosing started at the ages of 18 and 19 days. It can be inferred that this result was attributable to excessive stress as well as the lack of maternal care by licking the pups and the decrease in food consumption results from earlier weaning, because rats are usually weaned at the age of 21 days [6].

In conclusion, the results reported here suggest that the uterotrophic assay using immature Crj:CD(SD)IGS rats should be carried out by the first dosing of estrogenic chemicals at the ages of 20-22 days under a weaning condition.

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Table 1. The Aging Effect of 17-Ethinyl Estradiol Administration on Body Weight Change in Non-weaned Rats.

Dose	Start of dosage			Necropsy
(μ g/kg)	18-day	19-day	20-day	21-day
Control	37.2 ± 2.2	40.1 ± 2.5	42.6 ± 2.8	45.9 ± 3.3
0.02	37.2 ± 2.1	39.9 ± 1.9	42.2 ± 2.2	45.7 ± 3.1
0.2	37.1 ± 2.4	40.0 ± 2.5	42.0 ± 2.9	45.3 ± 3.4
2	37.3 ± 2.2	39.7 ± 2.3	42.0 ± 2.4	45.3 ± 2.6
20	37.3 ± 2.2	40.1 ± 2.4	42.0 ± 2.5	45.3 ± 2.9
200	37.1 ± 2.3	39.4 ± 2.5	41.3 ± 2.6	44.3 ± 2.8
Dose	Start of dosage			Necropsy
(μ g/kg)	19-day	20-day	21-day	22-day
Control	40.9 ± 2.5	43.4 ± 2.8	47.0 ± 3.6	51.3 ± 4.2
0.02	40.8 ± 2.6	43.4 ± 2.9	46.7 ± 3.5	50.8 ± 3.9
0.2	40.9 ± 2.5	43.5 ± 2.8	46.8 ± 3.6	51.0 ± 3.4
2	40.9 ± 2.5	43.2 ± 2.5	46.2 ± 3.0	50.0 ± 3.2
20	40.9 ± 2.5	43.0 ± 3.0	46.1 ± 3.4	49.8 ± 3.7
200	41.0 ± 2.6	42.9 ± 2.8	44.7 ± 2.7	46.6 ± 3.0
Dose	Start of dosage			Necropsy
(μ g/kg)	20-day	21-day	22-day	23-day
Control	43.1 ± 1.9	47.3 ± 2.7	52.2 ± 3.1	56.8 ± 3.8
0.02	43.1 ± 1.7	47.0 ± 2.0	51.3 ± 2.4	55.8 ± 2.9
0.2	43.0 ± 2.1	46.2 ± 2.5	50.9 ± 2.6	55.1 ± 2.9
2	43.0 ± 2.3	46.9 ± 2.4	51.1 ± 2.8	55.1 ± 3.0
20	42.9 ± 2.6	45.5 ± 2.4	49.4 ± 2.2	53.1 ± 2.8*
200	43.0 ± 1.5	45.1 ± 1.3	48.0 ± 1.1**	50.4 ± 1.5**
Dose	Start of dosage			Necropsy
(μ g/kg)	21-day	22-day	23-day	24-day
Control	46.5 ± 6.3	50.7 ± 6.4	55.3 ± 6.8	60.0 ± 7.2
0.02	47.1 ± 5.6	50.4 ± 6.1	54.5 ± 6.7	58.4 ± 7.9
0.2	46.5 ± 5.8	49.9 ± 7.0	53.6 ± 7.4	57.5 ± 8.4
2	46.8 ± 5.6	50.5 ± 5.9	54.7 ± 6.2	59.0 ± 7.0
20	46.5 ± 6.0	49.4 ± 6.6	53.7 ± 6.5	57.7 ± 6.7
200	46.9 ± 5.4	49.1 ± 5.4	52.1 ± 5.6	54.4 ± 5.7
Dose	Start of dosage			Necropsy
(μ g/kg)	22-day	23-day	24-day	25-day
Control	55.1 ± 5.7	58.0 ± 4.7	62.3 ± 4.9	65.8 ± 4.1
0.02	55.4 ± 7.2	57.8 ± 6.3	62.5 ± 6.8	64.8 ± 4.7
0.2	55.4 ± 5.6	58.3 ± 4.5	62.8 ± 5.1	65.9 ± 3.9
2	54.6 ± 5.9	57.8 ± 5.3	62.4 ± 5.5	65.8 ± 4.9
20	53.4 ± 5.3	55.1 ± 4.9	58.9 ± 5.2	62.2 ± 3.9
200	53.7 ± 4.4	55.2 ± 3.9	58.7 ± 4.4	60.5 ± 1.5*

Data represent mean±SD. Significantly different from each control group, ** : $p<0.01$, * : $p<0.05$

Table 2. The Aging Effect of 17-Ethinyl Estradiol Administration on Body Weight Change in Weaned Rats.

Dose	Start of dosage			Necropsy
(μ g/kg)	18-day	19-day	20-day	21-day
Control	39.8 ± 2.3	38.3 ± 2.7	40.5 ± 3.7	45.7 ± 4.1
0.02	39.8 ± 2.5	38.2 ± 2.6	40.8 ± 3.7	45.5 ± 4.2
0.2	39.8 ± 2.0	37.8 ± 2.2	39.9 ± 2.5	45.3 ± 2.9
2	40.1 ± 2.4	37.8 ± 2.1	39.2 ± 2.7	44.0 ± 3.3
20	40.2 ± 3.1	37.6 ± 3.2	38.9 ± 2.5	43.3 ± 3.2
200	39.7 ± 1.8	36.6 ± 1.7	36.1 ± 2.5**	40.3 ± 3.3**
Dose	Start of dosage			Necropsy
(μ g/kg)	19-day	20-day	21-day	22-day
Control	43.6 ± 2.5	42.3 ± 3.1	45.5 ± 3.9	50.4 ± 3.7
0.02	43.6 ± 2.9	42.0 ± 2.9	45.6 ± 3.1	51.1 ± 3.2
0.2	43.5 ± 2.7	41.7 ± 3.0	44.3 ± 3.8	49.2 ± 4.0
2	43.5 ± 2.4	41.7 ± 3.5	44.6 ± 4.5	50.5 ± 4.6
20	43.5 ± 2.5	41.3 ± 3.0	43.6 ± 3.5	48.1 ± 3.6
200	43.5 ± 2.6	40.8 ± 2.8	41.1 ± 3.7	45.1 ± 3.6*
Dose	Start of dosage			Necropsy
(μ g/kg)	20-day	21-day	22-day	23-day
Control	46.9 ± 3.1	48.4 ± 3.4	52.8 ± 3.1	57.8 ± 3.4
0.02	46.7 ± 2.3	48.4 ± 2.6	53.2 ± 2.6	58.0 ± 2.8
0.2	46.8 ± 2.8	47.8 ± 3.3	52.6 ± 2.9	57.3 ± 3.2
2	46.8 ± 4.0	47.7 ± 4.4	52.3 ± 4.5	57.1 ± 4.8
20	46.8 ± 2.4	46.7 ± 3.5	51.0 ± 3.4	55.8 ± 3.1
200	46.7 ± 2.3	45.9 ± 2.8	48.9 ± 2.9	52.8 ± 2.6**
Dose	Start of dosage			Necropsy
(μ g/kg)	21-day	22-day	23-day	24-day
Control	45.0 ± 4.2	46.5 ± 4.3	50.4 ± 4.6	54.9 ± 4.9
0.02	44.9 ± 3.6	46.9 ± 4.5	50.7 ± 5.3	54.9 ± 5.3
0.2	44.5 ± 3.0	45.4 ± 3.7	49.5 ± 3.6	54.4 ± 3.6
2	44.6 ± 3.1	43.8 ± 3.1	48.1 ± 3.1	53.0 ± 3.5
20	44.6 ± 3.3	42.6 ± 4.5	45.4 ± 5.4	47.8 ± 7.7**
200	44.4 ± 2.9	45.3 ± 3.3	48.2 ± 3.1	51.6 ± 3.1
Dose	Start of dosage			Necropsy
(μ g/kg)	22-day	23-day	24-day	25-day
Control	49.4 ± 4.5	52.4 ± 5.4	56.4 ± 5.6	60.6 ± 5.5
0.02	49.6 ± 4.2	52.6 ± 5.1	55.9 ± 5.0	60.3 ± 5.2
0.2	49.8 ± 4.6	53.0 ± 4.9	56.4 ± 5.8	60.6 ± 5.5
2	49.4 ± 5.2	52.5 ± 6.8	56.0 ± 7.0	60.9 ± 7.4
20	49.1 ± 5.6	51.4 ± 6.4	54.9 ± 6.7	58.7 ± 6.7
200	49.5 ± 4.4	50.7 ± 4.4	53.6 ± 4.2	56.9 ± 3.8

Data represent mean±SD. Significantly different from each control group, ** : $p<0.01$, * : $p<0.05$

Table 3. Aging and Weaning Effects of 17-Ethinyl Estradiol Administration on Uterus Weight (mg)

Dose	Non-weaned rats		Weaned rats	
(μ g/kg)	Wet wt.	Blotted wt.	Wet wt.	Blotted wt.
18 days old at the start of dosage				
Control	42 ± 13	39 ± 12	26 ± 2	23 ± 3
0.02	38 ± 10	35 ± 10	29 ± 4	26 ± 2
0.2	42 ± 16	37 ± 13	30 ± 5 *	27 ± 5 *
2	109 ± 22**	91 ± 15**	89 ± 26**	76 ± 14**
20	165 ± 43**	104 ± 13**	138 ± 23**	101 ± 11**
200	209 ± 58**	107 ± 13**	195 ± 66**	105 ± 15**
19 days old at the start of dosage				
Control	33 ± 5	30 ± 5	29 ± 3	27 ± 3
0.02	31 ± 6	29 ± 6	29 ± 3	26 ± 3
0.2	37 ± 6	34 ± 6	31 ± 5	29 ± 5
2	105 ± 24**	88 ± 12**	102 ± 13**	89 ± 10**
20	162 ± 39**	106 ± 10**	166 ± 58**	114 ± 17**
200	215 ± 62**	104 ± 8 **	223 ± 30**	109 ± 7 **
20 days old at the start of dosage				
Control	39 ± 6	37 ± 6	32 ± 3	29 ± 3
0.02	40 ± 7	37 ± 6	32 ± 4	29 ± 4
0.2	46 ± 24	42 ± 19	32 ± 4	29 ± 4
2	114 ± 23**	96 ± 14**	100 ± 36**	87 ± 23**
20	233 ± 58**	133 ± 15**	206 ± 50**	127 ± 15**
200	243 ± 58**	125 ± 15**	222 ± 59**	125 ± 10**
21 days old at the start of dosage				
Control	40 ± 14	37 ± 13	30 ± 5	28 ± 4
0.02	42 ± 16	38 ± 14	31 ± 6	28 ± 5
0.2	43 ± 8	37 ± 7	35 ± 7	32 ± 7
2	119 ± 46**	97 ± 24**	83 ± 12**	78 ± 11**
20	225 ± 42**	141 ± 18**	192 ± 52**	114 ± 18**
200	200 ± 50**	134 ± 16**	254 ± 44**	122 ± 11**
22 days old at the start of dosage				
Control	41 ± 10	38 ± 9	36 ± 15	33 ± 15
0.02	42 ± 9	38 ± 7	37 ± 6	33 ± 5
0.2	46 ± 11	43 ± 11	40 ± 5 **	37 ± 5 *
2	76 ± 9 **	71 ± 9 **	105 ± 48**	84 ± 22**
20	264 ± 31**	147 ± 19**	214 ± 54**	129 ± 14**
200	253 ± 73**	142 ± 17**	277 ± 58**	135 ± 12**

Data represent mean±SD. Significantly different from each control group, ** : $p<0.01$, * : $p<0.05$

Table 4. Aging and Weaning Effects of 17-Ethinyl Estradiol Administration on Ovary Weight (mg)

Dose (μ g/kg)	Ages at start of dosage				
	18 days old	19 days old	20 days old	21 days old	22 days old
Non-weaned rats					
Control	10 ± 2	12 ± 3	16 ± 4	15 ± 3	19 ± 3
0.02	9 ± 2	13 ± 3	15 ± 2	16 ± 4	20 ± 2
0.2	8 ± 2	10 ± 2	14 ± 2	14 ± 5	21 ± 4
2	8 ± 1	9 ± 3	12 ± 2	14 ± 3	19 ± 4
20	8 ± 2	12 ± 2	12 ± 4	14 ± 3	20 ± 3
200	9 ± 2	12 ± 3	13 ± 3	13 ± 3	19 ± 3
Weaned rats					
Control	12 ± 3	13 ± 3	16 ± 3	16 ± 4	18 ± 4
0.02	11 ± 4	13 ± 2	17 ± 2	16 ± 3	19 ± 4
0.2	10 ± 2	10 ± 2	15 ± 1	15 ± 3	16 ± 4
2	8 ± 3	11 ± 2	13 ± 3	12 ± 3	16 ± 3
20	9 ± 2	12 ± 4	14 ± 2	13 ± 2	14 ± 4
200	10 ± 4	11 ± 3	14 ± 3	13 ± 3	17 ± 3

Data represent mean ± SD.

Comparison of Reproductive and Developmental Toxicity Parameters of Crj:CD (SD) IGS rats Among 3 Different Breeding Centers (1)
- Data regarding reproductive function and embryo-fetal development -

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ABSTRACT. In order to obtain background data for reproductive and developmental toxicity studies using Crj:CD (SD) IGS rats and to know differences in data from studies due to the difference of breeding centers, we compared data such as body weight and food consumption of males and females, organ weight (testes, epididymides, seminal vesicle and prostate), estrous cycle, reproductive performance, the numbers of corpora lutea, implantations, live fetuses and embryo-fetal deaths, body weight of live fetuses, sex and external, visceral and skeletal abnormalities and variations.
There were no differences in data regarding reproductive function or embryo-fetal development in the rats from 3 different breeding centers, except that body weight and food consumption in rats from Hino breeding center tended to be low in comparison with those from Atsugi and Tsukuba breeding centers. — Key words: Crj:CD (SD) IGS rats, Embryo-fetal development, Reproductive parameters

CD(SD)IGS-2001: 98-104

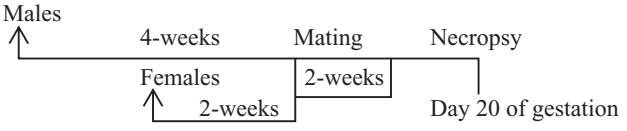
INTRODUCTION

We examined the differences in Crj:CD (SD) IGS rats from the Atsugi breeding center, Tsukuba breeding center and Hino breeding center regarding reproductive and developmental toxicity parameters such as reproductive function of males and females and embryo-fetal development using rats from Atsugi breeding center, Tsukuba breeding center and Hino breeding center.

MATERIALS AND METHODS

Twenty male (7 weeks of age) and 20 female (6 weeks of age) Crj:CD (SD) IGS rats each were obtained from Atsugi breeding center, Tsukuba breeding center and Hino breeding center of Charles River Inc. The animals were housed in an animal room which was maintained at a temperature of 23±3℃ and a relative humidity of 50±20%, air ventilation at 10-15 times per hour, and 12-hour light cycle. The animals were housed individually in wire mesh cages except for the mating period. Pellet diet (NMF: Oriental Yeast, Co., Ltd.) and tap water were provided *ad libitum*.

Experimental design was as follows:



Males were weighed twice a week and on the day of necropsy. Females were weighed twice a week prior to mating and during the mating period and on days 0, 4, 6, 8, 10, 12, 16, 18 and 20 of gestation. Food consumption was determined on the day of body weight measurement except for day 2 of pre-mating period and day 1 of gestation. The estrous cycles of all females were examined daily by the vaginal smear for 2 weeks prior to mating until successful mating. Females were paired with males on a one-to-one basis at approximately 12 weeks of age for a maximum of 2 weeks. The day on which the presence of vaginal plugs or sperm in the vaginal smear was observed was designated as day 0 of gestation. The indices of copulation and fertility were determined. Males were sacrificed by exsanguination under ether anesthesia after completion of the mating period. The testes, epididymides, prostate and seminal vesicle were weighed. The right epididymis was used for the sperm examination in which total numbers of sperm and numbers of immotile sperm, live and dead sperm, and sperm with abnormalities and the motility, survival index and the incidence of sperm with abnormalities were determined.

Females were sacrificed by exsanguination under ether anesthesia on day 20 of gestation. For pregnant animals, their ovaries and uterus were removed, and the numbers of the corpora lutea, implantations, the numbers of live fetuses and resorbed or dead fetuses were counted and the indices of implantation and embryo-fetal death were determined. The live fetuses were examined for external abnormalities including the oral cavity. Their body weights were measured after sex determination. Approximately one half of the fetuses in each litter were fixed in Bouin's solution and examined for visceral abnormalities [1, 2]. Of the remaining half of the fetuses in each litter, clear skeletal specimens stained with Alizarin-red S were prepared and examined for skeletal abnormalities, variations and progress of ossification [3].

For statistical analyses, parametric data such as body weight were analyzed by Student's t-test or Aspin-Welch's t-test. Non-parametric data such as the indices regarding dams and fetuses were analyzed by the Wilcoxon rank sum method except that data such as copulation, fertility and sex ratio were analyzedby the Chi-square test. Statistical analyses were made between data from Atsugi breeding center and data from Tsukuba breeding center or Hino breeding center at two-tailed 5 and 1% levels of significance.

RESULTS AND DISCUSSION

1. Males

Body weight is shown in Table 1. Males from Hino breeding center showed low body weight change and significant differences from males of Atsugi breeding center were noted on days 1, 15, 18, 22, 25 and 29. Males from Tsukuba breeding center showed body weight similar to those from Atsugi breeding center and no

significant differences from males of Atsugi breeding center were noted. No significant differences from males of Atsugi breeding center were noted in body weight gain from day 1 to day 81 in males from Tsukuba breeding center or Hino breeding center. Therefore, it was concluded that there were no differences in body weight gain among the 3 breeding centers.

Food consumption is shown in Table 2. Although food consumption on day 18 of males from Tsukuba breeding center showed significantly high values in comparison with males from Atsugi breeding center, it was concluded that there were no differences in food consumption among the 3 breeding centers.

No macroscopic abnormalities in the major organs and tissues of the thoracic or abdominal cavities were observed except that unilateral softening of the testis was observed in 1 out of 20 animals from Atsugi breeding center. Organ weights and the results of sperm examination are shown in Tables 3 and 4. No significant differences from the animals of Atsugi breeding center were noted in the testes, epididymides, prostate or seminal vesicle weights in the animals from Tsukuba or Hino breeding center. In the results of the sperm examination, there were no significant differences between the animals from Atsugi breeding center and animals from Tsukuba or Hino breeding center on the number of sperm, mortality of sperm, survival index or incidence of sperm with abnormalities. Therefore, it was concluded that there were no differences in organ weight or the result of the sperm examination among the 3 breeding centers.

2. Females

Body weight is shown in Table 5. Females from Hino breeding center showed a tendency towards low body weight in the pre-mating period and gestation period and significant differences from Atsugi breeding center were noted in body weight on and after day 4 of gestation and body weight gains from day 0 to day 6 of gestation and from day 0 to day 20 of gestation. Females from Tsukuba breeding center showed body weight similar to those from Atsugi breeding center and no significant differences from females of Atsugi breeding center were noted in body weight or body weight gain. Food consumption is shown in Table 6. Food consumption in the pre-mating period showed values similar in the animals from each breeding center. Food consumption during the gestation period showed low values in the animals from Hino breeding center and significant differences from Atsugi breeding center were noted on days 4, 6, 10, 12 and 14 of gestation. On the other hand, food consumption of the animals from Tsukuba breeding center showed values similar to those from Atsugi breeding center.

Estrous cycle, the results of mating and fertility are shown in Table 7. Estrous cycle and indices of copulation or fertility showed similar values among the 3 breeding centers. Therefore, it was concluded that there were no differences in these parameters among the 3 breeding centers.

No macroscopic abnormalities in the major organs and tissues of the thoracic or abdominal cavities were observed in any dam from the 3 breeding centers. Findings at cesarean section are shown in Table 8. The numbers of corpora lutea, implantations and live fetuses, implantation index and index of embryo-fetal death showed similar values among the 3 breeding centers. In parameters obtained from fetuses, no significant differences from Atsugi breeding center were noted in sex ratio or body weights of the live fetuses in Tsukuba or Hino breeding center. No fetuses with external abnormalities were observed in any fetus from dams of the 3 different breeding centers. Findings obtained from visceral and skeletal examinations of live fetuses are shown in Tables 9 and 10. Visceral abnormalities or variations were observed in a few fetuses from each breeding center. No fetuses with skeletal abnormalities were noted in the fetuses from any breeding center. Skeletal variations were observed in a few fetuses from each breeding center. Therefore, it was concluded that there were no differences in the incidences of visceral and skeletal abnormalities or variations among the 3 breeding centers. Regarding ossification, no big differences were noted in any parameters among the 3 breeding centers and it was concluded that there were no differences in the progress of the ossification among the 3 breeding centers.

From the above results, there were no differences in data regarding reproductive function or embryo-fetal development in the rats from the 3 different breeding centers, except that body weight and food consumption in rats from Hino breeding center tended to be low in comparison with those from Atsugi and Tsukuba breeding centers. However, we need to obtain more of these kinds of data in order to conduct reproduction studies using the rats because the sample size of the rats was too small in this experiment.

ACKOWLEDGMENTS

I thank the members of the reproduction section in the Gotemba Laboratory of Bozo Research Center Inc.

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

Breeding Center			Atsugi	Tsukuba	Hino
No. of animals			20	20	20
	Days	1	346.7 ± 18.7	354.0 ± 14.0	334.1* ± 15.4
		4	362.4 ± 21.0	369.8 ± 16.9	350.0 ± 16.9
		8	383.5 ± 23.4	390.3 ± 21.0	370.7 ± 19.8
		11	401.2 ± 26.3	405.1 ± 25.4	384.8 ± 22.7
		15	418.6 ± 28.6	419.2 ± 27.8	398.5* ± 24.5
		18	433.1 ± 30.1	432.8 ± 29.5	411.4* ± 27.1
		22	449.8 ± 34.4	446.6 ± 30.9	427.2* ± 26.0
		25	460.2 ± 35.0	457.3 ± 29.2	435.1* ± 27.5
		29	472.6 ± 37.8	468.9 ± 31.3	448.2* ± 29.2
		32	473.0 ± 40.1	468.1 ± 29.6	449.6 ± 29.1
		36	487.5 ± 42.9	482.8 ± 30.8	464.4 ± 28.9
		39	496.4 ± 44.4	494.2 ± 32.7	474.6 ± 30.5
		43	512.0 ± 47.1	506.4 ± 33.6	487.0 ± 32.5
		46	520.8 ± 46.8	514.2 ± 35.9	495.5 ± 33.2
		50	530.9 ± 46.7	523.7 ± 35.8	506.6 ± 36.4
		53	536.0 ± 46.8	525.8 ± 38.1	511.4 ± 36.7
		57	541.5 ± 48.1	533.1 ± 39.0	517.6 ± 38.0
		60	549.3 ± 50.5	540.2 ± 38.7	525.5 ± 39.0
		64	555.8 ± 50.0	545.4 ± 40.7	531.7 ± 40.4
		67	561.9 ± 50.9	550.2 ± 40.6	536.6 ± 40.5
		71	568.5 ± 52.2	557.0 ± 41.9	542.0 ± 41.9
		74	574.0 ± 55.3	562.1 ± 41.7	547.7 ± 43.8
		78	581.1 ± 56.8	568.3 ± 41.6	555.3 ± 44.3
		81	587.6 ± 56.9	576.8 ± 44.6	561.6 ± 43.8
	Gain	1-81	240.9 ± 41.5	222.8 ± 37.1	227.6 ± 33.2

Unit: g, Mean ± S.D.
*: $p<0.05$ (Significant difference from Atsugi Breeging Center)

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

Breeding Center			Atsugi	Tsukuba	Hino
No. of animals			20	20	20
	Days	2	28.0 ± 2.6	28.1 ± 3.2	27.0 ± 2.7
		4	29.8 ± 3.7	29.9 ± 2.5	29.2 ± 2.6
		8	29.9 ± 3.1	29.5 ± 2.9	29.6 ± 2.5
		11	30.1 ± 3.4	30.3 ± 3.5	29.8 ± 2.9
		15	31.1 ± 3.5	29.7 ± 2.8	29.8 ± 3.2
		18	29.0 ± 3.2	31.6* ± 3.7	29.3 ± 3.4
		22	30.9 ± 3.6	30.3 ± 2.4	30.2 ± 3.0
		25	29.4 ± 3.0	29.8 ± 2.8	28.7 ± 2.6
		29	29.4 ± 3.4	29.9 ± 2.6	29.7 ± 2.5

Unit: g / rat / day, Mean ± S.D.
*: $p<0.05$ (Significant difference from Atsugi Breeging Center)

Table 3. Organ weight of Crj:CD(SD)IGS male rats

Breeding Center		Atsugi	Tsukuba	Hino
No. of males		20	20	20
Body weight	(g)	588.0 ± 57.0	576.0 ± 46.0	562.0 ± 44.0
Testes	(g)	3.65 ± 0.31	3.55 ± 0.16	3.62 ± 0.30
	(g/100g B.W.)	0.63 ± 0.08	0.62 ± 0.05	0.65 ± 0.06
Epididymides	(mg)	1408 ± 111	1356 ± 108	1346 ± 84.0
	(mg/100g B.W.)	242 ± 35	237 ± 24	241 ± 19
Prostate	(g)	1.53 ± 0.21	1.39 ± 0.15	1.46 ± 0.23
	(g/100g B.W.)	0.26 ± 0.04	0.24 ± 0.03	0.26 ± 0.05
Seminal vesicle (g)		1.68 ± 0.23	1.57 ± 0.20	1.51* ± 0.17
	(g/100g B.W.)	0.29 ± 0.05	0.27 ± 0.04	0.27 ± 0.03

Mean ± S.D.
*: $p<0.05$ (Significant difference from Atsugi Breeging Center)

Table 4. Sperm examination of Crj:CD(SD)IGS male rats

Breeding Center	Atsugi	Tsukuba	Hino
No. of males	20	20	20
No. of sperm (Mean ± S.D.) [1]	491.1 ± 77.2	475.1 ± 55.6	488.1 ± 68.2
Motility (%, Mean ± S.D.) [2]	74.9 ± 4.6	73.2 ± 5.0	75.3 ± 9.0
No. of sperm (Mean ± S.D.)	154 ± 32	143 ± 19	147 ± 20
No. of immotile sperm (Mean ± S.D.)	38 ± 6	38 ± 5	35 ± 7
Survival Index (%, Mean ± S.D.) [3]	81.9 ± 16.4	85.8 ± 5.8	85.1 ± 4.9
No. of counts	9560	10000	10000
No. of live sperm	8111	8581	8514
No. of dead sperm	1449	1419	1486
Abnormal sperms (%, Mean ± S.D.) [4]	1.1 ± 4.0	0.5 ± 0.5	0.6 ± 0.8
No. of sperm	3878	4000	4000
No. of sperm with abnormalities	24	19	22
Normal	3854	3981	3978
No hook	1	0	1
Amorphous	23	19	21

1) No. of sperm ($\times 10^6$/g caudal epididymis)
2) (No. of sperm - No. of immotile sperm / No. of sperm) × 100
3) (No. of sperm - No. of stained sperm / No. of sperm) × 100
4) (No. of abnormal sperm / No. of sperm) × 100

Table 5. Body weight of Crj:CD(SD)IGS female rats during the per-mating and gestation periods

Breeding Center			Atsugi	Tsukuba	Hino
Pre-mating period					
No. of animals			20	20	20
	Days	1	239.3 ± 21.6	231.6 ± 16.9	226.6 ± 14.9
		4	237.7 ± 21.5	232.8 ± 17.5	226.6 ± 13.8
		8	244.9 ± 21.0	238.8 ± 17.7	234.6 ± 14.4
		11	250.1 ± 23.4	241.5 ± 19.2	239.8 ± 17.9
		15	256.0 ± 23.1	246.9 ± 18.3	243.9 ± 18.3
	Gain	1-15	16.7 ± 7.4	15.3 ± 6.6	17.3 ± 8.9
Gestation period					
No. of dams			18	18	20
Days of gestation		0	262.5 ± 26.3	257.2 ± 15.3	249.9 ± 16.1
		4	287.8 ± 26.7	279.6 ± 16.5	270.1** ± 18.0
		6	295.4 ± 26.5	288.3 ± 16.9	275.6** ± 17.9
		8	305.7 ± 26.8	297.2 ± 17.5	283.9** ± 18.0
		10	314.6 ± 26.1	306.6 ± 17.8	293.3** ± 20.2
		12	325.8 ± 28.3	316.0 ± 19.1	302.4** ± 19.0
		14	335.3 ± 28.7	325.0 ± 18.0	312.0** ± 19.0
		16	350.6 ± 28.7	342.7 ± 18.3	327.7** ± 20.1
		18	379.8 ± 31.5	371.6 ± 20.0	355.9** ± 21.6
		20	413.0 ± 35.5	404.7 ± 21.8	389.0* ± 23.4
	Gains	0-6	32.9 ± 6.1	31.1 ± 5.8	25.7** ± 5.5
		6-18	84.3 ± 10.1	83.3 ± 6.2	80.3 ± 7.5
		18-20	33.2 ± 6.9	33.1 ± 5.7	33.2 ± 4.1
		0-20	150.5 ± 17.1	147.5 ± 12.3	139.1* ± 10.9

Unit: g, Mean±S.D.
*: $p<0.05$, **: $p<0.01$ (Significant difference from Atsugi Breeging Center)

Table 6. Food consumption of Crj:CD(SD)IGS female rats during the pre-mating and gestation periods

Breeding Center			Atsugi	Tsukuba	Hino
Pre-mating period					
No. of animals			20	20	20
	Days	2	17.6 ± 4.1	17.4 ± 4.4	16.2 ± 4.0
		4	21.2 ± 5.2	21.6 ± 3.5	20.0 ± 3.2
		8	20.0 ± 3.4	21.2 ± 3.1	20.6 ± 2.4
		11	20.0 ± 2.8	20.8 ± 3.1	20.0 ± 3.3
		15	21.1 ± 3.0	21.1 ± 2.9	20.5 ± 2.9
Gestation period					
No. of Dams			18	18	20
Days of gestation		1	21.9 ± 2.6	21.0 ± 3.1	20.2 ± 2.6
		4	25.4 ± 2.6	24.8 ± 3.0	22.9** ± 2.6
		6	26.1 ± 2.8	25.9 ± 3.2	23.9* ± 2.2
		8	26.8 ± 3.4	25.9 ± 2.8	25.1 ± 2.8
		10	27.1 ± 2.8	25.3 ± 3.0	24.6* ± 2.3
		12	27.5 ± 3.2	26.2 ± 3.2	25.4* ± 2.3
		14	26.7 ± 2.2	25.4 ± 2.6	24.9* ± 2.1
		16	27.9 ± 2.2	27.4 ± 2.5	26.2 ± 3.3
		18	28.7 ± 3.0	27.8 ± 2.7	27.5 ± 2.4
		20	28.4 ± 2.8	27.0 ± 2.7	26.9 ± 2.2

Unit: g / rat / day, Mean±S.D.
*: $p<0.05$, **: $p<0.01$ (Significant difference from Atsugi Breeging Center)

Table 7. Reproductive function in Crj:CD(SD)IGS male and female rats

Breeding Center	Atsugi	Tsukuba	Hino
Estrous cycle (days, Mean±S.D.)	4.3±0.7	4.1±0.2	4.1±0.2
Copulation and fertility data			
No. of pairs	20	20	20
No. copulated	20	18	20
No. of pregnant females	18	18	20
Copulation Index (%) [1)]	100.0	90.0	100.0
Fertility Index (%) [2)]	90.0	100.0	100.0

1) (No. of animals with successful copulation / No. of animals mated)×100
2) (No. of pregnant animals / No. of females with successful copulation)×100

Table 8. Findings at cesarean section in Crj:CD(SD)IGS dams

Breeding Center		Atsugi	Tsukuba	Hino
No. of dams		18	18	20
Data from dams				
No. of corpora lutea		288	289	316
	(Mean±S.D.)	16.0 ± 1.7	16.1 ± 1.4	15.8 ± 2.0
No. of implantions		279	281	306
	(Mean±S.D.)	15.5 ± 1.4	15.6 ± 1.4	15.3 ± 1.8
Implantation index				
	(%, Mean±S.D.) [1)]	97.1 ± 4.1	97.3 ± 4.3	96.9 ± 4.1
No. of live fetuses		255	266	296
	(Mean±S.D.)	14.2 ± 1.8	14.8 ± 1.4	14.8 ± 1.8
Males		132	120	140
	(Mean±S.D.)	7.3 ± 2.6	6.7 ± 1.9	7.0 ± 2.3
Females		123	146	156
	(Mean±S.D.)	6.8 ± 1.9	8.1 ± 2.2	7.8 ± 2.4
No. of embryo-fetal deaths		24	15	10
	(%, Mean±S.D.) [2)]	8.4 ± 10.0	5.2 ± 5.8	3.1 ± 5.5
Early		24	15	9
Late		0	0	1
No. of live fetuses examined		255	266	296
Sex ratio (Male/Female)		1.07	0.82	0.90
Body weight of live fetuses	(g)			
Males	(Mean±S.D.)	4.10 ± 0.30	4.03 ± 0.21	4.04 ± 0.25
Females	(Mean±S.D.)	3.86 ± 0.27	3.82 ± 0.19	3.83 ± 0.19
No. of live fetuses with external		0	0	0
abnormalities	(%, Mean±S.D.) [3)]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

1) (No. of implantations / No. of corpora lutea)×100
2) (No. of resorptions and dead fetuses / No. of implants)×100
3) (No. of fetuses with external abnormalities / No. of live fetuses examined)×100

Table 9. Visceral examination of live fetuses from Crj:CD(SD)IGS dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of dams	18	18	20
No. of fetuses examined	123	128	140
No. of fetuses with visceral abnormalities	1	4	0
(%, Mea n±S.D.) [1]	0.9 ± 3.9	2.8 ± 11.8	0.0 ± 0.0
Dilation of renal pelvis and ureter	1	4	0
(%, Mean±S.D.)	0.9 ± 3.9	2.8 ± 11.8	0.0 ± 0.0
No. of fetuses with visceral variations	2	1	1
(%, Mean±S.D.) [2]	1.5 ± 4.3	0.9 ± 3.9	0.7 ± 3.2
Thymic remnant in the neck	2	1	0
(%, Mean±S.D.)	1.5 ± 4.3	0.9 ± 3.9	0.0 ± 0.0
Left umbilical artery	0	0	1
(%, Mean±S.D.)	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 3.2

1) (No. of fetuses with visceral abnormalities / No. of fetuses examined)×100
2) (No. of fetuses with visceral variations / No. of fetuses examined)×100

Table 10. Skeletal abnormalities and variations of live fetuses from Crj:CD(SD)IGS dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of dams	18	18	20
No. of fetuses examined	132	137	156
No. of fetuses with abnormalities	0	0	0
(%, Mean±S.D.) [1]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
No. of live fetuses with variations	19	25	16
(%, Mean±S.D.) [2]	14.6 ± 17.8	18.9 ± 29.1	10.4 ± 18.6
Cervical rib	1	1	0
(%, Mean±S.D.)	0.8 ± 3.4	0.7 ± 2.9	0.0 ± 0.0
Wavy rib	1	0	0
(%, Mean±S.D.)	0.6 ± 2.6	0.0 ± 0.0	0.0 ± 0.0
14th rib	17	24	14
(%, Mean±S.D.)	13.2 ± 18.4	18.2 ± 29.4	9.0 ± 18.8
Splitting of thoracic vertebral body	0	1	2
(%, Mean±S.D.)	0.0 ± 0.0	0.7 ± 2.9	1.3 ± 4.1

1) (No. of fetuses with skeletal abnormalities / No. of fetuses examined)×100
2) (No. of fetuses with skeletal variations / No. of fetuses examined)×100

Comparison of Reproductive and Developmental Toxicity Parameters of Crj:CD (SD) IGS Rats Among 3 Different Breeding Centers (2)
- Data Regarding Maternal Function and Prenatal and Postnatal Development of F_1 Rats -

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ABSTRACT. In order to obtain background data for reproductive and developmental toxicity studies of Crj:CD (SD) IGS rats and to know differences in data from the studies due to the difference of breeding centers, we compared data such as body weight and food consumption of dams, delivery index, gestation period, the numbers of implantations, stillborn index, the numbers of liveborn pups, live birth index, sex ratio, external abnormalities of liveborn pups and their viability, body weight and external differentiation after birth, reflex function, behavior (open field test and water-filled multiple maze test) and reproductive function.

There were no differences in data regarding maternal function and prenatal and postnatal development of F_1 rats in the rats from 3 different breeding centers, except that body weight of F_1 rats of Hino breeding center after weaning tended to be low in comparison with those from Atsugi and Tsukuba breeding centers. — Key words: Crj:CD (SD) IGS rats, Maternal function, Prenatal and postnatal development

CD(SD)IGS-2001: 105-110

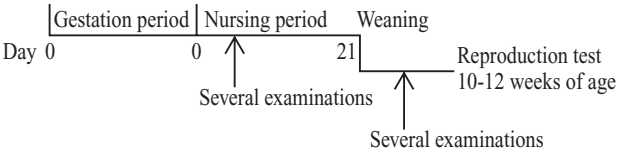
INTRODUCTION

We examined the differences in Crj:CD (SD) IGS rats from the Atsugi breeding center, Tsukuba breeding center and Hino breeding center regarding reproductive and developmental toxicity parameters such as maternal functions and prenatal and postnatal development of F_1 rats using rats from Atsugi breeding center, Tsukuba breeding center and Hino breeding center.

MATERIALS AND METHODS

Thirty male (11 weeks of age) and 30 female (10 weeks of age) Crj:CD (SD) IGS rats each were obtained from each of Atsugi breeding center, Tsukuba breeding center and Hino breeding center of Charles River Inc. The animals were housed in an animal room which was maintained at a temperature of 23±3℃ and a relative humidity of 50±20%, air ventilation at 10-15 times per hour, and 12-hour light cycle. The animals were housed individually in wire mesh cages except for the mating period. The females on and after day 17 of gestation were housed in plastic Econ cages with bedding until weaning and weanlings were housed 2 animals of the same sex per litter in the same wire mesh cages. Pellet diet (NMF: Oriental Yeast, Co., Ltd.) and tap water were provided *ad libitum*.

Experimental design was as follows:



Females were weighed on days 0, 4, 7-17 and 20 of gestation, and on days 0, 4, 7, 11, 14, 17 and 21 of lactation. Food consumption was determined on days 1, 4, 8, 11, 14, 17, 19 and 20 of gestation, and on days 2, 4, 7, 11, 14, 17 and 21 of lactation. Females were allowed to deliver. They were observed for the presence or absence of abnormalities in delivery, and the duration of the gestation and the delivery index were determined. They were allowed to nurse their pups for 21 days after delivery and their nursing behavior was observed daily. They were necropsied on day 22 of lactation and the number of implantation sites was counted.

On the day of birth, the numbers of liveborn and stillborn pups were counted and liveborn pups were examined for sex and the presence or absence of external abnormalities. Body weight of nurslings was measured on days 0, 4, 7, 11, 14, 17 and 21 after birth. On day 4 after birth, the nurslings were culled to 8 pups (4 males and 4 females, as a rule). They were examined for physical development: pinna detachment on day 4 after birth, abdominal hair growth and eruption of the lower incisors on days 11 and 14 after birth and opening of the eyelids on days 14 and 17 after birth. For the ontogeny of the sensory and reflex functions during the nursing period, 2 males and 2 females in each litter were examined for surface righting reflex on day 10 after birth, air righting reflex on day 15 after birth and pupillary, Preyer's and pain reflexes on day 21 after birth. The stillborn index, birth index, survival index on days 4 and 21 after birth were determined.

On day 21 after birth, 2 males and 2 females from each litter were selected for various examinations after weaning and the remainders were necropsied. After weaning, an open field test and a multiple water T-maze test were conducted on 1 male and 1 female from each litter at 5 weeks of age and at 7-8 weeks of age, respectively. For the open field test, a Behavioral Tracing Analyzer (BTA-2A: Muromachi Kikai Co., Ltd.) was used and latency, the amount of ambulation and the numbers of rearing, grooming and defecation were recorded for 3 minutes once a day. For the water T-maze test, the animals were examined using Biel's water maze in 3 trials a day and the time taken to the goal and the number of errors were recorded. The reproductive performance of the weanlings was examined in 1 male and 1 female from each litter at 10-12 weeks of age. Males and females were housed together overnight on a one-to-one basis avoiding sibling mating. All females with confirmed copulation were weighed on days 0, 4, 7, 11 and 14 of gestation and necropsied by exsanguination under ether anesthesia on day 14 of gestation. For pregnant animals, the numbers of the corpora lutea, implantations, the numbers of live or dead embryos were counted. From the results of the reproductive performance, the

indices of copulation, fertility, implantation and dead embryos were determined.

For statistical analyses, parametric data such as body weight were analyzed by Student's t-test or Aspin-Welch's t-test. Non-parametric data such as the indices regarding dams and F_1 generation were analyzed by the Wilcoxon rank sum method except that data such as copulation, fertility and sex ratio were analyzed by the Chi-square test. Statistical analyses were made between data from Atsugi breeding center and data from Tsukuba breeding center or Hino breeding center at two-tailed 5 and 1% levels of significance.

RESULTS AND DISCUSSION

1. Dams

Body weight is shown in Table 1. Body weight of dams from Hino breeding center showed low values throughout the gestation and lactation periods and significant differences from Atsugi breeding center were noted except for day 17 of lactation. Dams from Tsukuba breeding center showed values similar to those from Atsugi breeding center and no significant differences from dams of Atsugi breeding center were noted. Dams from Hino breeding center showed low body weight gain during the gestation period in comparison with dams from Atsugi breeding center. However, body weight gain in these dams showed the same values as those in dams from Atsugi and Tsukuba breeding center. Therefore, it was concluded that there were no differences in body weight during the lactation period among the 3 breeding centers. Food consumption is shown in Table 2. Food consumption showed the similar values in dams from each breeding center except for a significantly high value on day 8 of gestation in dams from Tsukuba breeding center and a significantly low value on day 21 of lactation in dams from Hino breeding center. Therefore, it was concluded that there were no differences in food consumption among the 3 breeding centers.

Delivery data on dams and examination of F_1 pups are shown in Table 3. No significant differences from Atsugi breeding center were noted in delivery index, gestation period, the numbers of implantations, liveborns pups and stillborn pups, incidence of stillborn pups or live birth index in Tsukuba or Hino breeding center. Therefore, it was concluded that there were no differences in the delivery data among the 3 breeding centers. No macroscopic abnormalities in the major organs and tissues of the thoracic or abdominal cavities were observed in any dam from the 3 breeding centers.

2. F_1 animals

The results of observation at birth and viability of F_1 pups during the lactation period are shown in Tables 4 and 5. The sex ratio and body weight of F_1 pups at birth showed similar values for each breeding center and no significant differences from Atsugi breeding center were noted in these parameters in Tsukuba or Hino breeding center. Viability index on day 4 after birth and weaning index on day 21 after birth showed similar values for each breeding center. Therefore, it was concluded that there were no differences in data of the observation at birth or viability of the F_1 pups during the lactation period among the 3 breeding centers. Body weight of F_1 rats during the lactation period and the period after weaning is shown in Tables 6-1 and 6-2. Body weight of males and females from each breeding center showed similar values during the lactation period and no significant differences from Atsugi breeding center were noted in Tsukuba or Hino breeding center. Body weight after weaning (on and after day 28 after birth) tended to be low in males and females of Hino breeding center and significant differences from Atsugi breeding center were noted from days 42 to 70 after birth in males and on days 28, 42 and 49 after birth in females. On the other hand, males and females of Tsukuba breeding center showed values similar to F_1 animals of Atsugi breeding center. Therefore, although body weight at birth and during the lactation period showed similar values in F_1 pups of each breeding center, body weight of F_1 rats of Hino breeding center after weaning showed low vales. No differences from F_1 rats of Atsugi breeding center were noted in the results of the physical development, reflex responses, open field test or water-filled multiple T-maze test in F_1 rats of Tsukuba or Hino breeding center. The results of mating and fertility in F_1 rats are shown in Table 7. The copulation index was 94.7 to 100% in F_1 rats of each breeding center and the fertility index in F_1 rats of each breeding center were 100%. Therefore, it was concluded that there were no differences in the copulation index or fertility index among the 3 breeding centers. Reproductive and embryo parameters in F_1 rats are shown in Table 8. There were no significant differences among the 3 breeding centers in the numbers of corpora lutea, implantations, dead embryos or live embryos or implantation index, and it was concluded that there were no differences in these parameters among the 3 breeding centers.

From the above results, there were no differences in data regarding maternal function or prenatal and postnatal development of F_1 rats in the rats from the 3 different breeding centers, except that body weight of F_1 rats of Hino breeding center after weaning tended to be low in comparison with those from Atsugi and Tsukuba breeding centers. However, we need to obtain more of these kinds of data in order to conduct reproduction studies using the rats because the sample size of the rats was too small in this experiment.

ACKOWLEDGMENTS

I thank the members of the reproduction section in the Gotemba Laboratory of Bozo Research Center Inc.

Table 1. Body weight of Crj:CD(SD)IGS dams during the gestation period

Breeding Center			Atsugi	Tsukuba	Hino
No. of dams			20	20	20
Days of gestation					
		0	266.8 ± 10.3	276.1* ± 10.1	257.5* ± 10.2
		4	293.6 ± 12.8	301.1 ± 10.6	279.5** ± 10.6
		7	307.4 ± 15.5	315.7 ± 12.1	291.7** ± 11.5
		8	310.4 ± 14.5	320.2* ± 11.3	296.2** ± 11.6
		9	314.7 ± 16.0	323.2 ± 11.9	300.5** ± 12.2
		10	319.7 ± 16.4	329.3 ± 11.2	303.4** ± 12.4
		11	325.8 ± 16.5	335.5 ± 11.3	309.3** ± 12.9
		12	330.8 ± 17.0	341.0 ± 10.8	314.2** ± 14.4
		13	335.8 ± 18.1	344.5 ± 11.4	318.1** ± 12.9
		14	341.5 ± 17.4	349.6 ± 10.6	323.3** ± 12.9
		15	348.3 ± 19.5	358.6 ± 11.3	330.7** ± 14.1
		16	357.4 ± 21.0	369.1 ± 11.8	339.4** ± 15.8
		17	367.9 ± 21.9	380.8* ± 11.6	351.4** ± 14.8
		20	381.8 ± 23.0	394.2* ± 12.0	363.9** ± 14.4
	Gain	0-20	114.3 ± 16.2	118.1 ± 10.5	106.4 ± 9.3
Days of lactation					
		0	321.8 ± 21.2	330.6 ± 13.3	305.4** ± 16.1
		4	341.4 ± 18.9	350.2 ± 12.8	329.7* ± 13.0
		7	353.0 ± 16.5	358.5 ± 12.3	335.1** ± 11.2
		11	355.7 ± 18.4	359.0 ± 16.3	339.4** ± 12.8
		14	347.7 ± 20.7	347.3 ± 19.9	329.8 ± 12.9
		17	338.4 ± 16.6	347.0 ± 16.5	326.8 ± 16.6
		21	323.2 ± 15.6	334.3* ± 14.0	311.4* ± 13.9
	Gain	0-21	1.4 ± 15.3	3.7 ± 17.5	6.0 ± 13.0

Unit: g, Mean±S.D.
*: $p<0.05$, **: $p<0.01$ (Significant difference from Atsugi Breeging Center)

Table 2. Food consumption of Crj:CD(SD)IGS dams during the gestaion and lactation periods

Breeding Center		Atsugi	Tsukuba	Hino
No. of dams		20	20	20
Days of gestation				
	1	22.3 ± 2.3	23.6 ± 2.4	21.2 ± 2.0
	4	25.9 ± 2.6	27.1 ± 2.4	25.6 ± 2.0
	8	26.3 ± 2.5	28.8** ± 2.3	26.3 ± 2.8
	11	27.9 ± 2.8	28.5 ± 2.9	25.8 ± 3.0
	14	27.3 ± 2.8	28.9 ± 1.7	26.6 ± 2.6
	17	28.5 ± 3.5	30.1 ± 1.7	28.5 ± 3.3
	19	24.9 ± 2.9	26.5 ± 2.4	25.2 ± 3.7
	20	24.9 ± 2.3	26.3 ± 1.9	25.4 ± 2.4
Days of lactation				
	2	30.4 ± 6.5	33.6 ± 5.1	32.3 ± 6.2
	4	47.1 ± 4.5	47.4 ± 5.2	45.6 ± 3.4
	7	51.2 ± 4.2	50.1 ± 4.7	49.4 ± 3.0
	11	57.5 ± 4.4	55.2 ± 4.3	54.6 ± 4.5
	14	59.5 ± 5.2	55.8 ± 7.8	56.3 ± 4.9
	17	58.8 ± 6.0	60.4 ± 5.8	58.9 ± 6.8
	21	72.8 ± 5.9	71.5 ± 7.1	67.8* ± 5.5

Unit: g, Mean±S.D.
*: $p<0.05$, **: $p<0.01$ (Significant difference from Atsugi Breeging Center)

Table 3. Delivery data of Crj:CD(SD)IGS dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of pregnant females	20	20	20
No. of females with live pups	20	20	20
Delivery index (%) [1]	100.0	100.0	100.0
Gestation period (Mean±S.D.)	22.1 ± 0.4	22.0 ± 0.4	21.9 ± 0.3
No. of implantations	305	319	290
(Mean±S.D.)	15.3 ± 1.4	16.0 ± 1.5	14.5 ± 1.3
No.of stillborn pups	1	2	0
(%, Mean±S.D.) [2]	0.3 ± 1.5	0.6 ± 1.8	0.0 ± 0.0
No.of liveborn pups	285	302	270
(Mean±S.D.)	14.3 ± 1.6	15.1 ± 1.8	13.5 ± 1.4
Live birth index (%, Mean±S.D.) [3]	93.5 ± 6.4	94.7 ± 6.6	93.1 ± 5.4

1) (No.of females which delivered live pups / No. of pregnant females)×100
2) (No. of stillborn pups / No. of stillborn and liveborn pups)×100
3) (No. of liveborn pups / No. of implantation)×100

Table 4. External ovservation of F_1 liveborn pups at birth from Crj:CD(SD)IGS dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of dams	20	20	20
No. of males	146	150	135
(Mean±S.D.)	7.3 ± 2.4	7.5 ± 2.8	6.8 ± 2.0
No. of females	139	152	135
(Mean±S.D.)	7.0 ± 2.2	7.6 ± 2.8	6.8 ± 2.3
Sex ratio (Male/Female)	1.05	0.99	1.00
Body weight (g, Mean±S.D.)			
Males	6.5 ± 0.5	6.5 ± 0.5	6.6 ± 0.4
Females	6.2 ± 0.4	6.1 ± 0.4	6.2 ± 0.5
External abnormalities	0	0	0
(%, Mean±S.D.) [1]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

1) (No. of liveborn pups with external abnormalities / No. of liveborn pups)×100

Table 5. Viability index of F_1 pups from Crj:CD(SD)IGS dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of dams	20	20	20
Before culling			
No. of live pups on day 0	285	302	270
(Mean±S.D.)	14.3 ± 1.6	15.1 ± 1.8	13.5 ± 1.4
No. of live pups on day 4	282	297	266
(Mean±S.D.)	14.1 ± 1.6	14.9 ± 1.7	13.3 ± 1.5
Viability index (%, Mean±S.D.) [1]	99.0 ± 2.5	98.5 ± 3.9	98.8 ± 3.2
After culling			
No. of live pups on day 4	160	160	160
(Mean±S.D.)	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0
No. of live pups on day 21	160	157	160
(Mean±S.D.)	8.0 ± 0.0	7.9 ± 0.7	8.0 ± 0.0
Weaning index (%, Mean ± S.D.) [2]	100.0 ± 0.0	98.1 ± 8.4	100.0 ± 0.0

1) (No. of live pups on day 4 / No. of liveborn pups on day 0)×100
2) (No. of live pups on day 21 / No. of liveborn pups on day 4)×100

Table 6-1. Body weight of F_1 males from Crj:CD(SD)IGS dams

Breeding Center		Atsugi	Tsukuba	Hino
No. of dams		20	20	20
Days of after birth				
	0	6.5 ± 0.5	6.5 ± 0.5	6.6 ± 0.4
	4	10.2 ± 1.0	9.8 ± 0.9	10.3 ± 1.0
	7	16.8 ± 1.4	16.6 ± 1.7	16.8 ± 1.2
	11	26.6 ± 1.8	26.2 ± 2.4	26.1 ± 1.5
	14	33.4 ± 2.3	33.0 ± 2.7	32.6 ± 1.7
	17	39.7 ± 2.4	39.5 ± 3.0	38.6 ± 1.9
	21	52.6 ± 4.2	51.4 ± 4.4	51.0 ± 3.2
	28	93.4 ± 7.6	91.0 ± 8.9	90.6 ± 4.9
	35	155.2 ± 11.3	152.6 ± 13.8	148.5 ± 9.2
	42	222.0 ± 12.8	217.6 ± 16.1	210.1* ± 12.5
	49	288.8 ± 15.9	284.4 ± 20.1	272.2** ± 15.1
	56	350.3 ± 18.6	344.1 ± 22.5	327.9** ± 17.2
	63	393.8 ± 20.9	386.2 ± 26.2	367.4** ± 20.5
	70	434.9 ± 24.4	427.5 ± 28.3	404.9** ± 25.8

Unit: g, Mean±S.D.
*: $p<0.05$; **$p<0.01$(Significant difference from Atsugi Breeding Center)

Table 6-2. Body weight of F_1 females from Crj:CD(SD)IGS dams

Breeding Center		Atsugi	Tsukuba	Hino
No. of dams		20	20	20
Days of after birth				
	0	6.2 ± 0.4	6.1 ± 0.4	6.2 ± 0.5
	4	9.7 ± 0.8	9.3 ± 0.8	9.7 ± 1.0
	7	15.9 ± 1.3	15.4 ± 1.1	15.7 ± 1.4
	11	25.5 ± 2.0	24.8 ± 1.7	24.7 ± 2.1
	14	32.2 ± 2.4	31.3 ± 2.1	31.1 ± 2.2
	17	38.3 ± 2.7	37.4 ± 2.2	36.8 ± 2.4
	21	50.5 ± 4.0	48.4 ± 3.4	48.4 ± 3.5
	28	84.7 ± 8.2	81.5 ± 7.9	78.9* ± 6.8
	35	132.9 ± 8.9	128.1 ± 12.6	126.7 ± 7.7
	42	170.1 ± 9.5	163.9 ± 14.5	162.2* ± 7.9
	49	198.1 ± 11.2	192.3 ± 15.6	188.4* ± 9.5
	56	221.0 ± 14.0	217.4 ± 15.6	212.0 ± 10.5
	63	242.4 ± 15.6	239.5 ± 18.1	232.0 ± 10.4
	70	259.4 ± 16.5	254.6 ± 18.1	248.2 ± 12.4

Unit: g, Mean±S.D.
*: $p<0.05$(Significant difference from Atsugi Breeding Center)

Table 7. Copulation and fertility results in Crj:CD(SD)IGS F_1 rats

Breeding Center	Atsugi	Tsukuba	Hino
No. of pairs	20	19	20
No.of copulated	20	18	19
No. of pregnant females	20	18	19
Copulation index (%) [1)]	100.0	94.7	95.0
Fertility index (%) [2)]	100.0	100.0	100.0

1) (No. of animals with successful copulation / No. of animals mated)×100
2) (No. of pregnant animals / No. of females with successful copulation)×100

Table 8. Findings on day 14 of gestation in Crj:CD(SD)IGS F_1 dams

Breeding Center	Atsugi	Tsukuba	Hino
No. of F_1 dams	20	18	19
No. of corpora lutea	312	272	298
(Mean±S.D.)	15.6 ± 1.8	15.1 ± 1.6	15.7 ± 2.3
No. of implantations	292	259	282
(Mean±S.D.)	14.6 ± 2.4	14.4 ± 1.6	14.8 ± 2.0
Implantation index (%, Mean±S.D.) [1)]	93.8 ± 12.5	95.2 ± 4.2	94.9 ± 5.2
No. of dead embryos	19	22	18
(%, Mean±S.D.) [2)]	5.8 ± 8.7	8.5 ± 11.0	6.3 ± 6.5
No. of live embryos	273	237	264
(Mean±S.D.)	13.7 ± 2.0	13.2 ± 2.1	13.9 ± 2.0

1) (No. of implantations / No. of corpora lutea)×100
2) (No. of dead embryos / No. of implantations)×100

Spontaneous Developmental Abnormalities in Crj:CD(SD)IGS Rats Uses of "the Terminology of Developmental Abnormalities in Common Laboratory Mammals", Edited by the International Federation of Teratology Society

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ABSTRACT. To obtain background data of incidence of spontaneous congenital anomalies in Crj:CD(SD)IGS rats, 356 fetuses from 26 dams were examined. Anomalies and variations were categorized based on the Terminology of Developmental Abnormalities in Common Laboratory Mammals edited by the International Federation of Teratology Society (IFTS), then were compared with previous data obtained in 1997 in our institute [1]. Two fetuses exhibited anomalies, while no congenital anomalies had been found in the previous study. One showed cleft palate accompanied with small thymus, membranous ventricular septum defect and narrowed pulmonary trunk, and the other showed overriding aorta. However, no differences were observed in incidences of skeletal and visceral variations, weights of fetuses and placentas, viability of fetuses and numbers of fetuses, implantation sites and corpus luteum. — Key words: congenital anomalies, IFTS, terminology

CD(SD)IGS-2001: 111-114

INTRODUCTION

Crj:CD(SD)IGS is one of the rat strains which have been commonly used for the safety evaluation of drugs and chemicals. We have used IGS rats in the reproductive and developmental toxicity studies in Hatano Institute of Food and Drug Safety Center since 1997. When IGS rats had been initiated to supply, we collected background data of IGS rats for various observation items, which were adopted for endpoints of the reproductive and developmental toxicity studies including incidence of spontaneous congenital anomalies [1]. In the present study, we examined identical number of fetuses to the previous study to collect recent background data of IGS rats on incidence of spontaneous congenital anomalies. To share the data between laboratories, we categorized anomalies found in the present and previous studies based on the Terminology of Developmental Abnormalities in Common Laboratory Mammals edited by the International Federation of Teratology Society (IFTS) Committee on International Harmonization on Nomenclature in Developmental Toxicology (IFTS glossary) [2].

MATERIALS AND METHODS

Thirty female Crj:CD(SD)IGS rats were obtained from Charles River Japan, Inc. (Atsugi Breeding Center, Kanagawa, Japan) at 9 weeks of age. Fertile proven males of the same strain were also used for the mating partner. These animals were maintained under a specific lighting condition (lights on 07:00-19:00) in an animal husbandry facility, which was controlled for standards of temperature and humidity at 23-25°C and 50-65%, respectively. The animals were housed individually in cages with wire-meshed floor (220w×270d×190h mm) and were supplied with pellet chow (CE-2, CLEA Japan Inc.) and water (tap water) *ad libitum*. After 1-week acclimatization to the environment, the animals were mated, and the day when spermatozoa were confirmed in the vagina was defined as gestational day (GD) 0.

The pregnant females were individually housed, and were daily monitored for general conditions. Body weight was measured on GD 0 and daily on GDs 7-20. Food consumption was measured daily during GDs 6-20.

On GD20, dams were sacrificed by bleeding under ether inhalation, and were macroscopically examined for the thoracic and abdominal organs. Then, the ovary was dissected for counting the number of corpus luteum, and the uterus was counted for the numbers of implantation sites, live fetuses and dead embryos/fetuses. The uterus, which was removed all of the conceptus, was reconfirmed for the number of implantation sites by the method of Salewski [3].

Live fetuses and accompanied placentas were weighed, and the fetuses were examined for external morphology and sex under a stereomicroscopy. About half of live fetuses in each dam were processed for skeletal specimens [4], and was examined for skeletal morphology and the number of osscified sacrococcygeal vertebrae. The other fetuses were fixed in 10% formalin solution and Bouin's solution to observe visceral morphology [5,6].

Dead embryos/fetuses were categorized in early resorbed embryos, placental remnants and macerated fetuses, then viability indices, such as implantation rate (number of implantation sites/number of corpus luteum, %) and intrauterine mortality (number of dead embryos/fetuses/number of implantation sites, %), were calculated.

RESULTS AND DISCUSSION

By cohabitation with males as long as 4 days, 26 females conceived.

There were no abnormalities in general condition, and body weight increased normally (Tables 1 and 2 and Fig. 1). Food intake of dams was slightly increased when body weight increased remarkably (Table 3). Since dietary condition was different between the present and the previous studies [1], changes in body weight and food intake during the gestation period could not be compared between the studies.

Reproductive findings at the cesarean section are indicated in

Table 4. Numbers of corpus luteum pregnancy and implantations were 13-20 (average 15.5) and 10-19 (average 14.6), respectively, and were identical to those in the previous study [1]. Intrauterine mortality, 0-50.0% (average 6.5%), and number of live fetuses, 5-18 (average 13.7), were also similar to those in the previous study [1]. Body weights of normal fetuses, 1.37-4.39g (average 3.75g) in males and 2.15-4.17g (average 3.60g) in females, were identical to the previous study [1] as shown in Table 5.

In the present study, cleft palate, an external anomaly, was found in one fetus (Table 6). The fetus was accompanied several visceral anomalies, such as small thymus, membranous ventricular septum defect and narrowed pulmonary trunk. In addition to this case, there was one fetus with visceral anomaly, such as overriding aorta.

There were no fetuses with skeletal anomaly, however, skeletal variations, such as misshapen sternebra, unossified sternebra, dumbbell-ossification of thoracic centrum, bipartite ossification of thoracic centrum, short rib, full supernumerary rib, short supernumerary rib and unossified thoracic centrum, lumbar arch, lumbar centrum, sacral arch or sacral centrum, were observed in the present study [1]. Among these variations, the term of unossified sternebra was defined unossification of the 5th and 6th sternebra, since the 1st to 4th sternebra were usually not ossified on 20 days after conception (Table 7).

While the findings found in the present study were identical to those observed in the previous study [1], but frequency of fetuses with skeletal variations was slightly lower than that in the previous study.

The number of ossified sacrococcygeal vertebrae is one of the indices to know the degree of ossification. In the present study, the number of ossified sacrococcygeal vertebrae was 0-9 (average 7.5), and was similar to the previous study [1].

As for visceral variations, malpositioned thymus, dilatated renal pelvis, dilatated ureter and malpositioned umbilical artery were observed in the present study. Incidence of fetuses with visceral variations was similar to that in the previous study [1].

The previous study [1] revealed there were large differences between SD rats and IGS rats in the numbers of corpus luteum, implantations and live fetuses and fetal and placental weights. These values obtained in the present study were similar to those obtained from IGS rats in the previous study [1].

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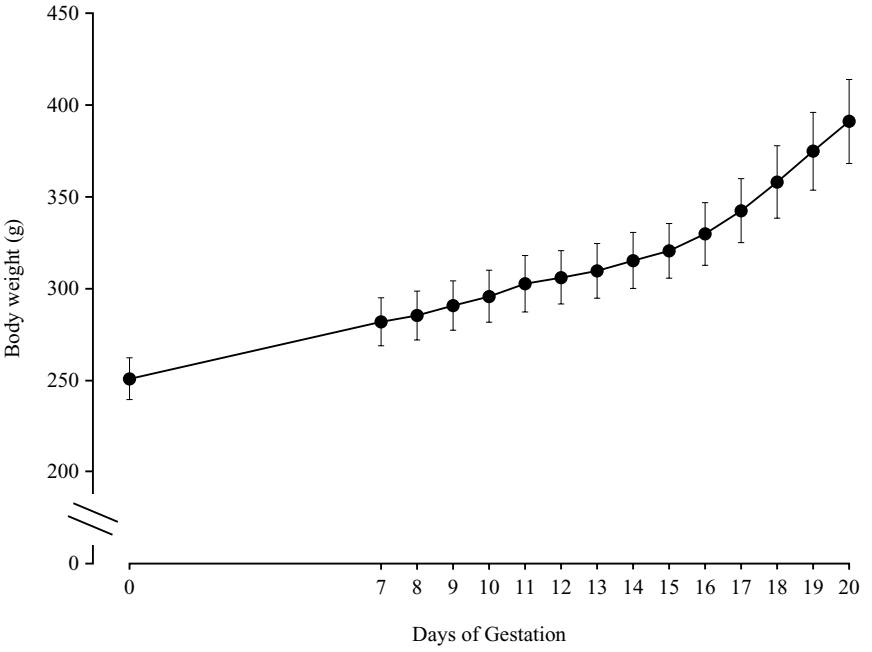

Fig 1. Body weight of dams during gestation.
Each point represents mean body weight of 26 pregnant females, and vertical bars indicate standard deviation.

Table 1. Body weight gain of dams during gestation

Days of gestation		
0～7	31.2±6.2	(26)
7～8	3.4±3.3	(26)
8～9	5.3±3.6	(26)
9～10	5.0±3.2	(26)
10～11	6.8±3.6	(26)
11～12	3.5±3.8	(26)
12～13	3.7±3.9	(26)
13～14	5.6±4.0	(26)
14～15	5.2±2.5	(26)
15～16	9.1±3.7	(26)
16～17	12.7±3.3	(26)
17～18	15.5±4.9	(26)
18～19	16.6±3.5	(26)
19～20	16.2±3.2	(26)

Parameter: Mean±S.D. (g)
( ): Number of dams.

Table 2. Cumulative body weight gain of dams during gestation

Days of gestation		
0～7	31.2±6.2	(26)
0～8	34.6±5.8	(26)
0～9	39.9±6.6	(26)
0～10	44.8±7.2	(26)
0～11	51.7±7.5	(26)
0～12	55.2±7.6	(26)
0～13	58.9±7.9	(26)
0～14	64.5±9.0	(26)
0～15	69.7±8.9	(26)
0～16	78.8±10.2	(26)
0～17	91.5±10.7	(26)
0～18	106.9±13.2	(26)
0～19	123.5±14.0	(26)
0～20	139.7±15.7	(26)

Parameter: Mean±S.D. (g)
( ): Number of dams.

Table 3. Food consumption of dams during gestation

Days of gestation		
6～7	25.0±2.8	(26)
7～8	24.6±2.0	(26)
8～9	25.3±2.5	(26)
9～10	25.3±2.3	(26)
10～11	25.4±2.3	(26)
11～12	26.3±2.4	(26)
12～13	26.4±2.2	(26)
13～14	25.5±2.3	(26)
14～15	25.5±2.1	(26)
15～16	26.5±2.9	(26)
16～17	27.4±2.3	(26)
17～18	28.1±2.8	(26)
18～19	28.0±2.4	(26)
19～20	27.3±2.7	(26)

Parameter: Mean±S.D. (g)
( ): Number of dams.

Table 4. Summary of reproductive data of dams at the cesarean section on day 20 of gestation

Number of pregnant females	26
Number of corpora lutea/dam	
Total	402
Mean±S.D.	15.5±2.0
Number of implantations/dam	
Total	379
Mean±S.D.	14.6±2.1
Implantation rate(%) A)	
Mean±S.D.	94.5±8.9
Number of intrauterine death	
Total	22
Mean±S.D.	0.9±1.4
Number of early resorption/dam	
Total	0
Number of placental remnants/dam	
Total	22
Mean±S.D.	0.8±1.3
Number of dead fetus/dam	
Total	1
Mean±S.D.	0.0±0.2
Intrauterine mortality(%) B)	6.5±11.1
Number of fetuses	
Total	356
Males/Females	192 / 146
Mean±S.D.	13.7±2.6
Sex ratio(%) C)	
Mean±S.D.	49.5±10.9

A) Implantation rate=(Number of implantations in each dam/Number of corpora lutea in each dam)×100
B) Intrauterine mortality=(Number of intrauterine deaths in each dam/Number of implantations in each dam)×100
C) Sex ratio=(Number of male fetuses in each dam/Total number of fetuses in each dam)×100

Table 5. Fetal and placental weight

Number of pregnant females	26	
Fetal weight		
Males	3.75±0.38	(26)
Females	3.60±0.34	(26)
Placental weight		
Males	0.48±0.08	(26)
Females	0.46±0.06	(26)

Parameter: Mean±S.D. (g)
( ): Number of dams.

Table 6. Findings of fetuses

Year	2000		1997 [a]	
External malformations	0.4±1.8 [b]	(1/356) [c]	0.0±0.0 [b]	(0/393) [c]
Cleft palate	0.4±1.8	(1/356)		
Skeletal malformations	0.0±0.0	(0/187)	0.0±0.0	(0/206)
Skeletal variations	11.3±13.1	(20/187)	21.9±21.4	(47/206)
Misshapen sternebra	0.6±2.8	(1/187)		
Unossified sternebra	4.8±9.9	(7/187)		
Dumbbell ossification of thoracic centrum	1.6±4.5	(5/187)	9.2±17.1	(21/206)
Bipartite ossification of thoracic centrum			0.4±2.3	(1/206)
Short rib	0.6±3.3	(1/187)		
Full supernumerary rib			14.5±14.5	(30/206)
Short supernumerary rib	4.4±11.4	(9/187)		
Unossified (thoracic centrum, lumbar arch, lumbar centrum, sacral arch, sacral centrum)	0.6±2.8	(1/187)		
Number of ossified sacrococcygeal vertebrae	7.5±0.5		7.9±0.4	
Visceral malformations	1.1±3.9	(2/169)	0.0±0.0	(0/187)
Small thymus	0.4±2.2	(1/169)		
Membranous ventricular septum defect	0.4±2.2	(1/169)		
Overriding aorta	0.6±3.3	(1/169)		
Narrowed pulmonary trunk	0.4±2.2	(1/169)		
Visceral variations	8.3±13.0	(12/169)	9.0±15.9	(18/187)
Malpositioned thymus	4.6±8.1	(8/169)	5.0±12.8	(1/187)
Dilated renal pelvis	3.9±10.9	(4/169)	3.6±8.8	(7/187)
Dilated ureter			0.6±3.1	(1/187)
Malpositioned umbilical artery	0.4±2.2	(1/169)		

a) Adopted from Matsumoto et al.[1] by modifying based on the "Terminology of Developmental Abnormalities in Common Laboratory Mammals", Edited by the International Federation of Teratology Society[2].
b) Mean±S.D.(%)
c) Number of fetuses positive/Number of fetuses examined

Table 7. Frequency of fetuses with unossified sternebrae

Position of the unossified sternebra	Frequencies of fetuses (%)	
First	1.9±7.2 [a]	(2/187) [b]
Second	4.8±9.9	(7/187)
Third	1.9±7.2	(2/187)
Forth	2.6±9.0	(3/187)
Fifth	78.3±21.6	(146/187)
Sixth	89.6±19.5	(160/187)

a) Mean±S.D. in 26 dams
b) Number of fetuses positive/Number of fetuses examined

Comparison of Reproductive Parameters Between Crj:CD(SD)and Crj:CD(SD)IGS Rats

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ABSTRACT. A reproductive study (breeding study) using international genetic 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, necroptic 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-2001: 115-118

INTRODUCTION

A reproductive study (breeding study) using international genetic 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, observation of clinical signs 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. In addition, 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.

In addition, for 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 moving sperms and non-moving 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.

In addition, 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. For 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 138.9 g, which was slightly larger than the BG (86.7 to 135.7 g). The weight gain for females was 29.5 g from 2 weeks before the start of mating to the day before the start of mating and 150.0 g from Days 0 to 20 of gestation, which were almost within the range of the BG.

The necroptic findings are shown in Table 2. Light yellow nodes were observed in the epididymis. 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 3.6 times, which was slightly lower than the BG (3.73 to 3.81 times). In addition, the estrous cycle was 3.98 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 both 100%, and the number of days required for copulation was 2.20 days. These values were almost within the range of the BG.

The results of the sperm test examination are shown in Table 5. The number of sperm was 498.9×10^6, the sperm motility rate was 94.7%, and the sperm form anomalies index was 1.38%, all of which were almost the same as the BG.

The results of the examination at cesarean section are shown in Table 6. The number of corpor lutea and the number of implantations were 16.15 and 15.40, respectively, both of which were within the range of the BG. In addition, the pre-implant loss rate was 4.64%, which tended to show a slightly lower value than the BG (3.34 to 16.37%). Total fetal mortality was 5.19%, 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 4.87%, 0.32% and 14.60, respectively, which were the same as those of the BG. The mean sex ratio was 0.85, and the mean F1 body weight was 3.67 g for males and 3.46 g for females, both of which were within the range of the BG.

The results of the external examination are shown in Table 7. The incidence of edema was 0.34%, which was close to that of the BG.

The results of the visceral examination are shown in Table 8. Dilatation of the ureter occurred in 9.33%, dilatation of the renal pelvis and ureter in 0.67% and thymic reminamt in the neck in 1.33%. For dilatation of the ureter, the incidence tended to be slightly higher than that of the BG (0 to 8.66%) for this strain, but the incidences of other 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, cervical rib, lumbar rib, and splitting of the sternebra 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 vertebral bodies of the cervical vertebra in the vertebral bone was 0.80, which showed earlier ossification than the BG (0.10 to 0.49). In the sternal bone, the degrees of ossification of the fifth and sixth sterna were 78.72% and 97.87%, 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% for the left distal phalanx and 0.29% 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)
Days of pre-mating		
male（4w）	138.9	86.7～135.7
female（2w）	29.5	16.6～32.7
Days of gestation		
	150.0	122.9～149.1

Table. 2 Necropsy findings in rats

	Crj:CD(SD)IGS		Crj:CD(SD)
Organs and findings	Male	Female	—
Number of animals	20	20	5～36
Genital system			
Epididymis			
nodule, light yellow	1	0	0

Table 3. Estrous cycle in female rats

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of animals examined	20	
Count of estrus	3.60±0.50[a)]	3.73～3.81
Estrous cycle	3.98±0.33	3.98～5.72

a) Mean±S.D.

Table 4. Mating and pregnancy in rats

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of mated animals		
Male	20	11～25
Female	20	11～25
No. of copulated animals		
Male	20(100)	83.33～100
Female	20(100)	83.33～100
No. of impregnated	20(100)	89.47～100
No. of pregnant animals	20(100)	85.1 ～100
Duration of mating(days)	2.20±1.01	2.07～2.95

( ) : %

Table 5. Examination of sperm in male rats

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of animals examined	20	
Count of sperm ($\times 10^6$/g)	498.9±71.5[a)]	495.5～557
Sperm motility (%)	94.7±3.1	88.0～95.3
Sperm form anomalies index (%)	1.38±1.50	1.41

a) Mean±S.D.

Table 6. Findings at cesarean section in Fo dams and fetuses

	Crj:CD(SD)IGS	Crj:CD(SI
No. of dams	20	6～25
No. of corpora lutea	323(16.15±1.46)[a)b)]	15.32～18.
No. of implants	308(15.40±2.21)[a)b)]	13.5 ～17.
No. of pre-implant loss	15(4.64)[c)]	3.34～16.
No. of total dead fetuses	16(5.19)[d)]	3.50～8.4
Early resorptions	15(4.87)[d)]	3.50～8.8
Late resorptions	1(0.32)[d)]	0～0.2
Dead fetuses	0	0～0.2
No. of live fetuses	292(14.60±2.89)[a)b)]	12.82～16.
Sex ratio of live fetuses	0.85(134/158)	0.71～1.3
Body weight(g) of live fetuses		
Male	3.67±0.23[a)]	3.28～3.8
Female	3.46±0.20	3.10～3.6

a) Mean±S.D.
b) No. per dam
c) % for No. of corpora lutea
d) % for No. of implants

Table 7. External examination of F1 fetuses

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of fetuses examined	292	80～374
No. of fetuses with external anomalies		
Edema	1(0.34)	0～0.29

( ) : %

Table 8. Visceral examination in F1 fetuses

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of fetuses examined	150	33～151
No. of fetuses with visceral anomalies	17(11.33)	0.79～11.11
Ventricular septal defect	0	0～0.79
Dilatation of the ureter	14(9.33)	0～8.66
Dilatation of the renal pelvis and ureter	1(0.67)	0～1.68
Thymic remnant in the neck	2(1.33)	0～6.30

( ) : % for No. of fetuses examined

Table 8. Visceral examination in F1 fetuses

	Crj:CD(SD)IGS	Crj:CD(SD)
No. of fetuses examined	150	33～151
No. of fetuses with visceral anomalies	17(11.33)	0.79～11.11
Ventricular septal defect	0	0～0.79
Dilatation of the ureter	14(9.33)	0～8.66
Dilatation of the renal pelvis and ureter	1(0.67)	0～1.68
Thymic remnant in the neck	2(1.33)	0～6.30

( ) : % for No. of fetuses examined

Table 10. Skeletal examination(progress of ossification) in F1 fetuses

		Crj:CD(SD)IGS	Crj:CD(SD)
No. of fetuses examined		141	58～245
Vertebrae			
Cervical	arch(R)	7.00±0.00[a)]	7.00
	arch(L)	7.00±0.00	7.00
	body	0.80±1.03	0.10～0.49
Thoracic	arch(R)	13.00±0.00	12.98～13.00
	arch(L)	13.00±0.00	12.98～13.00
	body	13.00±0.00	12.92～13.00
Lumbar	arch(R)	6.00±0.00	5.99～6.03
	arch(L)	6.00±0.00	6.00～6.03
	body	6.00±0.00	5.99～6.03
Sacrocaudal		5.97±0.25	6.00～6.03
	arch(L)	5.93±0.23	5.50～5.99
	body	7.88±0.39	7.64～8.04
Sternebrae			
1st		100	99.56～100
2nd		100	97.10～100
3rd		100	99.51～100
4th		100	98.76～100
5th		78.72	55.26～85.27
6th		97.87	81.12～98.63
Metacarpus(R)		79.01	69.78～79.96
Metacarpus(L)		77.87	69.41～79.91
Phalanges of forelimb			
Proximal (R)		12.77	0.67～31.70
Proximal (L)		11.77	0.75～34.23
Middle (R)		0	0～0.11
Middle (L)		0	0
Distal (R)		100	97.04～100
Distal (L)		100	97.97～100
Metatarsus (R)		80.28	79.66～86.17
Metatarsus (L)		80.71[b)]	79.74～86.87
Phalanges of hindlinb			
Proximal (R)		0	0.75～34.23
Proximal (L)		0.29[b)]	0.67～31.70
Middle (R)		0	0
Middle (L)		0	0
Distal (R)		99.57	96.10～98.96
Distal (L)		99.57[b)]	99.10～100

a) Mean±S.D.
b) Technical error.

EFFECTS OF VALPROIC ACID ON FERTILITY AND REPRODUCTIVE ORGANS IN MALE RATS

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ABSTRACT. Crj:CD(SD)IGS rats were orally administered valproic acid at doses of 250, 500 or 1000 mg/kg/day for 4, 7 or 10 weeks.
At each dose, one group of male rats was euthanized after 4-week dosage (4-week dose group) and the other two were mated with untreated females after 4 (7-week dose group) or 7 (10-week dose group) weeks of treatment with valproic acid and their fertility was evaluated. Females were euthanized on day 14-17 of gestation, and numbers of corpora lutea, implantations and live and dead fetuses were recorded. After 4, 7 or 10 weeks of treatment, males were euthanized, genital organs were weighed, the number of sperm in the cauda epididymis was counted, sperm motion analyzed, and histopathological examination of testes performed.
The male rats of the 1000 mg/kg dose group died or were moribund 3 or 4 days after the start of treatment. No effects on fertility of male rats were observed up to the 500 mg/kg 10-week dose group. Treatment for 4 weeks at 500 mg/kg/day decreased epididymis weight. After 7 weeks at 500 mg/kg/day, the weights of epididymis, seminal vesicles and prostate were decreased, and the number of sperm heads per cauda epididymis and percentage of motile sperm were reduced. In the 500 mg/kg 10-week dose group, the weight of testis was decreased. On histopathological examination of the testis, degeneration of seminiferous tubules and loss or exfoliation of spermatids were observed, and the ratio of retention of step 19 spermatids in stage IX-XI was increased in the 500 mg/kg 4-, 7- and 10-week dose groups.
These results suggest that analysis of sperm motion and histopathological evaluation of testes are sensitive methods for assessing toxicity of valproic acid on male reproductive organs. — Key words: Valproic acid, Sperm analysis, Reproductive toxicity, Rats.

CD(SD)IGS-2001: 119-126

INTRODUCTION

Valproic acid is widely used as an anticonvulsant drug. It has teratogenic and hepatotoxic effects in man and in experimental animals (Esaki *et al*., 1975; Nau and Löscher, 1984; Vorhees *et al*., 1987), and adverse effects on male fertility such as decreased testicular and epididymal weight, degeneration of seminiferous tubules, decrease of fertility rate and sperm motility following subchronic exposure (Miyagawa *et al*., 1971; Cohn *et al*., 1982; Walker *et al*., 1990). We evaluated the effects of valproic acid on male fertility and assessed the sensitivity of parameters such as reproductive performance, organ weights, histopathological examination, and sperm motion using Cellsoft CASA^{tm}.

MATERIALS AND METHODS

Animals and husbandry

Crj:CD(SD)IGS male and female rats were purchased from Charles River Ltd., Japan and acclimated to laboratory conditions for 7 days. They were provided a diet of regular rodent chow (CRF-1, Charles River Ltd., Japan.) and tap water *ad libitum* during the study. Animals were randomly assigned to groups by computer-generated numbers based on body weights. Each group contained 10 animals. Except during the mating period, male and female rats were housed individually in suspended stainless steel wire mesh cages. Throughout the study, rats were housed in a room maintained at 23±2℃ temperature, 55±10% humidity, and on a 12:12-hr light/dark cycle.

Chemical preparation

Valproic acid (Lot Nos. 67F0250, 96H0962, 67H1987) was purchased from Sigma Chemical Co., (St. Louis, MO, USA) and dissolved in distilled water.

Experimental protocols

Valproic acid at dose levels of 250, 500 or 1000 mg/kg/day or vehicle was given by gavage to male rats (starting at 9 weeks of age) for 4, 7, or 10 weeks. These dose levels were based on a report by Miyagawa *et al*. (1971). In this report, atrophy of testis and impairment of spermatogenesis were observed with doses of 1250mg/kg for 24 days or 540mg/kg for 180 days.

Clinical signs of male and female rats were recorded daily, and individual body weights of male rats were recorded weekly. In the 7- and 10-week dose groups, each male was cohabited overnight with one untreated female (from 4 or 7 weeks after the start of treatment, respectively) for up to 14 nights. Copulation was determined by daily vaginal smear inspection. The day when sperm was observed in a vaginal smear was designated as day 0 of gestation, and the mating was discontinued (Figure 1).

Examinations of female rats

Sperm-positive females were euthanized by exsanguination under ether anesthesia on gestation days 14 to 17, and the numbers of corpora lutea, implantations, and live and dead fetuses were counted.

Examination of male rats

After the treatment period, animals were euthanized by exsanguination under ether anesthesia. Sperm motion parameters were measured with a Cellsoft sperm analysis system (Cryo Resources, Ltd., New York). About 5 mm of vas deferens was removed and placed in a medium (phosphate buffered saline containing 1% bovine serum albumin at 37℃) to diffuse sperm. After 10 min of incubation, the sperm movement was recorded on videotape. One hundred or more spermatozoa per sample were analyzed. Parameters used in the analysis were percentage of motile sperm, velocity, linearity, beat/cross frequency, maximum

Note: This paper is reprinted from original paper (The Journal of Toxicological Sciences; 25(2), 85-93, 2000)

and mean amplitude of lateral head displacement, average radius, percentage of circular cells over motile cells and percentage of circular cells over all cells. Testes (bilateral), epididymides (bilateral), seminal vesicles and ventral prostates were removed and weighed. The right cauda epididymis was homogenized in 0.9% NaCl, containing 0.1% Triton-X solution, and the number of sperm heads was counted using a Thoma hematocytometer (Kayagaki Irika Kogyo Co., Ltd., Japan) with a coverglass.

Histopathological examination

The testes were fixed in formalin-sucrose-acetic acid solution (5% sucrose solution:formalin:acetic acid= 15:5:0.8) for 5 days and transferred to 10% phosphate buffered formalin. These tissues were embedded in paraffin, sectioned at approximately 2 μm thickness, and stained with hematoxylin and eosin. Testis sections were also stained with PAS-hematoxylin.

Retention of step 19 spermatids in stage IX-XI was found in the preliminary inspection. This change has reported with nitrofurazone, which is a testicular toxicant (Nishimura *et al*., 1995). Therefore, tubules presenting this change were also counted in cross-sections.

Statistical analyses

Parametric data were analyzed by one-way ANOVA and compared with the control group by Dunnett's test (Yoshimura, 1988). Nonparametric data were analyzed by the Kruskall-Wallis' test (Yoshimura, 1988), and compared with the control group by Steel's test (Nagata and Yoshida, 1997). Categorized data were analyzed by χ-square test (Yoshimura, 1988).

RESULTS

Clinical signs, mortality and body weight

In the 500 and 1000 mg/kg dose group, decreases of spontaneous activity and bradypnea were observed. The male rats of the 1000 mg/kg dose group were found dead (2/15) or moribund (7/15) within 3 or 4 days after the start of treatment. Therefore, treatment was discontinued in this group.

None of the male rats in the 250 and 500 mg/kg dose groups died during the treatment. In the 500 mg/kg group, body weight was lower than the control group from day 56 to 70 (Figure 2).

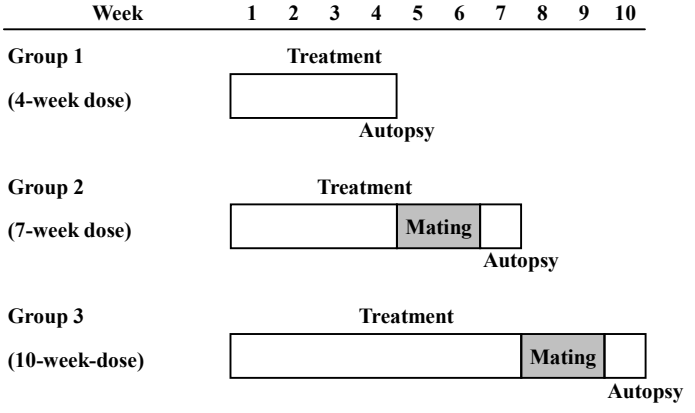

Figure 1. Experimental design.

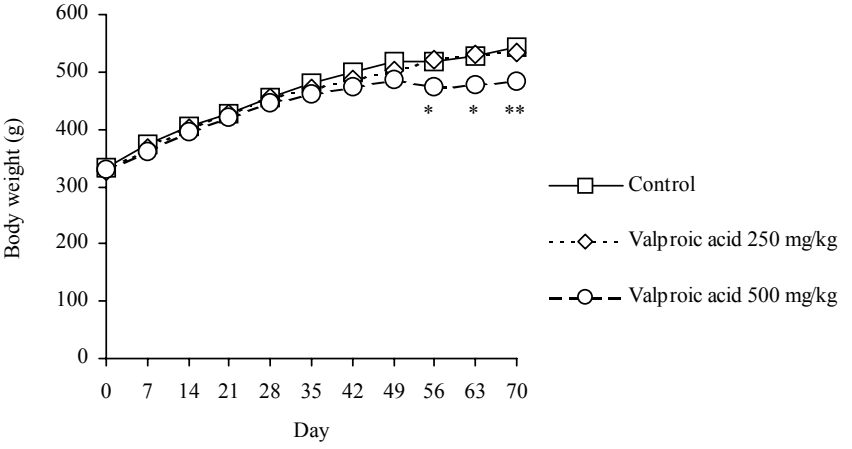

Figure 2. Body weight changes of male rats orally administered valproic acid.
*, **: Significantly different from control group value at $p<0.05$ and $p<0.01$, respectively.

Reproductive performance

In the 7- and 10-week dose groups (mated after 4 and 7 weeks treatment, respectively), at 250 and 500 mg/kg, there was no observable effect of valproic acid on copulation and fertility indices, number of implants and live and dead fetuses (Table 1).

Organ weights

In the 4-week dose group, the weight of epididymis was decreased in the 500 mg/kg dose group. In the 7 -week dose group, the weights of epididymis, seminal vesicle and prostate of the 500 mg/kg group were decreased. In the 10-week dose group, the weights of testis, epididymis and prostate of the 500 mg/kg group were decreased. No changes were observed in the 250 mg/kg group in the 4-, 7- or 10-week dose groups (Table 2).

Sperm analysis

In the 500 mg/kg group in the 7- and 10-week dose groups, the numbers of sperm heads per cauda epididymis were decreased to 1.023×10^8 and 0.975×10^8, respectively, compared with 1.319×10^8 and 1.264×10^8 in their respective control groups.

In the 500 mg/kg group in the 7- and 10-week dose groups, the percentages of motile sperm were also decreased, being 72.38% and 72.23% respectively, compared with 87.06% and 86.57% in their respective control groups. The other parameters (velocity, linearity, beat/cross frequency, maximum and mean amplitude of lateral head displacement, average radius, percentage of circular cells over motile cells and percentage of circular cells over all cells) were not affected.

No changes were observed in the 250 mg/kg group in the 4-, 7- and 10-week dose groups (Table 3, 4).

Histopathological examination

In the histopathological examination of the testis, the ratios of retention of step 19 spermatids in stage IX-XI were higher in the 500 mg/kg groups in the 7- and 10-week dose groups than control groups, and there was a tendency toward increase in the 500 mg/kg group in the 4-week dose group. In some males, degeneration of seminiferous tubules and loss or exfoliation of spermatids were observed in the 500 mg/kg dose group. In these males, round or elongated spermatids were decreased or had disappeared, and in severely affected males, spermatocytes were also affected. No changes were observed in the 250mg/kg group in the 4-, 7- and 10-week dose groups (Table 5, 6).

DISCUSSION

Valproic acid is reported to have toxic effects on male fertility such as decrease of testicular and epididymal weight, degeneration of seminiferous tubules, decrease of fertility rate and sperm motility following subchronic exposure (Miyagawa *et al*., 1971; Cohn *et al*., 1982; Walker *et al*., 1990).

In our study, decrease of epididymal weight and retention of step 19 spermatids in stage IX-XI, degeneration of seminiferous tubules and loss or exfoliation of spermatids in histopathological examination were observed in the 500 mg/kg group in the 4-, 7- or 10-week dose groups. Decrease of weight of accessory sex organs (seminal vesicle and prostate), number of sperm heads per cauda epididymis and percentage of motile sperm were observed in the 7- or 10-week dose groups, and testis weight was decreased in the 10-week dose group.

Retention of step 19 spermatids in stage IX-XI frequently occurs in Crj:CD(SD)IGS males (Matsumoto *et al*., 1999). The ratio of this change was relatively high in the control group, but the ratio of this change in the valproic acid 500 mg/kg-treated group was higher than the value of the control group. This change was observed with several testicular toxicants such as *cis*-platinum (Pogach *et al*., 1989), sodium dichloroacetate (Toth *et al*., 1992), cadmium (Hew *et al*. 1993), 2,5-hexandione (Hall *et al*., 1991) and nitrofurazone (Nishimura *et al*., 1995), many of them affecting Sertoli cells. The weights of accessory sex organs, such as epididymis, prostate and seminal vesicles are androgen-dependent, and may reflect the changes in the animal's androgen levels or testicular function (Zenick *et al*., 1994). These hormonal changes has been considered to cause dysfunction of Sertoli cells and induced retention of step 19 of spermatids in stage IX-XI (Kishimoto *et al*., 1995; Harada, *et al*., 1995). The same phenomenon is considered to occur with valproic acid. An assay of hormone levels following valproic acid treatment would be informative to characterization of effects of this compound.

Decreases in the percentage of motile sperm and the number of sperm heads have been reported with sodium dichloroacetate (Toth *et al*., 1992) and normal Crj:CD(SD) rats (Matsumoto *et al*., 1999). In these cases, other sperm motion parameters such as velocity, linearity, beat/cross frequency and amplitude of lateral head displacement were also decreased. However, since the percentage of motile sperm was only affected in this study, this parameter is appropriate to evaluate sperm motion. Retention of step 19 spermatids in stage IX-XI was also observed in these cases and considered to be associated with decreases in the percentage of motile sperm and the number of sperm heads. In sodium dichloroacetate ingestion, sperm were considered to be phagocytized by Sertoli cells (Toth *et al*., 1992). In our study, it is considered that the same phenomenon is one of the causes of the reduction of number of sperm.

Cohn *et al*. reported fertility index was reduced with valproic acid (1993). In our study, the fertility was not affected despite the apparent toxicity on the testis. The cause of the difference between their results and our study is not clear, but some differences in the study methods such as age and strain of used animals or administration route are considered one of the causes. Fertility is a weak indicator of toxicity on male reproductive organs (Takayama *et al*., 1995), and this parameter is not sensitive enough for evaluation of valproic acid-induced male reproductive organ toxicity in our study.

In conclusion, we detected the effects of valproic acid on sperm using a computer-aided sperm analysis system. In the 7-week dose group, the sperm motion analysis and histopathological examination revealed the effects of valproic acid, while fertility was not affected. In the sperm motion analysis by Cellsoft, the most sensitive parameter is percentage of motile sperm. Our results suggested that sperm motion analysis as well as histopathological evaluation is relevant for assessing the reproductive toxicity of valproic acid in male rats.

Table 1. Reproductive performance of male rats orally administered valproic acid. Implantation rate and fetal death rate was expressed as mean and S. D. of each litter's value.

7 weeks

Dose level		No. of pairs	No. of Copulations	No. of Pregnancies	No. of corpora lutea	No. of implantations	Implantation rate	No. of live fetuses	No. of dead fetuses	Fetal death rate
Control	Total	10	9	8	119	115		105	10	
	Mean				14.9	14.4	96.7	13.1	1.3	11.2
	S.D.				1.5	1.4	3.5	2.2	1.5	14.1
	%		90.0	88.9						
Valproic acid	Total	10	9	9	132	131		121	10	
250 mg/kg	Mean				14.7	14.6	99.3	13.4	1.1	12.5
	S.D.				1.2	1.1	2.1	2.6	2.3	28.7
	%		90.0	100.0						
Valproic acid	Total	10	10	10	159	150		148	2	
500 mg/kg	Mean				15.9	15.0	94.5	14.8	0.2	1.2
	S.D.				2.3	2.2	5.7	2.0	0.4	2.6
	%		100.0	100.0						

10 weeks

Dose level		No. of pairs	No. of Copulations	No. of Pregnancies	No. of corpora lutea	No. of implantations	Implantation rate	No. of live fetuses	No. of dead fetuses	Fetal death rate
Control	Total	9	7	5	69	67		63	4	
	Mean				13.8	13.4	97.1	12.6	0.8	6.9
	S.D.				1.1	1.1	3.9	1.5	0.8	7.5
	%		77.8	71.4						
Valproic acid	Total	10	8	8	118	116		112	4	
250 mg/kg	Mean				14.8	14.5	98.5	14.0	0.5	4.2
	S.D.				1.8	1.5	2.8	1.9	1.1	9.6
	%		80.0	100.0						
Valproic acid	Total	10	10	9	112	90		84	5	
500 mg/kg	Mean				14.0	11.3	77.5	10.5	0.6	5.5
	S.D.				1.6	5.1	31.7	4.7	0.7	6.4
	%		100.0	90.0						

Table 2. Organ weights of male rats orally administered valproic acid.
*, **: Significantly different from respective control group values at $p<0.05$ and $p<0.01$, respectively.

4 weeks

Dose level		Body weight g	Testis g	Epididymis g	Seminal vesicle g	Prostate g
Control	Number of male rats	10	10	10	10	10
	Mean	436.07	3.140	1.146	1.603	0.623
	S.D.	33.60	0.300	0.120	0.201	0.165
Valproic acid	Number of male rats	10	10	10	10	10
250 mg/kg	Mean	451.33	3.109	1.156	1.611	0.528
	S.D.	50.56	0.294	0.134	0.309	0.129
Valproic acid	Number of male rats	10	10	10	10	10
500 mg/kg	Mean	424.57	2.994	1.010 *	1.393	0.524
	S.D.	55.30	0.309	0.092	0.163	0.138

7 weeks

Dose level		Body weight g	Testis g	Epididymis g	Seminal vesicle g	Prostate g
Control	Number of male rats	10	10	10	10	10
	Mean	524.68	3.201	1.252	1.848	0.670
	S.D.	48.26	0.513	0.148	0.311	0.161
Valproic acid	Number of male rats	10	10	10	10	10
250 mg/kg	Mean	490.15	3.281	1.209	1.549	0.523
	S.D.	62.11	0.354	0.110	0.209	0.147
Valproic acid	Number of male rats	10	10	10	10	10
500 mg/kg	Mean	507.88	3.051	1.099 *	1.482 *	0.439 **
	S.D.	62.20	0.485	0.125	0.338	0.132

10 weeks

Dose level		Body weight g	Testis g	Epididymis g	Seminal vesicle g	Prostate g
Control	Number of male rats	9	9	9	9	9
	Mean	542.68	3.543	1.349	1.838	0.704
	S.D.	52.91	0.377	0.137	0.385	0.148
Valproic acid	Number of male rats	10	10	10	10	10
250 mg/kg	Mean	531.49	3.440	1.308	1.757	0.597
	S.D.	42.59	0.200	0.123	0.358	0.173
Valproic acid	Number of male rats	10	10	10	10	10
500 mg/kg	Mean	483.36 **	3.229 *	1.077 **	1.533	0.463 **
	S.D.	17.31	0.260	0.111	0.293	0.064

Table 3. Sperm head count of cauda epididymis of male rats orally administered valproic acid.
*, **: Significantly different from respective control group values at $p<0.05$ and $p<0.01$, respectively.

Dose level		No. of sperm heads ($\times 10^8$/epididymis)		
		4 weeks	7 weeks	10 weeks
Control	Number of male rats	10	10	9
	Mean	1.278	1.319	1.404
	S.D.	0.258	0.286	0.091
Valproic acid	Number of male rats	10	10	10
250 mg/kg	Mean	1.278	1.236	1.299
	S.D.	0.176	0.101	0.176
Valproic acid	Number of male rats	10	10	9
500 mg/kg	Mean	1.088	1.023 *	0.975 **
	S.D.	0.264	0.264	0.139

Table 4. Sperm motion analysis of cauda epididymis of male rats orally administered valproic acid.
**: Significantly different from respective control group values at $p<0.01$. ALH: Amplitude of lateral head displacement, BCF: beat/cross frequency

4 weeks

Dose level		Number of rats examined	Percentage of motile sperm	Velocity	Linearity	ALH max	ALH mean	BCF	Average radius	Percentage of circular cells	
										over motile cells	over all cells
Control	Mean	9	80.82	519.62	2.34	37.56	28.54	7.33	25.46	6.49	5.30
	S.D.		13.09	89.42	0.25	6.13	4.64	0.58	6.41	2.14	2.10
Valproic acid	Mean	10	80.55	486.26	2.36	36.74	27.49	7.14	24.63	6.14	5.34
250 mg/kg	S.D.		22.30	53.01	0.21	5.17	4.55	0.53	6.88	2.74	2.33
Valproic acid	Mean	10	81.79	505.37	2.16	35.86	27.61	6.96	26.26	4.93	4.34
500 mg/kg	S.D.		19.40	68.82	0.56	7.84	6.16	0.60	5.14	2.00	1.70

7 weeks

Dose level		Number of rats examined	Percentage of motile sperm	Velocity	Linearity	ALH max	ALH mean	BCF	Average radius	Percentage of circular cells	
										over motile cells	over all cells
Control	Mean	9	87.06	533.78	2.59	39.03	29.88	7.24	25.50	7.21	6.29
	S.D.		3.13	31.49	0.21	2.03	1.55	0.63	3.90	1.64	1.46
Valproic acid	Mean	10	88.20	517.53	2.53	37.90	29.55	7.54	25.51	6.26	5.52
250 mg/kg	S.D.		5.11	42.97	0.25	2.65	2.20	0.48	6.40	2.71	2.44
Valproic acid	Mean	10	72.38 **	515.78	2.40	39.46	30.44	7.54	27.21	7.15	5.29
500 mg/kg	S.D.		13.47	31.89	0.31	4.06	2.32	0.47	5.55	2.59	2.23

10 weeks

Dose level		Number of rats examined	Percentage of motile sperm	Velocity	Linearity	ALH max	ALH mean	BCF	Average radius	Percentage of circular cells	
										over motile cells	over all cells
Control	Mean	9	86.57	523.50	2.46	37.48	28.60	7.11	27.04	5.27	4.51
	S.D.		4.50	51.67	0.19	3.36	2.95	0.39	7.43	2.73	2.28
Valproic acid	Mean	10	87.00	528.10	2.42	38.45	30.08	7.24	27.11	5.13	4.46
250 mg/kg	S.D.		5.10	35.19	0.15	2.69	1.96	0.28	10.27	1.20	1.02
Valproic acid	Mean	10	72.73 **	492.66	2.26	36.14	27.88	7.18	23.89	5.31	3.64
500 mg/kg	S.D.		16.66	46.74	0.31	3.07	2.52	0.37	6.20	2.90	2.17

Table 5. Incidence of retention of step 19 spermatids in stage IX-XI seminiferous tubules in male rats orally administered valproic acid.
*, **: Significantly different from respective control group values at $p<0.05$ and $p<0.01$, respectively.

Dose level		Incidence (%)		
		4 weeks	7 weeks	10 weeks
Control	Number of male rats	10	9	9
	Mean	29.92	26.44	29.98
	S.D.	10.74	18.80	14.46
Valproic acid	Number of male rats	10	10	10
250 mg/kg	Mean	20.96	33.84	27.51
	S.D.	10.65	16.57	10.69
Valproic acid	Number of male rats	10	10	10
500 mg/kg	Mean	38.80	54.36 **	57.47 *
	S.D.	22.87	27.75	25.38

Table 6. Histopathological findings of male rats orally administered valproic acid.

Dose level		4 weeks	7 weeks	10 weeks
Control	No. of males examined	10	10	9
	Degeneration of seminiferous tubules	0	1	0
	Loss or exfoliation of spermatids	0	0	0
Valproic acid	No. of males examined	10	10	10
250 mg/kg	Degeneration of seminiferous tubules	0	0	0
	Loss or exfoliation of spermatids	0	0	0
Valproic acid	No. of males examined	10	10	10
500 mg/kg	Degeneration of seminiferous tubules	1	2	1
	Loss or exfoliation of spermatids	1	2	3

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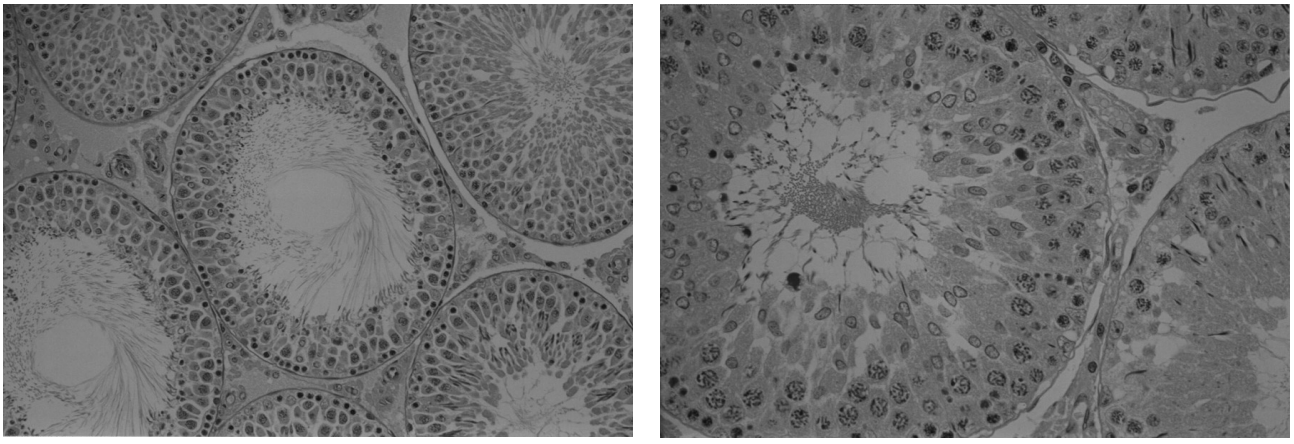

(A) (B)

Photo 1. Histopathological changes in male rats. Cross sections of testis (A). An animal treated with a 10-week 500 mg/kg dose of valproic acid shows loss or exfoliation of spermatids. Cross sections of testis of seminiferous tubules in stage X (B). In an animal treated with a 10-week 500 mg/kg dose of valproic acid, spermatids are still present at the adluminal region of the seminiferous epithelium.

Effects of Olive, Corn, Sesame or Peanut Oil on the Body Weights and Reproductive Organ Weights of Immature Male and Female Rats

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ABSTRACT. Olive, corn, sesame or peanut oil which have been used as vehicles in the immature rat uterotrophic assay or Hershberger assay, for detection of endocrine disrupting effects of environmental chemicals, was administered to ten immature female rats by subcutaneous injection from postnatal day (PND) 21 for 3 or 7 days, and each oil was also administered to ten male rats from PND 21 for 7 and 10 days. The body weights, and the weights of sex and sex accessory organs in female and male rats were measured. There were no significant differences in body weights of female rats between each oil group and the control group, while the body weight of male rats in the group given peanut or olive oil was significantly increased from 8 or 9 days after administration. There were no changes in the sex and sex accessory organ weights of female or male rats related to the endocrine disrupters. The results of the body weights and organ weights demonstrate that each oil is a suitable vehicle for the immature rat uterotrophic assay. However, each oil is suggested to be unsuitable for the Hershberger assay, because the analysis of changes of sex accessory organ weights in this assay might be confused by the increased body weights. — Key words: endocrine, oil, rat

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There is currently much concern that certain environmental chemicals may have the potential to disturb normal sexual differentiation and development in wildlife and humans [6,7]. Recently, the OECD proposed the immature rat uterotrophic assay or Hershberger assay as screening test methods for the detection of the endocrine disrupting effects of these chemicals [10]. Using the uterotrophic assay, estrogenic or anti-estrogenic effects of some chemicals have already been tested [2,4,8,9]. The Hershberger assay according to OECD methods has not yet been reported because the detailed method of this assay were not proposed by the OECD. Various oils such as sesame, corn or peanut oil have been used as the vehicle in the uterotrophic assay, and these oils are also being considered for use as the vehicle in the Hershberger assay. Namely, these oils are usually used as the vehicle in both assays. Some food plants have phytoestrogenic effects [1,5], and oils produced by these plants are considered to have the same effects. However, there are few reports on the effects of oils on the sex or sex accessory organs in female or male rats. Therefore, we examined the effects of oils such as olive, corn, sesame and peanut oil on the organ weights of the uterus and ovaries in immature female rats, and testes, prostate and seminal vesicle in immature male rats.

The chemicals were obtained from the following manufacturers: olive oil (Lot No.005RYP) and sesame oil (Lot No. 006RRK), Fujimi Pharmaceutical (Osaka, Japan); corn oil (Lot No. A4940) and peanut oil (Lot No.A6841), Katayama Chemical Industries, Ltd. (Osaka, Japan). Pregnant Crj:CD (SD) IGS rats at day 14 of gestation were purchased from Charles River Japan, Inc. (Shiga, Japan). At postnatal day (PND) 4, the litters were culled to eight per dam, and dams and pups were kept in polycarbonate pens until weaning. All rats were weaned at PND 20, and then housed individually in stainless steel, wire-mesh cages during the study. The immature rats were weighed, weight-ranked, and assigned randomly to each of the treated and control groups. Each oil treated group consisted of 10 rats. Rats were provided with tap water from the bottle and a commercial diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) *ad libitum* before weaning, and with tap water automatically and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) *ad libitum* after weaning. The animal room was maintained at a temperature of 23±2℃, relative humidity of 55±5%, and artificially illuminated with fluorescent light on a 12 h light/dark cycle (0600 - 1800 h). Female rats in oil groups were necropsied at 3 or 7 days after administration, and male rats were necropsied at 7 or 10 days after administration. The treatment periods were based on OECD proposals [10]. Untreated control groups of female and male rats were also established. Oils were daily injected subcutaneously between 9:30 and 10:30 at 5 ml/rat/day on the dorsal portion from PND 21. Body weight was recorded on a daily basis throughout the study. The animals were sacrificed approximately 24 h after the last administration by bleeding from the posterior vena cava under deep ether anesthesia. After necropsy, the uterus, ovaries, testes, ventral prostate and seminal vesicle were carefully dissected free of adhering fat and mesentery, and then weighed. Differences in body weight and the weight of each organ between each of the oil groups and the control group were assessed for statistical significance using the two-tailed Student's *t* test.

The test data are shown in Tables 1-4. In female rats, there were no significant differences in body weights between each of oil groups and the control. Abnormal clinical signs and premature vaginal opening were not detected in any female rat given oils. Male body weights in the groups given olive or peanut oil were significantly higher than in the control group from 8 and 9 days, respectively. Although there were no significant differences in uterine or ovarian weights between the groups given oils for 3 days and the control group, the absolute uterine weight or relative ovarian weight in the group given corn oil for 7 days was significantly lower than in the control group. In the male rats, the absolute and relative prostate weights in the group given peanut oil for 7 days and the absolute seminal vesicle weight in the group given peanut oil for 10 days were significantly higher than in the control group. Relative testes weights in the groups given corn, sesame or peanut oil for 10 days were significantly lower than in the control group.

Recently, the OECD proposed the uterotrophic assay for detecting chemicals having potential estrogenic or anti-estrogenic effects, and the Hershberger assay for detecting chemicals having potential androgenic or anti-androgenic effects [10]. In these assays, the changes of the sex or sex accessory organ weights are said to be good markers for the evaluation of

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endocrine disrupters [10]. Immature uterotrophic assays have been already performed, in which sesame oil [4,8], peanut oil [2,9] and corn oil [9] have been used as the vehicle. In *in vitro* assays, some food plants are known to have estrogenic effects such as phytoestrogen [1,5], and therefore the oils produced by these plants are suggested to have the same effects. In the future, because of OECD proposals the uterotrophic assay or Hershberger assay will become important screening tests for detecting endocrine disrupters. However, there are few reports on the effects of oil vehicles used in these tests on the sex or sex accessory organ weights. In the present immature rat uterotrophic assay, there were no significant differences in the body weights between each oil group and the control group after 3 or 7 day administration periods and the average values of the body weights among these groups were almost same. These facts demonstrate that each oil used in our study had no nutritional effect on the immature female rats during the present administration periods. In the present study, the absolute uterine weight and relative ovarian weight in the group given corn oil for 7 days were significantly lower than in the control group. The cause of these weight changes could not clarified, but these changes do not necessarily mean uterotrophic activity. Odum et al. [9] also reported that immature female Alpk:AP rats given peanut oil for 3 days did not show uterotrophic change. The results of the body weights and organ weights demonstrate that each oil is a suitable vehicle for the immature rat uterotrophic assay.

The body weights in the male groups given oils were increased with the administration periods. This result demonstrates that oils induce the increase of body weight during the present experimental periods. On the other hand, the relative testes weights in oil groups were lower than in the control group, and this change is suggested as having been caused by the increased body weight. Absolute and relative prostate weights in rats given peanut oil for 7 days and the absolute seminal vesicle weight in rats given peanut oil for 10 days were significantly higher than in control rats. These changes are not suggested as having been related to the oil, because the prostate weight in the group given peanut oil for 10 days was not increased and the relative seminal vesicle weight in the group given peanut oil for 10 days was not changed. Analysis of the changes of sex accessory organ weights in the Hershberger assay may be confused by the increased body weight. Therefore, oils are suggested as being unsuitable for the Hershberger assay, and other non-calorific vehicles should be used in this assay. Ashby et al. [3] proposed hydroxypropyl methoxycellulose as a non-carolific vehicle in the Hershberger assay. Furthermore, Ashby et al. [3] reported that the absolute weights of the prostate and seminal vesicle were increased from PND 21 to PND 36, but the relative weight of these organs were gradually decreased with aging. In the present study, both absolute and relative weights of the prostate and seminal vesicle in the control groups were increased with age. Disagreement between our results and Ashby's may be caused by the difference of the rat strain. It will be necessary to have adequate control data of sex or sex accessory organ weights in immature male rats for each laboratory.

ACKNOWLEDGMENT

This work was supported by a grant from Ministry of International Trade and Industry.

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Table 1. Body weight, and absolute uterine and ovarian weights of immature female rats administered olive, corn, sesame or peanut oil from 21 days of age for three or seven days

Treated days	Group	Initial body weight (g)	Final body weight (g)	Uterine weight (mg)	Ovarian weight (mg)
3	Untreated	49.2±3.4	58.3±3.3	37.3±9.9	20.3±5.3
	Olive oil	48.6±3.0	58.2±3.4	31.1±6.2	19.0±4.0
	Corn oil	48.2±3.1	58.1±3.3	33.3±7.4	19.6±5.4
	Sesame oil	48.9±3.3	57.8±4.2	31.9±6.8	18.0±3.9
	Peanut oil	48.4±2.4	57.9±3.0	33.3±3.2	18.7±3.7
7	Untreated	49.0±2.9	81.4±4.1	56.8±6.8	21.5±2.4
	Olive oil	48.2±2.5	82.5±3.2	54.5±11.1	20.6±4.0
	Corn oil	48.6±2.9	81.1±4.8	50.6±6.1*	19.2±2.9
	Sesame oil	49.0±3.4	80.8±6.0	51.4±12.0	19.6±2.6
	Peanut oil	48.9±2.8	82.9±6.0	54.1±8.8	20.8±3.2

No. of animals: 10 in each group.
Mean±SD.
*Significantly different from corresponding untreated control at $P < 0.05$ (Student's *t* test).

Table 2. Body weight, and relative uterine and ovarian weights of immature female rats administered olive, corn, sesame or peanut oil from 21 days of age for three or seven days

Treated days	Group	Initial body weight (g)	Final Body weight (g)	Uterine weight (mg/100g)	Ovarian weight (mg/100g)
3	Untreated	49.2±3.4	58.3±3.3	63.5±16.3	34.3±7.5
	Olive oil	48.6±3.0	58.2±3.4	53.4±10.4	32.8±7.6
	Corn oil	48.2±3.1	58.1±3.3	57.6±14.2	33.8±9.7
	Sesame oil	48.9±3.3	57.8±4.2	55.5±12.3	31.1±5.6
	Peanut oil	48.4±2.4	57.9±3.0	58.3±6.4	32.7±7.0
7	Untreated	49.0±2.9	81.4±4.1	70.0±9.7	26.5±3.0
	Olive oil	48.2±2.5	82.5±3.2	66.0±13.2	25.0±4.7
	Corn oil	48.6±2.9	81.1±4.8	62.7±8.8	23.6±2.9*
	Sesame oil	49.0±3.4	80.8±6.0	63.9±15.8	24.4±3.2
	Peanut oil	48.9±2.8	82.9±6.0	65.5±11.4	25.3±4.8

No. of animals: 10 in each group.
Mean±SD.
*Significantly different from corresponding untreated control at $P < 0.05$ (Student's *t* test).

Table 3. Body weight, and absolute testes, prostate and seminal vesicle weights of immature male rats administered olive, corn, sesame or peanut oil from 21 days of age for seven or ten days

Treated days	Group	Initial body weight (g)	Final body weight (g)	Testes (mg)	Prostate (mg)	Seminal vesicle (mg)
7	Untreated	50.0±3.0	85.6±4.2	619.3±101.1	36.2±11.1	36.5±11.1
	Olive oil	50.7±3.1	87.3±5.3	594.3±50.2	31.5±10.6	35.7±10.3
	Corn oil	50.6±3.8	86.2±5.6	600.9±92.1	44.4±7.8	32.2±8.1
	Sesame oil	49.5±3.3	87.3±5.4	605.5±65.7	44.6±10.9	34.6±6.0
	Peanut oil	50.3±3.4	88.1±5.1	594.2±83.9	47.7±10.6*	34.8±12.0
10	Untreated	49.9±2.1	112.7±3.8	877.1±41.8	58.0±7.9	45.5±11.1
	Olive oil	50.8±3.6	120.1±6.7**	887.5±79.2	55.8±12.9	53.8±9.6
	Corn oil	50.7±3.3	117.1±8.2	856.2±72.0	62.1±7.9	50.0±8.9
	Sesame oil	49.2±4.0	118.4±9.4	852.1±93.8	65.2±11.9	49.2±12.1
	Peanut oil	50.3±3.5	122.0±6.4**	889.7±55.3	60.4±12.3	54.6±7.5*

No. of animals: 10 in each group.
Mean±SD.
*Significantly different from corresponding untreated control at $P < 0.05$ (Student's *t* test).
**Significantly different from corresponding untreated control at $P < 0.01$ (Student's *t* test).

Table 4. Body weight, and relative testes, prostate and seminal vesicle weights of immature male rats administered olive, corn, sesame or peanut oil from 21 days of age for seven or ten days

Treated days	Group	Initial body weight(g)	Body weight (g)	Testes (mg/100g)	Prostate (mg/100g)	Seminal vesicle (mg/100g)
7	Untreated	50.0±3.0	85.6±4.2	658.1±82.6	38.9±13.0	38.7±10.7
	Olive oil	50.7±3.1	87.3±5.3	633.9±41.6	33.2±9.2	38.6±12.8
	Corn oil	50.6±3.8	86.2±5.6	643.3±78.7	47.7±8.4	34.5±7.9
	Sesame oil	49.5±3.3	87.3±5.4	638.9±61.9	47.0±11.5	36.6±6.7
	Peanut oil	50.3±3.4	88.1±5.1	623.9±71.1	49.9±9.7*	36.7±13.1
10	Untreated	49.9±2.1	112.7±3.8	779.2±42.3	51.5±7.2	40.4±10.1
	Olive oil	50.8±3.6	120.1±6.7**	738.9±54.6	46.4±10.2	44.9±7.8
	Corn oil	50.7±3.3	117.1±8.2	730.8±31.7**	53.0±6.5	43.1±9.4
	Sesame oil	49.2±4.0	118.4±9.4	720.6±65.9*	55.1±9.2	41.4±9.3
	Peanut oil	50.3±3.5	122.0±6.4**	730.7±49.4*	49.6±10.5	45.0±7.2

No. of animals : 10 in each group
Mean±SD.
*Significantly different from corresponding untreated control at $P < 0.05$ (Student's *t* test).
**Significantly different from corresponding untreated control at $P < 0.01$ (Student's *t* test).

CHAPTER 4

Carcinogenicity Related To

A REVIEW OF THE MORTALITY, BODYWEIGHT AND FOOD CONSUMPTION DATA FROM CD (SD) IGS RAT TUMORIGENICITY STUDIES COMPLETED IN 1998 TO 2001.

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ABSTRACT. At Huntingdon Life Sciences, data obtained from the Charles River International Genetic Standard (IGS) strain of Sprague-Dawley rat have been closely monitored since the introduction of this new strain designation in 1996. The mortality, bodyweight and food consumption data from the control groups of up to 27 IGS rat tumorigenicity studies have been reviewed to identify any possible trends with time over the period of 1998 to 2001. Low protein maintenance diet was used in all these dietary oral gavage studies. Statistical analysis of the terminal mortality against time has indicated a trend towards lower values in male rats, but not in female rats. However, the mortality pattern over 104 weeks for studies completed in 2001 is slightly decreased in both sexes in comparison to studies completed in 1998 or 1999-2000. There were no major differences over time for the bodyweight growth pattern, and the bodyweight gain and food consumption data analysed over the first year have only shown minor differences between the three comparison groups. From the results currently available, it can be concluded that the IGS rat continues to show a high mortality pattern in female rats, but there is indication of an improvement over time in the longevity of male rats. — Key words: Bodyweight, Crl:CD® BR (VAF) IGS rat, food consumption, mortality, tumorigenicity studies.

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INTRODUCTION

The Charles River International Genetic Standard (IGS) strain designation of Sprague-Dawley rat Crl:CD® BR (VAF) superseded the original strain designation of rat from 1996. At these laboratories, the data obtained from the IGS rat have been closely monitored and compared with data obtained from the original strain designation of Sprague-Dawley rat. A comparison of data obtained from the first 13 weeks of gang housed dietary studies has shown that there were no remarkable differences in the in-life, laboratory and organ weight parameters examined between the IGS and original strain of Sprague-Dawley rat [1, 2]. Subsequent assessments of the performance of the IGS rat in dietary and oral gavage tumorigenicity studies in comparison with the original strain designation of Sprague-Dawley rat have shown that there were no remarkable differences in the in-life parameters (mortality, bodyweight and food consumption) and tumour data between the two strain designations [3-6].

In this review, the mortality, bodyweight and food consumption data obtained from up to 27 IGS rat tumorigenicity studies completed over the period of 1998 to 2001 have been assessed to identify any possible trends with time.

MATERIALS AND METHODS

Animals:

Male and female Sprague-Dawley Crl:CD® BR (VAF) IGS rats obtained from Charles River UK breeding laboratories and maintained as control rats for tumorigenicity studies at Huntingdon Life Sciences. The rats were approximately 6 weeks of age at start of study, mainly housed 5 rats/cage (singly housed studies are indicated, where appropriate) and maintained under standard laboratory conditions, with target ranges of 19-23°C for temperature and 40-70% for relative humidity. A 12 hour light and 12 hour dark cycle was maintained. The animals were given a low protein rodent maintenance diet (Special Diets Services Rat and Mouse No. 1, typically 14.5% protein, 3% fat, 4% fibre) *ad libitum* throughout the study and tap water was also supplied *ad libitum* to the animals via water bottles.

Study Design:

The studies reviewed were dietary or oral gavage administered tumorigenicity studies that terminated between 1998 and 2001. The numbers of control groups reviewed were 25 male and 27 female groups. There were at least 50 males and 50 females in each control group.

Data presentation and analysis:

Mortality:

The mean terminal (Week 104) percentage mortality values are presented over the time periods of 1998, 1999-2000 and 2001 for the IGS rat studies, graphically (Figure 1) and in Table 1 (also detailing the route of administration and housing conditions). For comparative purposes, data from studies using the original strain of Sprague-Dawley rat are also presented in Figure 1 over selected time periods from 1987 to 1997. The distribution of the percentage mortality for each control group at study Week 104 is presented chronologically between 1998 and 2001 (Figures 2 and 3) for IGS rat studies. A regression analysis of mortality against time [7] was performed followed by a two-tailed t-test of the slope; P-values and the direction of slope are presented for gang-housed dietary studies only. Similar comparisons were also performed over the same period for all studies. The mortality pattern for IGS rat studies completed in 1998, 1999-2000 and 2001 is presented in Figures 4 and 5 for the period of Weeks 52 to 104 only, as mortality in the first year is very low.

Bodyweight:

The bodyweight growth pattern over the 104-week treatment period for IGS rat studies completed in 1998, 1999-2000 and 2001 is presented in Figure 6. The mean bodyweight gain values over Weeks 0 to 52 (the period of maximal growth) are also compared over the same time periods in Figure 7. A comparison based on a 't' distribution was made following analysis of variance to compare intergroup differences. For comparative purposes, the bodyweight gain (Weeks 0 to 52) values for gang and singly housed dietary and oral gavage administered studies are detailed in Table 2 over selected time periods.

Food consumption:

The mean weekly food consumption (g/rat/week) values are compared over the period of Weeks 1 to 52 for IGS rat studies completed in 1998, 1999-2000 and 2001 and presented in Figure 8. A comparison based on a 't' distribution was made following analysis of variance to compare intergroup differences. For comparative purposes, the mean food consumption (Weeks 1 to 52) values for gang and singly housed dietary and oral gavage administered studies are detailed in Table 3 over selected time periods.

RESULTS AND DISCUSSION

In order to place the Sprague-Dawley rat mortality values in historical perspective, the terminal mortality values are presented in Figure 1, over selected time periods from 1987 to 1997 for the original strain and from 1998 to 2001 for the IGS strain of rat. The trend towards increasing mortality is apparent, particularly from 1993 in female rats. However, since the first IGS studies completed 104 weeks in 1998, there appears to be a trend towards lower terminal mortality values for male IGS rat studies.

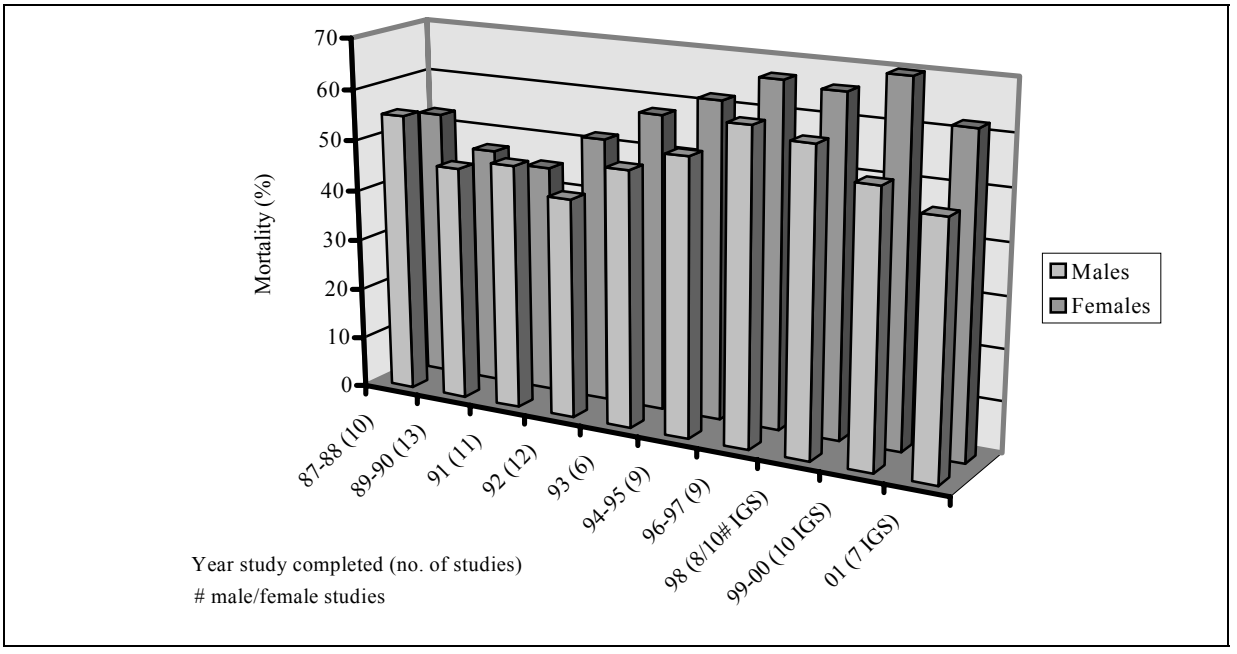

Figure 1. Mortality at Week 104 - a comparison over selected time periods

The individual terminal mortality values for the IGS rat studies were plotted against time over the period of 1998 to 2001 (Figures 3 and 4) and a regression analysis was performed. For gang housed dietary studies, a statistically significant trend towards lower mortality values was noted for male rats (P=0.019), but not for female rats (P=0.146). Similar findings were apparent when all study types were compared over this period (P=0.023 for males and P=0.253 for females).

The mortality pattern over the period of Weeks 52 to 104 is presented in Figures 4 (male rats) and 5 (female rats). It is apparent in both sexes that the mortality pattern for the IGS rat studies completed in 2001 is slightly decreased in comparison with studies completed in 1998 or 1999-2000.

For comparative purposes, the mean mortality, together with the Standard deviation and number of studies, are detailed in Table 1 for the different study types over selected time periods.

The bodyweight growth pattern over the 104-week study period for male IGS rats (Figure 6) indicates that there was a marginally lower pattern obtained for studies completed in 2001 in comparison with studies completed in 1998 or 1999-2000.

The bodyweight gain over Weeks 0 to 52 (Figure 7) was largely similar between the comparison groups and there was no apparent trend with time. However, the studies completed in 1999-2000 showed marginally higher mean values for males and females in comparison with the other groups, but statistical significance was only demonstrated in the females ($P \leq 0.05$). For comparative purposes, the bodyweight gain (Weeks 0 to 52) values are detailed in Table 2 for the different study types over selected time periods.

The mean weekly food consumption over the period of Weeks 1 to 52 (Figure 8) for the three comparison groups were similar for both sexes (P>0.05). For comparative purposes, the mean food consumption values (Weeks 1 to 52) are detailed in Table 3 for the different study types over selected time periods.

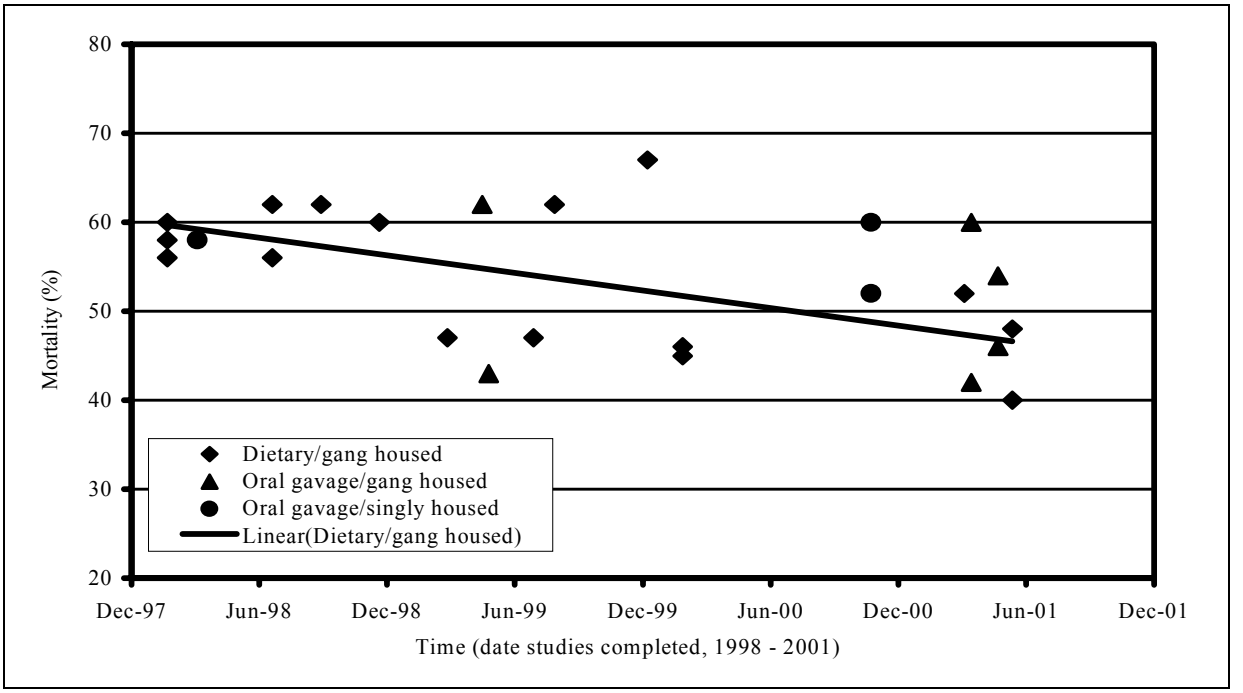

Figure 2. Mortality distribution at Week 104 - males

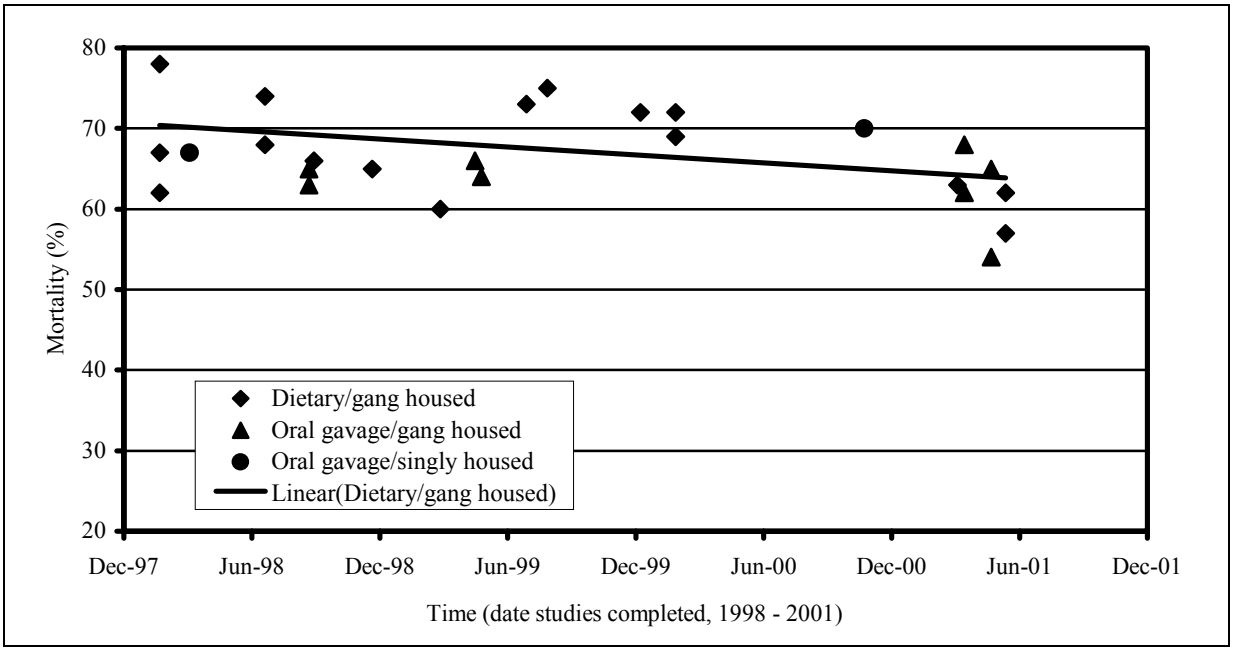

Figure 3. Mortality distribution at Week 104 - females

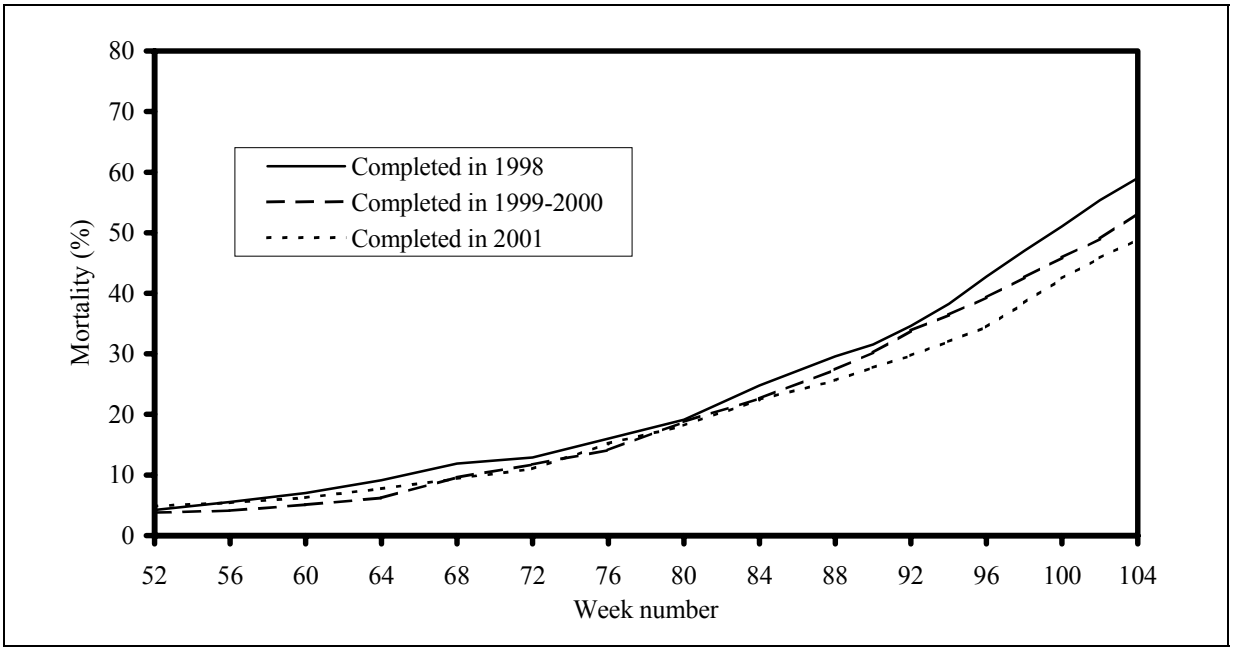

Figure 4. Mortality over Weeks 52 to 104 - males

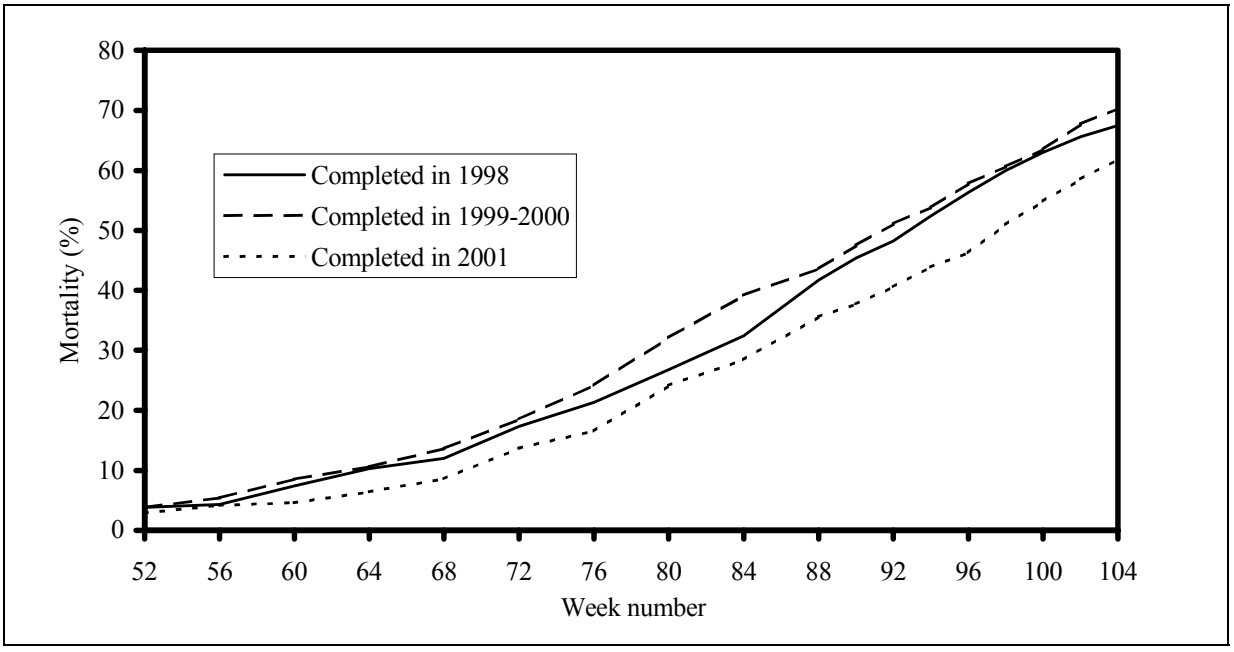

Figure 5. Mortality over Weeks 52 to 104 - females

Table 1. Mortality (%) at Week 104 - dietary and oral gavage rat studies

Studies completed		Dietary/ gang housed		Oral gavage/ gang housed		Oral gavage/ singly housed		All studies	
		M	F	M	F	M	F	M	F
1998	Mean	59	69		64	58	67	59	68
	SD	2.5	5.5		1.4	–	–	2.4	4.9
	n	7	7	0	2	1	1	8	10
1999-2000	Mean	52	70	53	65	56	76	53	70
	SD	9.6	5.3	13.4	1.4	5.7	8.5	8.8	6.1
	n	6	6	2	2	2	2	10	10
2001	Mean	47	61	51	62			49	62
	SD	6.1	3.2	8.1	6.0			7.0	4.7
	n	3	3	4	4	0	0	7	7
All studies	Mean	54	68	51	63	57	73	54	67
1998-2001	SD	7.9	6.0	8.7	4.2	4.2	7.9	7.7	6.2
	n	16	16	6	8	3	3	25	27

M Male rats, F Female rats, SD Standard deviation, n Number of studies

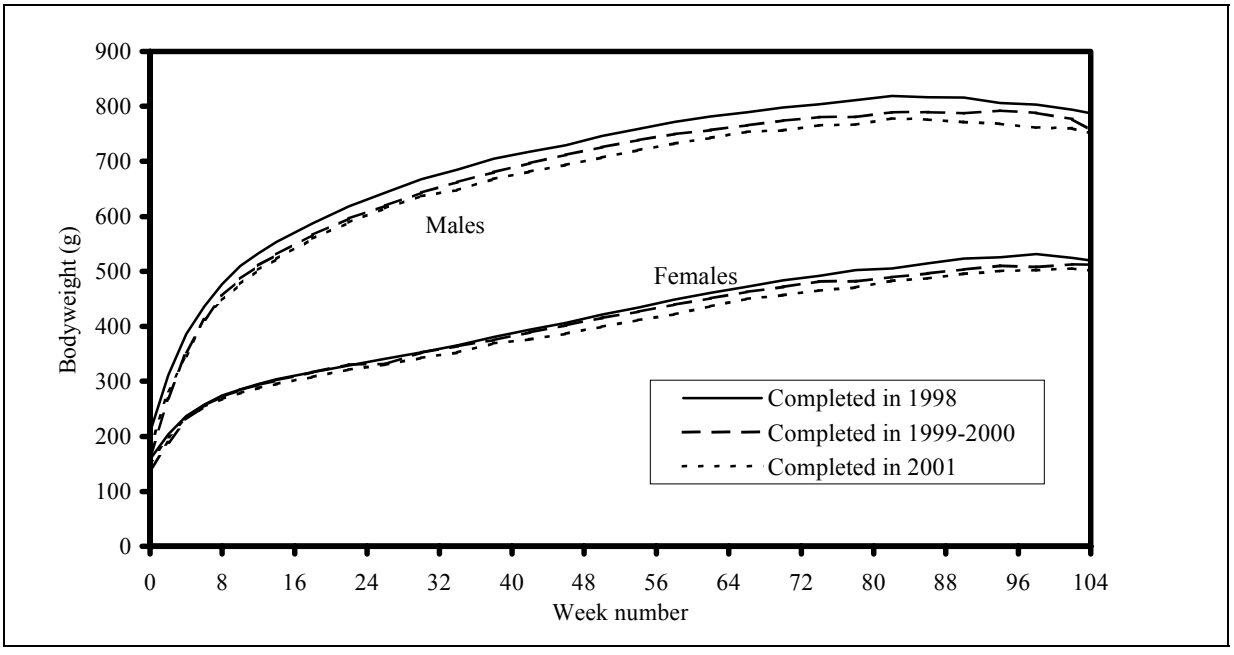

Figure 6. Bodyweight growth pattern

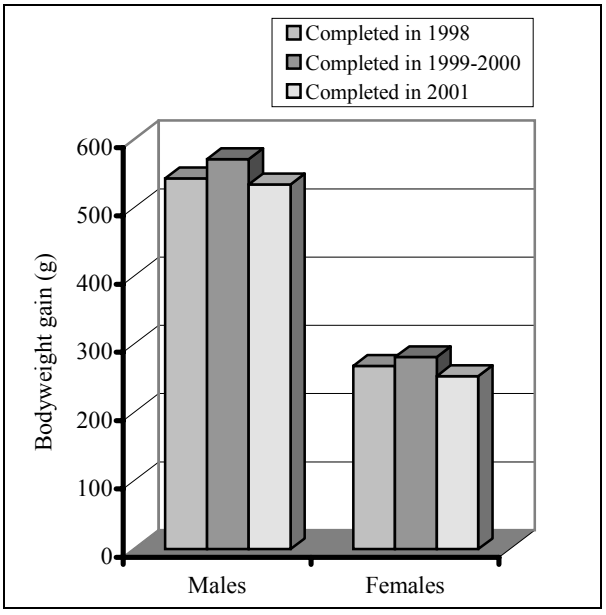

Figure 7. Bodyweight gain - Weeks 0 to 52

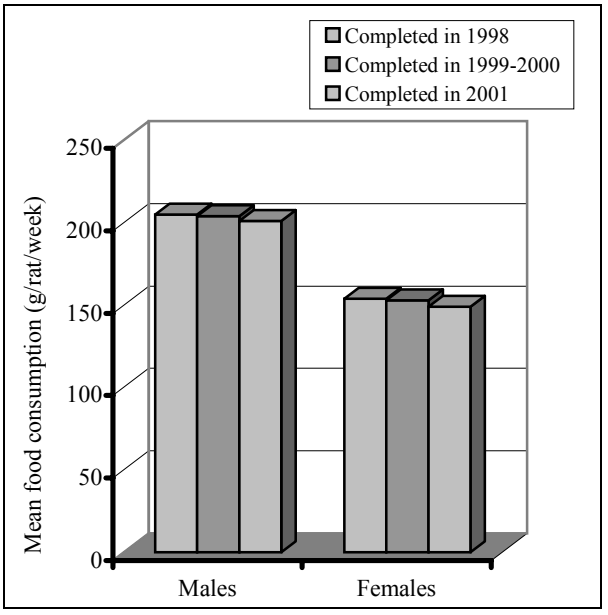

Figure 8. Food consumption - Weeks 1 to 52

Table 2. Bodyweight gain (g) over Weeks 0 to 52 - dietary and oral gavage studies

Studies completed		Dietary/ gang housed		Oral gavage/ gang housed		Oral gavage/ singly housed		All studies	
		M	F	M	F	M	F	M	F
1998	Mean	551	267		273	489	267	543	268
	SD	23.8	11.2		0.7	–	–	31.1	9.4
	n	7	7	0	2	1	1	8	10
1999-2000	Mean	571	279	586	277	553	290	571	281
	SD	42.5	22.0	14.5	1.1	21.2	4.9	34.6	17.1
	n	6	6	2	2	2	2	10	10
2001	Mean	534	264	535	245			534	253
	SD	26.0	41.9	48.4	29.3			37.4	33.4
	n	3	3	4	4	0	0	7	7
All studies 1998-2001	Mean	555	271	552	260	532	282	552	269
	SD	33.6	22.1	46.4	25.0	39.9	13.5	36.7	22.7
	n	16	16	6	8	3	3	25	27

M Male rats, F Female rats, SD Standard deviation, n Number of studies

Table 3. Mean food consumption (g/rat/week) over Weeks 1 to 52 – dietary and oral gavage studies

Studies completed		Dietary/ gang housed		Oral gavage/ gang housed		Oral gavage/ singly housed		All studies	
		M	F	M	F	M	F	M	F
1998	Mean	203	152		153	224	170	205	154
	SD	1.5	2.0		0.7	–	–	7.7	5.9
	n	7	7	0	2	1	1	8	10
1999-2000	Mean	197	149	211	152	217	166	204	153
	SD	4.5	3.7	17.0	11.3	5.7	0.7	11.4	8.4
	n	6	6	2	2	2	2	10	10
2001	Mean	198	147	204	151			201	149
	SD	3.2	5.3	2.2	2.9			4.3	4.3
	n	3	3	4	4	0	0	7	7
All studies	Mean	200	150	207	152	219	167	204	152
1998-2001	SD	4.2	3.8	8.5	4.7	5.7	2.6	8.6	6.6
	n	16	16	6	8	3	3	25	27

M Male rats, F Female rats, SD Standard deviation, n Number of studies

In conclusion, the results from the data currently available, for mortality pattern, bodyweight pattern and food consumption from tumorigenicity studies, indicate that the IGS rat has not differed remarkably over time. However, there is an indication of an improvement in the longevity of male rats.

As the survival in the female rats remains low, consideration has to be given for increasing group sizes to ensure sufficient survivors at termination, the use of diet restriction methods, or the use of alternative strains of rat [8, 9], to ensure acceptability of the tumorigenicity study to regulatory authorities.

ACKNOWLEDGEMENTS

Thanks are due to Richard Brammer of the Department of Statistics for the statistical analysis of mortality data. Thanks are also due Robert J. Harling for the Sponsorship of this project and Colin J. Hardy for reviewing the manuscript.

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Spontaneous Neoplastic Lesions and Causes of Death in Crj:CD(SD)IGS Rats Fed a Commercial Low Protein Diet in 52- and 104-week Studies

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ABSTRACT. To accumulate historical data on spontaneous neoplastic lesions, untreated Crj:CD(SD)IGS (IGS) rats used in the 52- and 104-week long-term studies were examined histopathologically. These rats were fed a low protein commercial diet (18% protein content) during the studies. In the 52-week study, histiocytic sarcoma (5.0%), pituitary adenoma (5.0%) and keratoacanthoma in the skin/suncutis (5.0%) were observed in males and pituitary adenoma (20.0%) and mammary fibroadenoma (5.0%) were detected in females. In the 104-week study, the most common neoplasm was pituitary ademona in both sexes (50.0% in male and 70.0% in female). In male rats, incidences of other major neoplasms (> 5.0%) were pheochromocytoma in the adrenal gland (18.0%), histiocytic sarcoma (8.0%), follicular cell adenoma and C-cell adenoma in the thyroid, and keratoacanthoma in the skin/subcutis (6.0% in each). In female rats, a total incidence of pituitary adenoma and carcinoma was 88.0%. Incidences of other major neoplasms (> 5.0%) were fibroadenoma (42.0%), adenocarcinoma (18.0%) and adenoma (12.0%) in the mammary gland, endometrial stromal polyp in the uterus (8.0%), C-cell adenoma in the thyroid and pheochromocytoma in the adrenal gland (6.0% in each). No animals died until the end of the 52-week study. In the 104-week study, the most common cause of death was pituitary tumors in both sexes. — Key words: Causes of death, Crj: CD(SD)IGS, Tumors, Rat.

CD(SD)IGS-2001: 138-142

INTRODUCTION

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. 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. The animals are expected to meet internationalization of research and development of new drugs. However, background data on spontaneous neoplastic lesions of IGS rats have not yet been fully accumulated. Therefore, we examined histopathologically spontaneous neoplastic lesions of the IGS rats and discuss the causes of death.

MATERIALS AND METHODS

Seventy male and 70 female IGS rats were purchased from Tsukuba Breeding Center (Charles River Japan, Inc., Chiba) at 4 weeks of age. The rats were housed individually in a wire-mesh cage (21×35×20 cm) and were maintained in a barrier-sustained room controlled at 21 - 23℃ and 30 - 63% relative humidity, and ventilated 10 times per hr, with a 12-hr light-dark cycle. 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., 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 (Exp. Anim. 36: 285-288, 1987).

After the 1-week acclimatization period, 20 male and 20 female IGS rats were used in the 52-week study and 50 male and 50 female IGS rats were used in the 104-week study. They were observed for clinical signs and mortality twice daily and weighed weekly. Food consumption for 24 hrs was measured weekly. During the study periods, all rats found dead or sacrificed in a moribund condition were necropsied as soon as possible. At the end of studies, all rats survived were euthanatized by ether inhalation and subjected to a complete necropsy. All organs, tissues and gross lesions were fixed in 10% neutral buffered formalin except for the eyeballs which were fixed in Davidson's solution and the testes which were fixed in Bouin's solution. These tissues were routinely trimmed, embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and examined histopathologically.

RESULTS

The incidences of neoplastic lesions in IGS rats are shown in Tables 1 and 2. No animals died in the course of the 52-week study. Mortality at the end of the 104-week study was 66.0% and 42.0% for males and females, respectively.

In the 52-week study, histiocytic sarcoma (5.0%), pituitary adenoma (5.0%) and keratoacanthoma in the skin/subcutis (5.0%) were observed in males and pituitary adenoma (20.0%) and mammary fibroadenoma (5.0%) were found in females.

In the 104-week study, the most common neoplasm was pituitary tumor (ademona/carcinoma) in both sexes (50.0% in males and 88.0% in females). In male rats, no pituitary carcinoma was observed. Incidences of other neoplasms, in order of decreasing incidence, were pheochromocytoma in the adrenal gland (18.0%), histiocytic sarcoma (8.0%), follicular cell adenoma and C-cell adenoma in the thyroid, and keratoacanthoma in the skin/subcutis (6.0% in each), astrocytoma in the brain and C-cell carcinoma in the thyroid (4.0% in each), thymoma, hepatocellular ademona, acinar cell adenoma in the pancreas, tubular cell carcinoma and liposarcoma in the kidney, cortical adenoma in the adrenal gland, basal cell tumor and sebaseous adenoma in the skin/subcutis (2.0% in each).

In female rats, thirty-five adenoma (70.0%) and 9 carcinoma (18.0%) were detected in the pituitary. Incidences of other neoplasms, in order of decreasing incidence, were fibroadenoma (42.0%), adenocarcinoma (18.0%) and adenoma (12.0%) in the mammary gland, endometrial stromal polyp in the uterus (8.0%), C-cell adenoma in the thyroid and pheochromocytoma

in the adrenal gland (6.0% in each), histiocytic sarcoma (4.0%), malignant lymphoma, mesenchymal tumor in the kidney, thecoma in the ovary, hemangioma, squamous cell carcinoma and leiomyosarcoma in the uterus, astrocytoma in the brain and spinal cord, squamous cell papilloma, basal cell tumor, hemangioma and lipoma in the skin/subcutis (2.0% in each).

The causes of death in IGS rats in the 104-week study are shown in Tables 3. The most common cause of death was pituitary tumors in both sexes (42.4% in males, 66.7% in females). Most of the rats with pituitary tumors exhibited clinically emaciation, ocular discharge or decreased locomotor activity before their death. Other major causes of death (> 5.0%) were histiocytic sarcoma in males (12.1%) and females (9.5%), chronic nephropathy/nephritis in males (12.1%), and cardiomyopathy in males (9.1%). Severe degrees of chronic nephropathy/nephritis and cardiomyopathy were found only in male rats without any lethal tumors. Liposarcoma in the kidney, squamous cell carcinoma in the uterus, basal cell tumor in the skin, and adenocarcinoma in the mammary gland were all malignant type tumors, which metastasizsed into other tissues and organs. Astrocytoma in a male rat showed invasive proliferation in the cerebrum and brain stem. Keratoacanthoma occurred in the head and neck regions of a male rat, resulting in food ingestion disturbance.

DISCUSSIONS

Spontaneously occurring tumors in the IGS rats in the 52- and 104-week studies were histopahtologically examined. In both sexes, pituitary tumors were the most common tumors in the 104-week study. This result was coincided with other 104-week studies of the IGS [2, 3, 5, 6, 8-10] rats and its original Sprague-Dawley strain (CD) of the rat in the Charles River Laboratories [1, 4, 7]. Carcinomas in the pituitary were differentiated from adenomas by invasion of adjacent tissues, such as the brain. In our study, the pituitary tumors (adenoma/carcinoma) were found in 50.0% of males and 88.0% of females. The incidences were within the range of the minimum and maximal values from other studies on the IGS rats (29.6 - 68.0% in males, 50.3 - 90.0% in females) [2, 3, 5, 6, 8-10] and on the CD rats (27.9 - 66.8% in males, 49.4 - 88.1% in females) [1, 3-5, 7, 10]. The next common tumors in the IGS rats were pheochromocytoma of the adrenal gland in males (18.0%) and fibroadenoma of the mammary gland in females (42.0%). In addition, most of the tumors with a higher incidence in the IGS rats, especially females, in the present study were related to the endocrine and reproductive systems and the mammary glands, resembling the tumors observed in the IGS and CD rats in other studies [1-10]. These results suggest that there are no noticeable differences of spontaneously occurring tumors in the IGS rats between our facility and other laboratories, and that there are no essential differences of the tumors between the IGS rats and the CD rats [1-10]. Moreover, there were no considerable differences of the tumor incidence between the IGS rats fed the low protein diet and the IGS rats fed a normal standard diet [2, 3, 5, 6, 8-10]. In the present study, the most common cause of death was pituitary tumors in the IGS rats, being coincided with another studies using IGS rats [8]. It is likely that the markedly high incidence of the pituitary tumors in the IGS rats may be closely related to their cause of death. On the basis of the above data, it is indicated that spontaneously occurring tumors in the IGS strain is comparable to those in the CD strain in the long-term carcinogenicity study.

ACKNOWLEDGMENTS

We are grateful to Y. Ogata, M. Kaneko, H. Suzaki, S. Ishikawa, Y. Amano, T. Fujii, and K. Satou for their technical assistance.

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Table 1. Incidences of neoplastic lesions in male Crj:CD(SD)IGS rats fed a low protein diet

Organs and tumors	Study period(wks)	52	104		
		KD	KD	FD	Total
No. of rats examined		20	17	33	50
Cardiovascular System		-	-	-	-
Hematopoietic System					
Multiple sites					
Histiocytic sarcoma		1(5.0)	0(0)	4(12.1)	4(8.0)
Thymus					
Thymoma		0(0)	1(5.9)	0(0)	1(2.0)
Respiratory System		-	-	-	-
Digestive System					
Liver					
Hepatocellular adenoma		0(0)	0(0)	1(3.0)	1(2.0)
Pancreas					
Acinar cell adenoma		0(0)	1(5.9)	0(0)	1(2.0)
Urinary System					
Kidney					
Tubular cell carcinoma		0(0)	0(0)	1(3.0)	1(2.0)
Liposarcoma		0(0)	0(0)	1(3.0)	1(2.0)
Reproductive System		-	-	-	-
Nervous System					
Brain					
Astrocytoma		0(0)	1(5.9)	1(3.0)	2(4.0)
Special Sense Organ		-	-	-	-
Endocrine System					
Pituitary					
Adenoma		1(5.0)	9(52.9)	16(48.5)	25(50.0)
Thyroid					
Follicular cell carcinoma		0(0)	1(5.9)	2(6.1)	3(6.0)
C-cell adenoma		0(0)	1(5.9)	2(6.1)	3(6.0)
C-cell carcinoma		0(0)	1(5.9)	1(3.0)	2(4.0)
Adrenal gland					
Cortical adenoma		0(0)	0(0)	1(3.0)	1(2.0)
Pheochromocytoma		0(0)	7(41.2)	2(6.1)	9(18.0)
Musculoskeletal System		-	-	-	-
Integumentary System					
Skin/subcutis					
Keratoacanthoma		1(5.0)	1(5.9)	2(6.1)	3(6.0)
Basal cell tumor		0(0)	1(5.9)	0(0)	1(2.0)
Sebaceous adenoma		0(0)	1(5.9)	0(0)	1(2.0)
Body Cavities		-	-	-	-

KD: Killed by design. FD: Found dead. -: No neoplastic lesions.
Values in parentheses represent percentages.

Table 2. Incidences of neoplastic lesions in female Crj:CD(SD)IGS rats fed a low protein diet

Organs and tumors	Study period(wks)	52	104		
		KD	KD	FD	Total
No. of rats examined		20	29	21	50
Cardiovascular System		-	-	-	-
Hematopoietic System					
Multiple sites					
Histiocytic sarcoma		0(0)	1(3.4)	1(4.8)	2(4.0)
Malignant lymphoma		0(0)	0(0)	1(4.8)	1(2.0)
Respiratory System		-	-	-	-
Digestive System		-	-	-	-
Urinary System					
Kidney					
Mesenchymal tumor		0(0)	0(0)	1(4.8)	1(2.0)
Reproductive System					
Ovary					
Thecoma		0(0)	1(3.4)	0(0)	1(2.0)
Uterus					
Endometrial stromal polyp		0(0)	4(13.8)	0(0)	4(8.0)
Hemangioma		0(0)	1(3.4)	0(0)	1(2.0)
Squamous cell carcinoma		0(0)	0(0)	1(4.8)	1(2.0)
Leiomyosarcoma		0(0)	1(3.4)	0(0)	1(2.0)
Nervous System					
Brain					
Astrocytoma		0(0)	0(0)	1(4.8)	1(2.0)
Spinal cord					
Astrocytoma		0(0)	1(3.4)	0(0)	1(2.0)
Special Sense Organ		-	-	-	-
Endocrine System					
Pituitary					
Adenoma		4(20.0)	19(65.5)	16(76.2)	35(70.0)
Carcinoma		0(0)	6(20.7)	3(14.3)	9(18.0)
Thyroid					
C-cell adenoma		0(0)	2(6.9)	1(4.8)	3(6.0)
Adrenal gland					
Pheochromocytoma		0(0)	3(10.3)	0(0)	3(6.0)
Pheochromocytoma, malignant		0(0)	1(3.4)	0(0)	1(2.0)
Musculoskeletal System		-	-	-	-
Integumentary System					
Skin/subcutis					
Squamous cell papilloma		0(0)	1(3.4)	0(0)	1(2.0)
Basal cell tumor		0(0)	0(0)	1(4.8)	1(2.0)
Hemangioma		0(0)	1(3.4)	0(0)	1(2.0)
Lipoma		0(0)	1(5.9)	0(0)	1(2.0)
Mammary gland					
Adenoma		0(0)	6(20.7)	0(0)	6(12.0)
Adenocarcinoma		0(0)	6(20.7)	3(14.3)	9(18.0)
Fibroadenoma		1(5.0)	16(55.2)	5(23.8)	21(42.0)
Body Cavities		-	-	-	-

KD: Killed by design. FD: Found dead. -: No neoplastic lesions.
Values in parentheses represent percentages.

Teble 3. Major causes of death in Crj:CD(SD)IGS rats fed a low protein diet

Sex	Major cause of death	Number of rats (%)
Male	Number of animals died	33
	Pituitary adenoma	14 (42.4)
	Histiocytic sarcoma	4 (12.1)
	Chronic nephropathy/nephritis (severe)	4 (12.1)
	Cardiomyopathy (severe)	3 (9.1)
	Liposarcoma (metastatic) in the kidney	1 (3.0)
	Astrocytoma in the brain	1 (3.0)
	Keratoacanthoma in the skin	1 (3.0)
	Cardiac thrombosis	1 (3.0)
	Undetermined	4 (12.1)
Female	Number of animals died	21
	Pituitary adenoma	11 (52.4)
	Pituitary carcinoma	3 (14.3)
	Histiocytic sarcoma	2 (9.5)
	Cardiomyopathy (severe)	1 (4.8)
	Squamous cell carcinoma (metastatic) in the uterus	1 (4.8)
	Basal cell tumor (metastatic) in the skin	1 (4.8)
	Adenocarcinoma (metastatic) in the mammary gland	1 (4.8)
	Malocculusion	1 (4.8)

CHAPTER 5

Pharmacology Related To

Diet Stimulation as a Synergistic Factor of Aggravation in a Pancreatic Bile Duct Ligation-induced Rat Pancreatitis Model

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ABSTRACT. We evaluated the association between aggravation of pancreatitis and multiple factors enhancing pancreatic exocrine secretion using a rat model of pancreatic bile duct ligation (PBDL)-induced pancreatitis. Under fasting and non-fasting conditions, a PBDL group, a second group treated by hepatic bile duct ligation (BDL) and a third group treated by pancreatic duct ligation (PDL) were compared in terms of serum amylase (S-amylase) activity. The S-amylase activity in the PBDL group was higher than in the sham group. In the PDL group, the increase in S-amylase activity was lower than in the PBDL group. In the BDL group, no increase in S-amylase activity was observed. Diversion of pancreatic and/or bile juice in these groups resulted in no increase of S-amylase activity. Truncal vagotomy or injection of an anticholinergic drug or a cholecystokinin (CCK) $_1$-receptor antagonist inhibited pancreatic exocrine secretion and S-amylase activity in the non-fasting PBDL group but not in the fasting PBDL group. These results suggest that retention of pancreatic juice in the pancreatic duct is necessary for the increase of S-amylase activity, and that dietary stimulation and impaired duodenal inflow of bile and pancreatic juice commonly enhance pancreatic exocrine secretion, acting synergistically as aggravating factors in pancreatitis. CCK and the vagus nerve system appears to be involved in enhancing pancreatic exocrine secretion with diet stimulation as an aggravating factor. — Key words: pancreatic bile duct ligation (PBDL), pancreatitis, cholecystokinin, serum amylase, fasting, non-fasting

CD(SD)IGS-2001: 143-149

INTRODUCTION

Acute pancreatitis is caused by autodigestion of the pancreas by pancreatic enzymes and is aggravated by enhanced pancreatic exocrine secretion.[1)] Pancreatic bile duct ligation (PBDL)-induced pancreatitis in rats, which is induced by ligation of the hepatic bile duct and the pancreatic duct at its entrance into the duodenum, is a pathological model of clinical pancreatic duct obstruction due to pancreatic stones or edema. In this model, pancreatitis is aggravated by enhancing pancreatic exocrine secretion by dietary stimulation, acetylcholine or gut hormones. Therefore, this PBDL model is considered to be important in clarifying the mechanism of aggravation of pancreatitis.[2, 3)]

Recent studies using the PBDL pancreatitis model have shown that impaired inflow of bile and pancreatic juice into the duodenum due to PBDL causes enhancement of pancreatic exocrine secretion by a regulation mechanism known as luminal feedback.[4-7)] However, there have been no detailed studies on associations among aggravating factors in PBDL pancreatitis.

In this study, we evaluated the association among various factors involved in PBDL pancreatitis and found that retention of pancreatic juice in the pancreatic duct is necessary for the development of pancreatitis, and that dietary stimulation and impaired duodenal inflow of bile and pancreatic juice enhance pancreatic exocrine secretion, acting as synergistic aggravating factors on pancreatitis. Cholecystokinin(CCK)[8)] and the vagus nerve system appear to be involved in enhancement of pancreatic exocrine secretion due to dietary stimulation.

MATERIALS AND METHODS

Animals

Male CD:SD (IGS) rats aged 7-10 weeks (Charles River Japan Inc., Kanagawa, Japan) were maintained at the experimental animal facility of our institution in polycarbonate cages (270 × 422 × 180 mm, Natsume, Tokyo, Japan) with beta chips on the floor (Charles River Japan Inc., Kanagawa, Japan). Temperature was maintained at 23 ± 3℃ and humidity at 55 ± 10% under ventilation by the return method, with illumination from 7:00 to 19:00. Solid stock food (CRF-1: Charles River Japan Inc., Kanagawa, Japan) and tap water were given *ad libitum*. The experiments in this studies were conducted in accordance with "the Guidelines of Zeria Pharmaceutical Animal Care and Use Committee".

Reagents

The CCK_1-receptor antagonist, Z-203, sodium (s)-3-[1-(2-fluorophenyl)-2,3-dihydro-3-[(3-isoquinolyl)-carbonyl] amino-6-methoxy-2-oxo-1-H-indole] propanoate, MW 521.48) was diluted with physiological saline (1%, product synthesized by Zeria Pharmaceutical Co., Ltd. Saitama, Japan) before use. Atropine (Sigma Chemicals, St. Louis, Missouri, U.S.A.) was dissolved in physiological saline before use.

Induction of PBDL pancreatitis

Experiments were performed in fasting and non-fasting groups. In the fasting group, rats were fasted for 16 h before initiation of experiments, with free access to water. In both groups, the abdomen was opened under ether anesthesia, and the hepatic bile duct and the pancreatic duct at its entrance into the duodenum were completely ligated with braid silk 5-0 (Matsuda Medical Engineering, Tokyo, Japan) (PBDL group). After suturing of the abdomen, the rats were placed back into maintenance cages. Neither the hepatic bile duct or the pancreatic duct were not ligated in the sham group. In both the fasting and non-fasting groups, some rats were treated only by common bile duct ligation, pancreatic juice being allowed to flow into the duodenum unhindered (BDL group). Others were treated only by pancreatic duct ligation, with polyethylene tube (Intramedic ®, PE10, Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) cannulation between the common bile duct and duodenum, allowing flow of bile into the duodenum (PDL group). In addition, in some PBDL rats, bile and pancreatic juice were allowed to flow via the pancreatic duct at its duodenal entrance by cannulation

*Note: This paper is reprinted from original paper (Bio. Pharm. Bull., 23(11), 1318-1322, 2000)

(diversion-PB group); and in some PDL, rats in which pancreatic juice was allowed to flow via the pancreatic duct at its duodenal entrance by cannulation (diversion-P group).

Vagotomy

Truncal vagotomy was performed immediately before PBDL. The abdomen was opened under ether anesthesia,and the vagal nerve running through the anteroposterior wall of the esophagus immediately below the diaphragm was transected according to the method of Li et al.[9)]

Effects of drugs on PBDL pancreatitis

Immediately after PBDL, atropine (0.3-3 mg/kg) was subcutaneously injected 6 times at 1 h intervals. Z-203 (0.01, 0.03, and 0.1 mg/kg)[10-12)] was injected into the caudal vein.

S-Amylase activity

Blood was collected at designated intervals, especially at 6 h after operation in each groups according to previous reports[4,10)] and the serum amylase (S-amylase) activity was measured using an Amylase B Test Wako kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Effects of pancreatic exocrine secretion on aggravation of pancreatitis

A median incision was made in the rat abdomen under urethane anesthesia (Sigma Chemicals, St. Louis, Missouri, U.S.A. 1.3 g/5 ml/kg, i.p.), and polyethylene tube cannulation (Intramedic ®, PE10, Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) was performed via the pancreatic duct at its entrance into the duodenum. The common bile duct was ligated on the liver side and pancreatic juice collected into a 1.5-ml centrifugation tube (Treff AG, Degersheim, Switzerland). After operation, pancreatic juice was collected for 1 h. The amount of pancreatic juice and the content of protein and amylase activity in the pancreatic juice were then measured. The BCA reaction reagent (Pierce, Rockford, Illinois, U.S.A.) was used for measurement of protein and the Amylase B-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for measurement of amylase activity. In addition, the effects of vagotomy, atropine, and the CCK_1-receptor antagonist on pancreatic exocrine secretion were evaluated.

Statistical analysis

All data are shown as the means ± standard error. Statistical analysis was performed using Super ANOVA v. 1.11 software (Abacus Concepts Inc., Berkeley, California, U.S.A.). The significant differences were tested by use of Dunnett's *t*-test for multiple group comparisons and Student's *t*-test for comparison between two groups. $P < 0.05$ were regarded as significant.

RESULTS

Serial changes in S-amylase activity in the PBDL pancreatitis model

In the non-fasting PBDL group, S-amylase activity began to increase 1 h after ligation and reached a peak after 6 h; the peak value was about 10 times that in the sham group. Subsequently, the increased S-amylase activity gradually decreased until 24 h after ligation. In the fasting PBDL group, the S-amylase activity 6 h after ligation was about 3 times that in the sham group (Fig. 1).

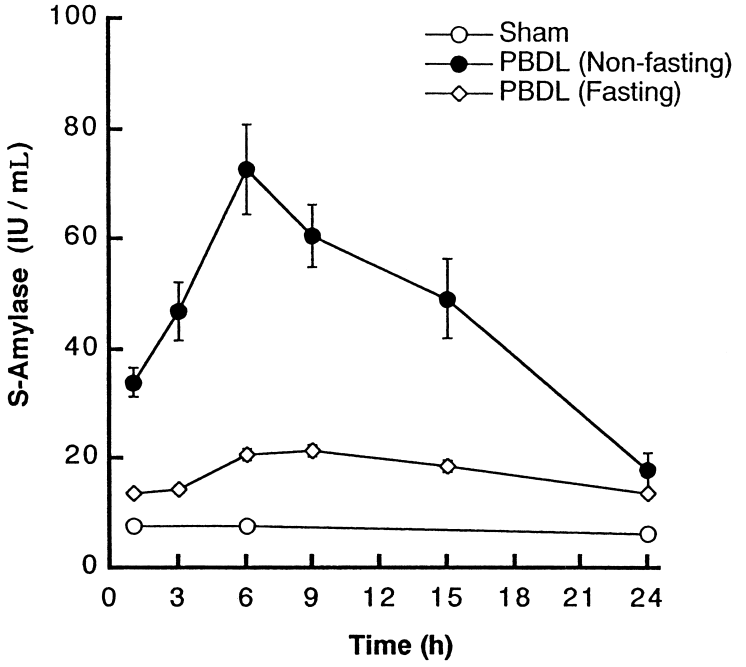

Figure 1. Changes in S-amylase Activity after PBDL Treatment under Non-fasting or Fasting Condition. The data represent the mean ± S.E.M. of five rats.

Aggravation factors in the PBDL pancreatitis model

S-amylase activity 6 h after the operation was compared among the BDL, PDL and PBDL groups. No increase in S-amylase activity was observed in the non-fasting and fasting BDL groups. The non-fasting and fasting PDL groups showed 60.5% and 42.5% inhibition of the increase in S-amylase activity, respectively, compared with the PBDL group (Fig. 2A). In the non-fasting diversion-PB or diversion-P group, no increase in S-amylase activity was observed (Fig. 2B).

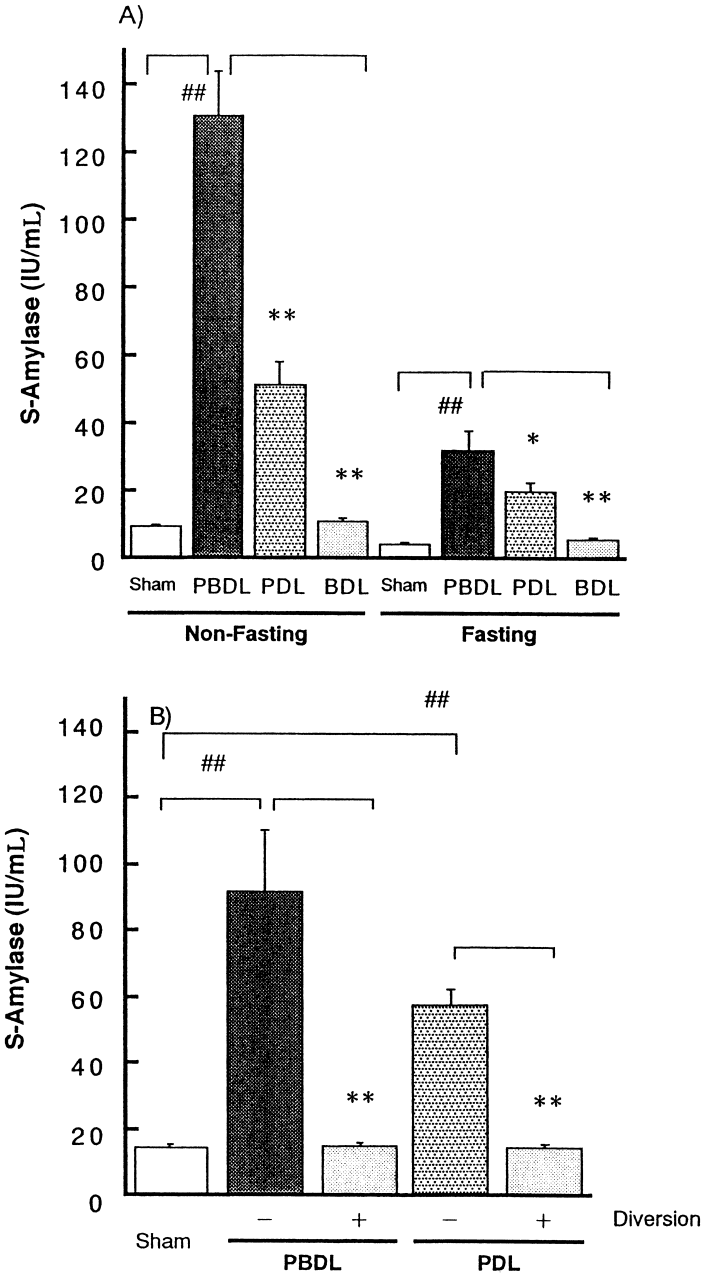

Figure 2. A) Comparison of S-amylase Activity in the PBDL, PDL and BDL Groups under Non-fasting or Fasting Condition. The data represent the mean ± S.E.M. of seven to eight rats. ##: Significantly different from the sham group at $p<0.01$ (Student's *t*-test); *$p<0.05$, **$p<0.01$: significantly different from the PBDL group.

B) Effect of Diversion of Pancreatic Juice to the Duodenum by Cannulation on S-amylase Activity in the PBDL and PDL Groups under Non-fasting Conditions. The data represent the mean ± S.E.M. of seven to eight rats. ##: Significantly different from the sham group at $p<0.01$ (Student's *t*-test); Significantly different (**$p<0.01$) from the PBDL group or the PDL group.

Involvement of the vagus nerve and CCK$_1$ receptors in the PBDL pancreatitis model

Truncal vagotomy or atropine injection significantly inhibited the increase in S-amylase activity 6 h after ligation in the non-fasting PBDL group (Figs. 3A, B). Injection of Z-203 (0.03-0.1 mg/kg) as a CCK1-receptor antagonist into the caudal vein significantly and dose-dependently inhibited the increase in S-amylase activity 6 h after ligation under non-fasting condition(Fig. 3C). In the fasting PBDL group, vagotomy or injection of atropine or Z-203 (Fig. 4) 6 h after ligation did not affect the increase in S-amylase activity.

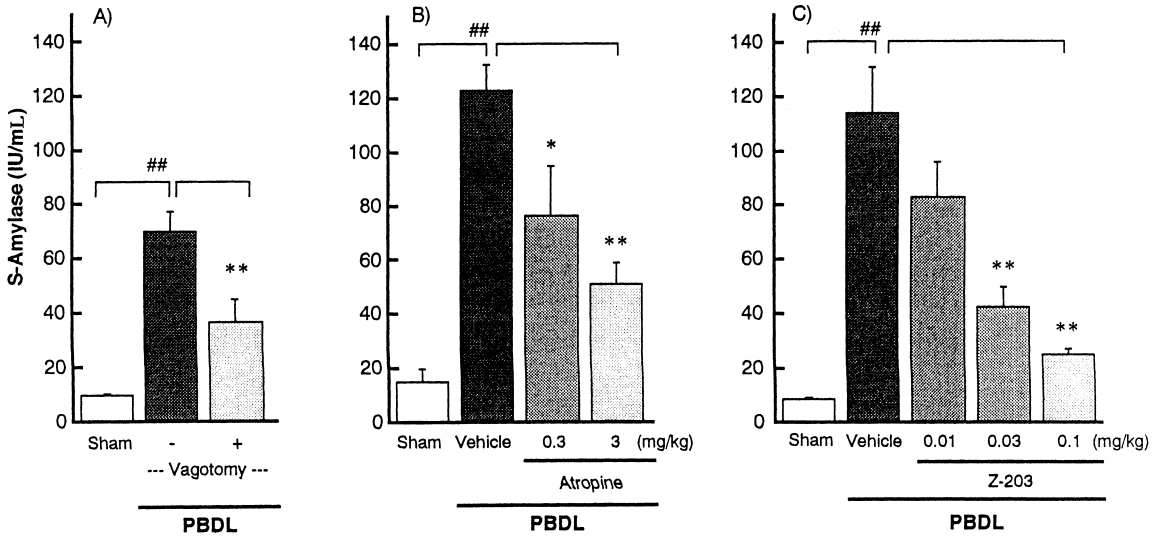

Figure 3. Effect of Truncal Vagotomy, Atropine and CCK$_1$-Receptor Antagonist Treatment on S-amylase Activity of the PBDL Group under Non-fasting Conditions. The data represent the mean ± S.E.M. of six rats. ##: Significantly different from the sham group at $p<0.01$ (Student's *t*-test); *$p<0.05$, **$p<0.01$: significantly different from the PBDL group.

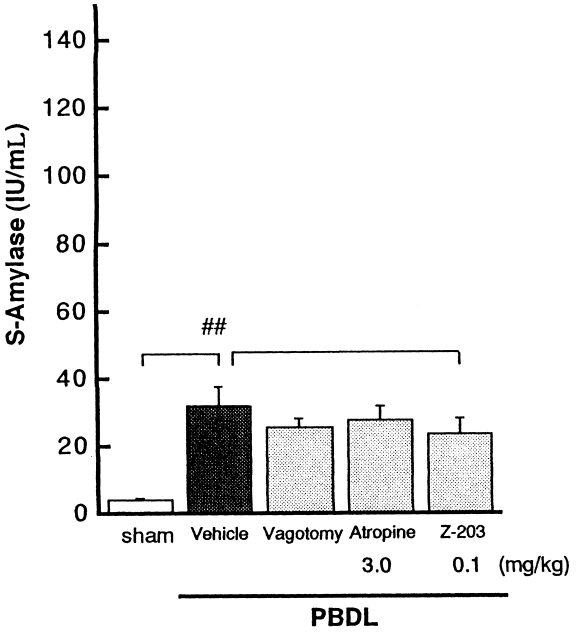

Figure 4. Effect of Truncal Vagotomy, Atropine and CCK$_1$-Receptor Antagonist Treatment on S-amylase Activity in the PBDL Group under Fasting Conditions. The data represent the mean ± S.E.M. of six rats. ##: Significantly different from the sham group at $p<0.01$ (Student's *t*-test)..

Effects of non-fasting on pancreatic exocrine secretion

Pancreatic exocrine secretion was compared between the fasting and non-fasting groups (Table 1). Compared with the fasting group, the non-fasting group showed increases in the amount of pancreatic juice, subsequent amylase activity and protein content, indicating enhanced pancreatic exocrine secretion. In the non-fasting group, vagotomy or injection of the CCK_1 receptor antagonist, or Z-203 (0.1 mg/kg, i.v.) significantly inhibited the increases in amylase activity and protein content. Injection of atropine (3 mg/kg, s.c., 1h intervals) significantly inhibited the increases in the amount of pancreatic juice, amylase activity and protein content (Table 1). In the fasting group, neither vagotomy nor injection of atropine or Z-203 affected the amount of pancreatic juice, amylase activity or protein content (Table 2).

Table 1. Effects of Truncal Vagotomy, Atropine and CCK_1 Receptor Antagonist Treatment on Pancreatic Exocrine Secretion under Non-fasting Condition

Group		Dose	Weight (mg/hr)	P-Amylase (IU/hr)	Protein (μg/hr)
Fasting	Vehicle		28 ± 3	129 ± 16	291 ± 35
Non-fasting	Vehicle		42 ± 5 ##	476 ± 174 ##	1021 ± 309 ##
	Vagotomy		34 ± 3	185 ± 23 *	508 ± 59 *
	Atropine	3mg/kg	22 ± 6 *	112 ± 20 **	349 ± 89 **
	Z-203	0.1mg/kg	34 ± 3	159 ± 30 *	391 ± 43 **

Results are expressed as the mean ± S.E.M. of 10 to 12 rats in each group. ## $P<0.01$: vs. Fasting-Vehicle group with Student's t-test.

* $P<0.05$, ** $P<0.01$: vs. Non-fasting-Vehicle group with Dunnett's test.

Weight: The weight of the pancreatic juice. P-Amylase: the amylase activity in the pancreatic juice. Protein: the protein levels in the pancreatic juice.

Table 2. Effects of Truncal Vagotomy, Atropine and CCK_1 Receptor Antagonist Treatment on Pancreatic Exocrine Secretion under Fasting Condition

Group		Dose	Weight (mg/hr)	P-Amylase (IU/hr)	Protein (μg/hr)
Fasting	Vehicle		32 ± 4	162 ± 22	413 ± 44
	Vagotomy		36 ± 4	193 ± 26	420 ± 69
	Atropine	3 mg/kg	31 ± 5	185 ± 28	427 ± 58
	Z-203	0.1 mg/kg	39 ± 5	209 ± 17	517 ± 26

Results are expressed as the mean ± SEM of 10 to 12 rats in each group. No significant difference were observed.

Weight: The weight of the pancreatic juice. P-Amylase: the amylase activity in the pancreatic juice. Protein: the protein levels in the pancreatic juice.

DISCUSSION

In this study, we evaluated the association among various factors considered to be involved in PBDL pancreatitis and found that several factors enhanced pancreatic exocrine secretion due to synergistically aggravated pancreatitis. We confirmed the enhancement of pancreatic exocrine secretion induced by diet stimulation via CCK and the vagus nerve system.

In the PBDL pancreatitis model, there are three types of impairment due to the ligation of pancreatic and bile ducts, i.e., impaired bile inflow into the duodenum, impaired inflow of pancreatic juice into the duodenum, and retention of pancreatic juice in the pancreatic duct. Impaired duodenal inflow of bile only can be selectively induced by BDL in which only the hepatic bile duct is ligated. Impaired duodenal inflow of pancreatic juice and retention of pancreatic juice in the pancreatic duct can also be selectively induced by PDL in which a bile bypass is added to PBDL.

Under non-fasting and fasting conditions, comparison among the PBDL, BDL, and PDL groups showed that factors resulting from these operative procedures act as synergistic aggravating factors in the PBDL pancreatitis model (Fig. 2A). The S-amylase activity in the PBDL group was higher than in the PDL group without impaired bile inflow into the duodenum, which suggests impaired bile inflow into the duodenum as an aggravating factor (Fig. 2A). In the BDL group with only impaired bile inflow into the duodenum, no increase in S-amylase activity was observed (Fig. 2A), indicating that impaired duodenal inflow of pancreatic juice and its retention in the pancreatic duct is involved in the development of pancreatitis. In both the PDL and PBDL groups, diversion of pancreatic juice into the duodenum resulted in no increase in S-amylase activity (Fig. 2B). This finding suggests that pancreatitis does not develop only in the presence of impaired duodenal inflow of pancreatic juice, and that retention of pancreatic juice in the pancreatic duct is necessary for the increase of S-amylase activity in the PBDL pancreatitis model.

Concerning the association between bile and pancreatic juice, Samuel et al.,[4] who infused bile and pancreatic juice obtained from rats into the duodenum of other rats treated by PBDL, suggested that impaired duodenal inflow of bile and pancreatic juice acts as a synergistic aggravating factor in the development of pancreatitis. In the PDL and PBDL groups, the S-amylase activity under non-fasting conditions increased much more compared with the fasting group. As is shown in Fig. 5, non-fasting and impaired duodenal inflow of bile and pancreatic juice are suggested to be synergistic factors aggravating hyperamylasemia in pancreatitis.

The above aggravating factors in the PBDL pancreatitis model,

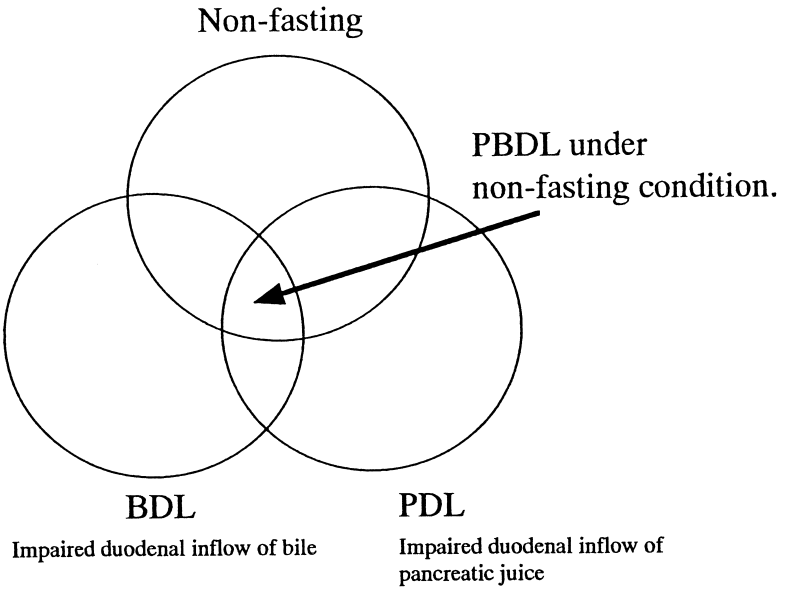

Figure. 5. Schematic Diagram of Synergistic Aggravation in PBDL-induced Pancreatitis.

i.e., impaired duodenal inflow of bile and pancreatic juice and non-fasting conditions, commonly enhance pancreatic exocrine secretion. Pancreatic exocrine secretion in rats[13-15] is regulated by bile and duodenal protease. Pancreatic exocrine secretion has been reported to increase when the supply of protease is interrupted because of impaired duodenal inflow of bile or pancreatic juice, or when protease activity is consumed by digestion of dietary protein. Therefore, under fasting conditions, it is thought that pancreatic exocrine secretion in the PBDL pancreatic model is enhanced by impaired duodenal inflow of bile and pancreatic juice. Under non-fasting conditions, it is suggested that pancreatic exocrine secretion in the PBDL pancreatitis model is enhanced by the synergistic action of ligation and non-fasting (Table 1). The differences in pancreatic exocrine secretion between the fasting and non-fasting PBDL groups shown in Tables 1 and 2 are due to differences between ligation alone and

ligation combined with non-fasting. This difference is estimated to reflect in S-amylase activity in the fasting and non-fasting PBDL groups.

A close association between enhanced pancreatic exocrine secretion and aggravation of pancreatitis was observed in this study. This association is also supported by the findings that both pancreatic exocrine secretion and S-amylase activity are inhibited by Z-203 as a CCK_1 receptor antagonist,[10-12] truncal vagotomy, or atropine as an anticholinergic drug in the non-fasting PBDL group but not in the fasting PBDL group (Fig. 3, Fig. 4). The differences in the effects of Z-203, truncal vagotomy, and atropine between the fasting and non-fasting groups indicate that all three treatments are ineffective against the effects of ligation under fasting conditions but effective under non-fasting conditions. Recently, enhancement of pancreatic exocrine secretion induced by dietary stimulation or CCK has been reported to be mediated by the vagus nerve system.[16-19] The results of this study connect the inhibitory action on pancreatic exocrine secretion to inhibitory action on the increase in S-amylase activity, and suggest that controlling CCK and the vagus nerve system as main factors enhancing pancreatic exocrine secretion can inhibit aggravation of pancreatitis.

In conclusion, non-fasting conditions and impaired duodenal inflow of bile and pancreatic juice enhance pancreatic exocrine secretion, acting as synergistic aggravating factors on PBDL-induced pancreatitis. CCK and the vagus nerve system appear to be involved dominantly in the enhancement of pancreatic exocrine secretion by non-fasting. These results suggest that the decrease of pancreatic secretion due to dietary stimulation may contribute to suppression of aggravation of pancreatitis.

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Cholecystokinin acts as an essential factor on exacerbation of a pancreatic bile duct ligation-induced rat pancreatitis model under non-fasting condition

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ABSTRACT. We examined the influence of 2 gut hormones involved in the enhancement of pancreatic exocrine secretion, secretin and cholecystokinin (CCK), in the exacerbation of pancreatitis. We also examined the role of the vagal system, which was considered to be a transmission route for these hormones. Our model of pancreatitis in the rat was prepared by pancreatic bile duct ligation (PBDL), which simultaneously ligated the pancreatic duct and the common bile duct. Serum amylase activity and histopathological changes in the pancreas were used as indices of pancreatitis. We also measured the volume of pancreatic juice, as well as the amylase activity and protein level of the pancreatic juice, as indices of increased pancreatic exocrine secretion. Two gut hormones were given 6 times at 1 h intervals. Administration of secretin (1-3 μ g/kg, s.c.) did not influence serum amylase activity in rats with PBDL-induced pancreatitis. However, food stimulation and administration of CCK-8 (1 μ g/kg, s.c.) increased serum amylase activity and promoted vacuolation of the pancreatic acinar cells in rats with PBDL-induced pancreatitis. Administration of atropine (3 mg/kg, s.c.) or a CCK_1-receptor antagonist, Z-203 (0.1 mg/kg, i.v.), inhibited food-stimulated or CCK-8 induced (1 μ g/kg, s.c.) enhancement of pancreatic exocrine secretion and exacerbation after the development of PBDL-induced pancreatitis. These results suggest that not secretin, which regulates the volume of pancreatic juice, but CCK, which regulates the secretion of pancreatic enzymes via the vagal system, plays an essential role in food-stimulated exacerbation after the development of pancreatitis. — Keywords: Pancreatic bile duct ligation (PBDL), pancreatitis, cholecystokinin

CD(SD)IGS-2001: 150-156

INTRODUCTION

Acute pancreatitis causes autodigestion of the pancreas by pancreatic enzymes that escape to the periacinar space. It is known that food-stimulated enhancement of pancreatic exocrine secretion causes exacerbation of pancreatitis (1). Previous studies using a pancreatic bile duct ligation (PBDL) model (2-7), which is prepared by simultaneously ligating the pancreatic duct and bile duct, and a pancreatic duct ligation (PDL) model (8-10) have reported that food stimuli exacerbate pancreatitis. To explain the mechanism behind this finding, the pancreatic duct obstruction and secretion theory (11-13) has been proposed: simple obstruction of the pancreatic duct very slightly increases pancreatic duct pressure, but obstruction with enhancement of pancreatic exocrine secretion increases the pancreatic duct pressure further, and pancreatic enzymes activation, causing exacerbation after the development of pancreatitis.

CCK and secretin are gut hormones that enhance food-stimulation-related pancreatic exocrine secretion. It has been suggested that these hormones are involved in the exacerbation after the development of pancreatitis(5, 12). In recent years, it has been shown that pancreatic exocrine secretion related to the presence of these gut hormones is mediated by the vagal system (14-19). It has been speculated that vagal stimuli are involved in the exacerbation of PBDL-induced pancreatitis, but the mechanism remains unclear. We examined the influence of 2 gut hormones involved in pancreatic exocrine secretion, secretin and CCK, after the development of PBDL-induced pancreatitis. We also examined the role of the vagal system, which could be a transmission route for these hormones. The ultimate aim is to clarify the mechanisms involved in exacerbation after the development of pancreatitis.

MATERIALS AND METHODS

Animals

Male CD:SD (IGS) rats (age: 7-10 w, Charles River Japan, Kanagawa) was used. The rats were acclimatized in the Laboratory Animal Institute of our laboratory under the following conditions: temperature, 23 ± 3°C; humidity, 55 ± 10%; ventilation, 80% return system; and lighting cycle, 7:00-19:00. Polycarbonate cages (270 × 422 × 185 mm, Natsume Seisakusho, Tokyo) and beta-chip floor mats (Northeastern Product Corp., USA) were used. Solid food (CRF-1, Charles River Japan, Kanagawa) and tap water were given *ad libitum*. The experiments in this study were conducted in accordance with "the Guidelines of the Zeria Pharmaceutical Animal Care and Use Committee".

Agents

Z-203, sodium (s)-3-[1-(2-fluorophenyl)-2,3-dihydro-3-[(3-isoquinolyl)-carbonyl]amino-6-methoxy-2-oxo-1-H-indole]propanoate, MW521.48)(20-22), solution (1%, dissolved in physiological saline and synthesized at our company) was diluted with physiological saline before use. Furthermore, atropine (Sigma Chemicals, U.S.A.),CCK-8 sulfate (CCK-8)and secretin (derived from pigs)(Peptide Institute, Osaka) were dissolved in physiological saline before use.

Preparation of an advanced pancreatitis model with the enhancement of pancreatic exocrine secretion

1) Influence of non-fasting conditions and gut hormones on pancreatic exocrine secretion

Non-fasted and fasted rats were anesthetized with urethane (Sigma Chemicals, 1.3 g/5 mL/kg, i.p.). We performed a midline laparotomy and cannulated the pancreatic duct on the side of the duodenal orifice. The hepatic side of the common bile duct was then ligated. At 1 h intervals, CCK-8(0.1-3 μ g/2 mL/kg) or secretin (0.3-3 μ g/2 mL/kg) was given subcutaneously to the fasted rats. At 1 h intervals, pancreatic juice was collected in a centrifuge tube (volume: 1.5 mL)(Treff Inc., Switzerland). The volume of pancreatic juice, as well as the level of protein and the amylase activity in the pancreatic juice was measured in the fasted and non-fasted rats. The protein levels were measured with BCA

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reactive reagent (Pierce, U.S.A). Amylase activity was measured with an amylase B-test Wako kit (Wako Pure Chemical Industry, Osaka, Japan).

CCK-8 (1 μg/2 mL/kg, s.c., 1 h intervals) was given to the fasted rats. At the same time atropine (3 mg/2 mL/kg,s.c., 1 h intervals) or the CCK_1-receptor antagonist Z-203 (0.1 mg/mL/kg,i.v.) was given in order to examine the effects of these drugs on pancreatic exocrine hypersecretion.

2) Preparation of a non-fasted PBDL-induced pancreatitis model

Two groups of rats: fasted group and non-fasted group was established. In the fasted group animals were fasted for 16 h before the experiment began, while water was given ad libitum. Laparotomy was performed under ether anesthesia, and the common bile duct on the hepatic side and the pancreatic duct on the duodenal orifice side were completely ligated with 5-0 braid silk (Matsuda Medical Industry, Tokyo). The abdomen was then sutured. Six hours after ligation, blood was collected, and the pancreas was extirpated. The serum amylase activity was measured (amylase B test Wako, Wako Pure Chemical Industry, Osaka). The extirpated pancreas was fixed in formalin. Sections were prepared and stained with hematoxylin and eosin (HE) for microscopy. The pancreatic head was examined under a light microscope. Any evidence of edema, autolysis, necrosis of acinar cells, hemorrhage and vacuolation of acinar cells was noted. Each parameter was evaluated by 3 grades: no change (−), moderate (+), and severe (＋).

3) Preparation of a PBDL-induced pancreatitis model loaded with gut hormones

The PBDL treatment described above in rats that had been fasted was performed. CCK-8 (0.1-3 μg/2 mL/kg,s.c.) or secretin (0.3-3 μg/2 mL/kg,s.c.) was administered 6 times at 1 h intervals. Six hours after the ligation, the serum amylase activity was measured. The extirpated pancreas was examined histopathologically, as described above.

4) Influence of agents on non-fasted or CCK-8 loaded model of PBDL-induced pancreatitis

Atropine (3 mg/2 mL/kg, s.c., 6 times at 1 h intervals) or Z-203 (0.1 mg/mL/kg,i.v.) was administered immediately after PBDL treatment under non-fasting conditions. We also administered CCK-8 (1 μg /2 mL/kg, s.c., 6 times at 1 h intervals) with atropine (0.3-3 mg/2 mL/kg, s.c.) and CCK-8 (1 μg /2 mL/kg,s.c., 6 times at 1 h intervals) with Z-203 (0.01-0.1 mg/mL/kg,i.v.).

Statistical analysis

All data were expressed as the means ± S.E.M. Super ANOVA v1.11 (Abacus Concepts Inc., USA) software for statistical analysis was used. The significance of differences was evaluated by Dunnett's test for multiple group comparisons and Student's *t*-test for comparison between two groups. $P < 0.05$ was regarded as significant.

RESULTS

Influence of non-fasting conditions and gut hormones on pancreatic exocrine secretion

Pancreatic exocrine secretion in non-fasted rats and fasted rats was compared. The volume of pancreatic juice, the pancreatic juice amylase activity and pancreatic juice protein levels were significantly higher in non-fasted rats than in fasted rats, suggesting enhancement of pancreatic exocrine secretion.

In non-fasted rats, administration of atropine (3 mg/2 mL/kg,s.c., 1 h intervals) significantly inhibited the increases in pancreatic juice volume, pancreatic juice amylase activity and pancreatic juice protein levels. Furthermore, administration of CCK_1-receptor antagonist Z-203 (0.1 mg/mL/kg,i.v.) significantly inhibited the increases in pancreatic juice amylase activity and

Table 1. Effects of atropine and CCK_1 receptor antagonist administration on pancreatic exocrine secretion under non-fasting conditions.

Group		Dose	Weight	P-Amylase	Protein
		(mg/kg)	(mg/hr)	(IU/hr)	(μg/hr)
Fasting	+Vehicle		28 ± 3	129 ± 16	291 ± 35
Non-fasting	+Vehicle		42 ± 5 ##	476 ± 174 ##	1021 ± 309 ##
Non-fasting	+Atropine	3.0	22 ± 6 *	112 ± 20 **	349 ± 89 **
Non-fasting	+Z-203	0.1	34 ± 3	159 ± 30 *	391 ± 43 **

Results are expressed as the means ± S.E.M. of 10 to 12 rats in each group.

P<0.01: vs. Fasting-Vehicle group with Student's t-test.

* p<0.05, ** p<0.01 : vs. Non-fasting-Vehicle group with Dunnett's test. P-Amylase: pancreatic juice amylase activity.

pancreatic juice protein levels in non-fasted rats (Table 1).

Secretin (0.3-3 μ g /2 mL/kg, s.c.) or CCK-8 (0.1-3 μ g/2 mL/kg, s.c.) was given to fasted rats at 1 h intervals, and the pancreatic exocrine secretion was measured. Administration of 1 μ g/kg or more of secretin increased the pancreatic juice volume. CCK-8 administration increased pancreatic juice volume, pancreatic juice amylase activity and pancreatic juice protein levels (Table 2). Administration of atropine (3 mg/2 mL/kg, s.c., 1 h intervals) with CCK-8 (1 μ g/2 mL/kg, s.c., 1 h intrevals) significantly inhibited the CCK-related increases in pancreatic juice volume and pancreatic juice amylase activity. Administration of Z-203 (0.1 mg/kg,i.v.) with CCK-8 (1 μ g /2 mL/kg, s.c., 1 h intervals) significantly inhibited increases in pancreatic juice volume, pancreatic juice amylase activity, and pancreatic juice protein levels (Table 3).

Table 2. Effects of gut hormone-stimulated pancreatic exocrine secretion in fasted rat.

Group	Dose (μg/kg)	Weight (mg/hr)	P-Amylase (IU/hr)	Protein (μg/hr)
Fasting		28 ± 3	101 ± 20	288 ± 41
Fasting +CCK-8	0.1	34 ± 3	103 ± 39	339 ± 81
	1	57 ± 13 *	468 ± 205 *	1350 ± 576 *
	3	47 ± 6	458 ± 124 *	1241 ± 297 *
Fasting		37 ± 10	46 ± 15	326 ± 108
Fasting +Secretin	0.3	73 ± 8	62 ± 11	545 ± 93
	1	89 ± 17 **	87 ± 33	986 ± 458
	3	103 ± 16 **	69 ± 4	439 ± 83

Results are expressed as the means ± S.E.M. of 6 to 10 rats in each group.

* P<0.05, ** P<0.01 : vs. Fasting group with Dunnett's test. P-amylase: pancreatic juice amylase activity.

Table 3. Effects of atropine and CCK_1 receptor antagonist administration on CCK-8 stimulated pancreatic exocrine secretion in fasted rat.

Group		Dose (mg/kg)	Weight (mg/hr)	P-Amylase (IU/hr)	Protein (μg/hr)
Fasting	+Vehicle		32 ± 4	141 ± 17	281 ± 30
Fasting +CCK-8	+Vehicle		63 ± 9 ##	1117 ± 289 ##	2257 ± 718 ##
	+Atropine	3.0	32 ± 4 **	504 ± 114 *	1002 ± 201
	+Z-203	0.1	37 ± 4 **	267 ± 43 **	692 ± 186 *

Results are expressed as the means ± S.E.M. of 10 to 12 rats in each group.

p<0.01: vs. Fasting-Vehicle group with Student's t-test.

* p<0.05, ** p<0.01 : vs. CCK-8 -Vehicle group with Dunnett's test.P-amylase: pancreatic juice amylase activity.

Exacerbation of pancreatitis in non-fasting PBDL-induced pancreatitis model

The serum amylase activity of the rats was measured 6 h after PBDL treatment under fasting and non-fasting conditions. In the PBDL group the serum amylase activity was 10.5 times higher than in the control group (Sham). In the PBDL group we observed pathological changes in the pancreas, including edema and necrosis of the pancreatic acinar cells. In addition to these findings, vacuolation of the pancreatic acinar cells was noted under non-fasting conditions. Administration of atropine (3 mg/2 mL/kg,s.c.,6 times at 1 h intervals) or Z-203 (0.1 mg/mL/kg, i.v.) inhibited the necrosis and vacuolation of the pancreatic acinar cells. Atropine significantly inhibited increase in serum amylase activity. The Z-203 also significantly inhibited serum amylase activity (Table 4).

Table 4. Serum amylase activity and histopathology findings after atropine or CCK_1 antagonist administration on food-stimulated PBDL pancreatitis model.

Group		S-amylase(IU/mL)	Histopathological findings				
			Edema	Autolysis	Acinar necrosis	Hemorrhage	Vacuolization
Control (Sham)		7.59 ± 0.24	0/5	0/5	0/5	0/5	0/5
Fasting	+Vehicle	38.85 ± 8.41	1/5	1/5	5/5	0/5	0/5
Non-fasting	+Vehicle	79.51 ± 7.52 ##	3/5	3/5	3/5	1/5	4/5 (1/5 ++) (3/5 +)
Non-fasting	+ Atropine	43.20 ± 3.65 **	1/5	1/5	1/5	1/5	1/5
Non-fasting	+Z-203	24.26 ± 1.16 **	3/5 (1/5 ++) (2/5 +)	2/5	0/5	0/5	0/5

After PBDL treatment under non-fasting condition, atropine (3 mg/kg, 6 times, s.c.) or Z-203(0.1 mg/kg, i.v.) was administreted. Serum amylase activity (S-amylase) are expressed as the means ± S.E.M. of five rats in each group.
P<0.01: vs Fasting+vehilcle group with Student's t-test. ** P<0.01: vs Non-fasting+vehilcle group with Dunnett's test.

Histopathological findings were examined at 6 hour after ligation. Pancreatic head site was examined under a light microscope. Each parameter was evaluated using 3 grades ; no change (-),moderate (+),severe (++). Figures showed the number of histopathological findings per total number of animals examined.

CCK-8 loading related exacerbation of on PBDL-induced pancreatitis

The serum amylase activity was measured 6 h after PBDL treatment under fasting conditions. The serum amylase activity in the fasted PBDL group was 3.5 times higher than in the control group. CCK-8 (1 μ g/2 mL/kg, s.c.) loading was performed at 1 h intervals after PBDL treatment. CCK-8 loading further increased serum amylase activity to 11.5 times that of the control group (Fig.1 (A)). On the other hand, secretin (0.3-3 μ g/2 mL/kg, s.c.) loading did not increase serum amylase activity after PBDL

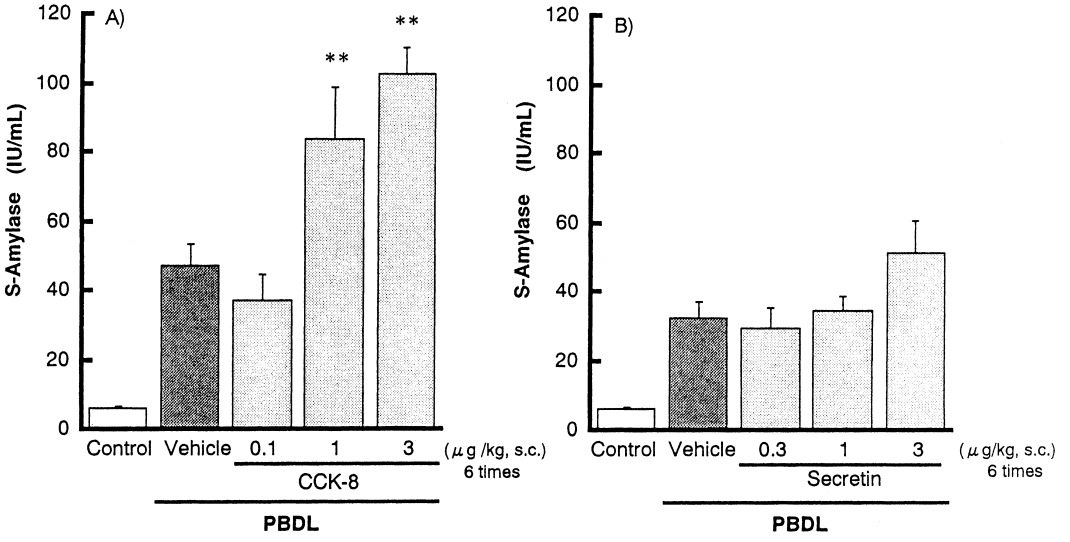

Figure 1. Effect of CCK-8 (A) or secretin (B) administration on serum amylase (S-Amylase) activity in the PBDL group under fasting conditions. The data represent the means ± S.E.M. of 4-6 rats. **$P < 0.01$: significantly different from the PBDL-Vehicle group.

treatment (Fig.1 (B)). In rats with PBDL-induced pancreatitis, atropine (3 mg/2 mL/kg, s.c., 6 times at 1 h intervals)(Fig.2(A)) or Z-203 (0.1 mg/mL/kg,i.v.) significantly inhibited increases in serum amylase activity in a dose-dependent manner after CCK-8 loading (Fig.2(B)). Histopathological examination of the pancreas after PBDL treatment under fasting conditions showed edema, autolysis and necrosis of the pancreatic acinar cells. CCK-8 loading induced vacuolation of the pancreatic acinar cells (Table 5).

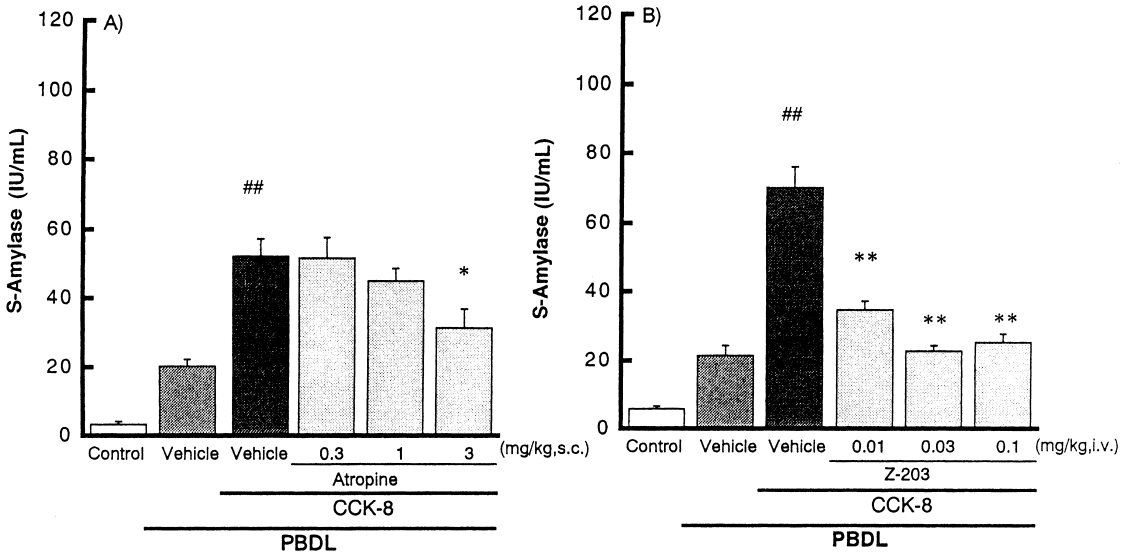

Figure 2. Effect of atropine(A) and CCK_1-receptor antagonist , Z-203(B) treatment on serum amylase (S-Amylase) activity in the CCK-8 stimulated PBDL group under fasting conditions. The data represent the means ± S.E.M. of 6 rats. ##$P < 0.01$: significantly different from the PBDL-Vehicle group (Student's *t*-test); *$P < 0.05$,**$P < 0.01$: significantly different from the CCK-8 stimulated PBDL group.

Table 5. Serum amylase activity and histopathology findings after gut hormone-stimulation on PBDL pancreatitis model.

Group	S-amylase(IU/mL)	Histopathological findings				
		Edema	Autolysis	Acinar necrosis	Hemorrhage	Vacuolization
Fasting	49.16 ± 14.23	4/4	3/4	4/4 (1/4 ++) (3/4 +)	2/4	0/4
Fasting + CCK-8	79.66 ± 4.23	4/4 (1/4 ++) (3/4 +)	1/5	3/4 (1/4 ++) (2/4 +)	1/4	4/4
Fasting +Secretin	45.32 ± 4.56	4/5	2/5	5/5 (3/5 ++) (2/5 +)	0/5	0/5

After PBDL treatment under fasting condition, CCK-8(1 μg/kg, 6 times, s.c.) or secretin(1 μg/kg, 6 times, s.c.) was administreted. Serum amylase activity (S-amylase) are expressed as the means ± S.E.M. of four-five rats in each group.

Histopathological findings were examined at 6 hour after ligation. Pancreatic head site was examined under a light microscope. Each parameter was evaluated using 3 grades ; no change (-), moderate (+), severe (++). Figures showed the number of histopathological findings per total number of animals examined.

DISCUSSION

We examined the influence of 2 gut hormones involved in pancreatic exocrine secretion (secretin and CCK) and the vagal system, which could be a transmission route for these hormones, in PBDL-induced pancreatitis. Serum amylase activity and histopathological changes in the pancreas were used as indices.

Among the rats with PBDL-induced pancreatitis, serum amylase activity differed between the non-fasted group and the fasted group. Vacuolation of the pancreatic acinar cells was a characteristic pathological feature in the non-fasted group. Administration of either atropine or a CCK_1-receptor antagonist, Z-203 (20-22), which inhibits the enhancement of pancreatic exocrine secretion under non-fasting conditions, inhibited both PBDL-related increases in serum amylase activity and vacuolation of the pancreatic acinar cells under non-fasting conditions. These differences may therefore reflect an exacerbation after the development of pancreatitis related to enhancement of pancreatic exocrine secretion.

The relationship between the enhancement of pancreatic exocrine secretion and vacuolation of the pancreatic acinar cells is unclear. However, in a pancreatitis model prepared by administration of a supramaximal dose of cerulein, and in an ethionine-induced pancreatitis model, it has been reported that vacuolation is caused by impaired segregation of zymogen granules and lysosomal hydrolases(23-25). These findings suggested that the similar impairment of segregation may be developed in this PBDL-induced model.

It is speculated that CCK, which increases the secretion of pancreatic enzymes, and secretin, which increases the volume of pancreatic juice secretion, are physiologically involved in the enhancement of pancreatic exocrine secretion induced by food stimuli. However, we compared the actions of these hormones with respect to their influence on pancreatic exocrine secretion and PBDL-induced pancreatitis. These results suggested that they had different effects. When CCK-8 was given at a dose (1-3 μg /2 mL/kg, s.c., 6 times at 1 h intervals) that dose not influence serum amylase activity in normal rats to rats with PBDL-induced pancreatitis under fasting conditions(data were not shown), it increased the serum amylase activity and induced vacuolation of the pancreatic acinar cells after PBDL-induced pancreatitis. Moreover, it has been reported that fasting lessen the severity of supramaximal cerulein-induced acute pancreatitis by reducing endogenous CCK release (26). Endogenous CCK may contribute to worsening for pancreatitis observed in the fed *ad libitum* rats. However, secretin did not have these effects, suggesting that CCK-related secretion of pancreatic enzymes is exacerbated after the development of pancreatitis. It has been reported that secretin inhibits CCK binding to acinar cells and exerts a preventive effect on supramximal cerulein-induecd acute pancreatitis in dogs and rats (27,28). Therefore, secretin is thought to be not harmful on PBDL-induced pancreatitis. In previous studies, because administration of food or CCK-8 loading at physiological concentration exacerbated acute pancreatitis, CCK was suggested to play an important permissive or contributory role in the development of acute pancreatitis (29,30). The exacerbation of PBDL-induced pancreatitis under non-fasting conditions resembled CCK-8 stimulation-related exacerbation of PBDL-induced pancreatitis under fasting conditions. Exacerbation of the pancreatitis by the addition of food or CCK stimulation in these models was inhibited by the administration of atropine, which inhibits the secretion of pancreatic enzymes, and by a CCK_1-receptor antagonist.

In conclusion, we suggest that exacerbation of pancreatitis under non-fasting conditions is associated with CCK-related secretion of pancreatic enzymes via the vagal system. Therefore, inhibition of CCK stimulation may be useful for controlling food-stimulus-related exacerbation after the development of pancreatitis.

Acknowledgments

We are grateful to Ms. T. Kinomoto for her competent technical assistance.

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Fasting exacerbate acute pancreatitis by occlusion of the common bile duct in rats

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ABSTRACT. We examined the effects of fasting and non-fasting on gallstone-related acute pancreatitis by the occlusion of the common bile duct (OCD). We prepared a rat OCD-induced pancreatitis model under both fasting and non-fasting conditions, and we measured amylase activity in ascites as well as production of inflammatory cytokines and chemokines. We also examined the pathology of the pancreas, myeloperoxidase (MPO) activity in some tissues and the mortality rates. In the fasted OCD group, ascites containing a large amount of amylase, interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and cytokine-induced neutrophil chemoattractant-1 (CINC-1) as well as marked hemorrhage and necrosis of the pancreatic acinar cells were observed. Pulmonary MPO activity increased 3.4-fold compared to the control group. In the non-fasted OCD group, there was no development of ascites. Slight necrosis of acinar cells and slight increases in pulmonary MPO activity were observed. In addition, in the fasted OCD group, the cumulative mortality rate was 50% 6 days after ligation. However, in the non-fasted OCD group, none of the animals died. These results suggest that gallstone-related severe pancreatitis depends on fasting-related structural and/or functional changes in the pancreas. Moreover, increased production of inflammatory cytokines and chemokines in ascites under fasting condition may be involved in multiple organ failure resulting from severe acute pancreatitis. — Key words: occlusion of the common bile duct (OCD), severe acute pancreatitis, cytokines, myeloperoxidase

CD(SD)IGS-2001: 157-163

INTRODUCTION

Most patients with acute pancreatitis show mild edematous pancreatitis with a good prognosis. However, when mild conditions develop into severe hemorrhagic or necrotic pancreatitis, multiple organ failure develops. The mortality rate of this type is known to be more than 30% (1,2). The pathogenesis of multiple organ failure related to severe acute pancreatitis is not understood. In recent years, several studies have investigated the involvement of tissue destruction by active oxygen (3) and elastase (4,5) released by neutrophils activated by various macrophage-derived inflammatory cytokines (6-8) and chemokines (9), phospholipid metabolites (leukotriene, thromboxane, platelet activating factor (PAF)) (10,11), and nitric oxide (NO) (12,13).

In clinical practice, gallstone-related pancreatitis is known as the main cause of acute pancreatitis. This condition can be modeled by infusing taurocholate into the pancreatic duct of rats (14,15). An alternative model of pancreatitis is induced by occlusion of the common bile duct (OCD): the duodenal orifice of the common bile duct or total hepatic bile duct of rats is ligated to promote regurgitation of endogenous bile into the pancreatic duct (16,17). The taurocholate-induced pancreatitis model produces severe necrotic pancreatitis but the OCD-induced model is considered to show mild edematous pancreatitis. It has been reported that pancreatic necrosis is induced by alcohol or by cholecystokinin (CCK) loading, which enhances pancreatic exocrine secretion (18). However, neither the factors related to severity nor the process of exacerbation has been examined.

In this study, we used a rat OCD-induced pancreatitis model to clarify factors that trigger severe pancreatitis and subsequent multiple organ failure.

MATERIALS AND METHODS

Animals

Male CD:SD (IGS) rats (7 - 10 weeks old, Charles River Japan, Kanagawa, Japan) were used. In the Laboratory Animal Institution of our laboratory, these rats were acclimated under the following conditions: temperature, 23 ± 3℃; humidity, 55 ± 10%; ventilation, 80% return system; and lighting cycle, 7:00-19:00. The animals were housed in polycarbonate cages (270 × 422 × 185 mm, Natsume Seisakusho, Tokyo, Japan) with a beta chip floor mat (Northeastern Product Corp., Warrensburg, NY, U.S.A) and were given solid food (CRF-1, Charles River Japan, Kanagawa, Japan) and tap water *ad libitum*. The experiments in this study were conducted in accordance with "the Guidelines of Zeria Pharmaceutical Animal Care and Use Committee".

Preparation of OCD-induced pancreatitis model

We established two groups, a fasted group and a non-fasted group. In the fasted group, rats were fasted for 16 h before the experiment, although water was given ad libitum. In another experiments, rats were placed in Bollman-type restraint cages and fasted for 16 h with or without subcutaneous administered cholecystokinin octapeptide sulfate (CCK-8, Peptide Institute, Inc., Osaka, Japan) loading (1 μ g/mL/kg/hr). In these groups, laparotomy was done under ether anesthesia, and the duodenal orifice of the bile duct was completely ligated with braid silk 5-0 (Matsuda Medical Industry, Tokyo, Japan). The abdomen was then sutured. Rats were housed in cages for breeding.

Measurement of serum and ascites amylase activity

In the fasted and non-fasted OCD groups, laparotomy was done under ether anesthesia 1, 3, 6, 9, and 15 h after ligation. Ascites and blood were collected. The volume of ascites was measured, and also serum and ascites amylase activity (Amylase B Test Wako, Wako Pure Chemical Industry, Osaka, Japan).

Measurement of TNF- α , IL-1 β , IL-6, and CINC-1 in ascites

In the fasted OCD group, the levels of inflammatory cytokines, such as tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β) and interleukin 6 (IL-6) (BioSource International, Inc., Camarillo, CA, U.S.A.) and a chemokine, cytokine-induced neutrophil chemoattractant-1 (CINC-1) (Panafirm laboratories Co., Ltd., Kumamoto, Japan) in ascites were measured by enzyme-linked immunosorbent assay (ELISA) at 1, 3, 6, 9, and 15 h after ligation. In the non-fasted OCD group, the levels of IL-1 β, IL-6 and

∗Note: This paper is reprinted from original paper (Jpn. J. Pharmacol., 84, 455-461, 2000)

CINC-1 in peritoneal cavity collected by 5mL of physiological saline were measured 6 h after ligation.

Pathological findings in the pancreas

In the fasted and non-fasted OCD groups, rats were killed by ether inhalation at 1, 3, 6, 9, and 15 h after ligation and the pancreases were removed. Specimens were fixed in formalin. Sections were prepared, and stained with hematoxylin and eosin (HE) for microscopy. The pancreatic head was examined under a light microscope for cellular infiltration, vacuolation of acinar cells, interstitial edema, necrosis of acinar cells and hemorrhage. Each factor was evaluated using four grades: no change(−), slight(+), moderate(＋), and severe(＋＋).

Measurement of organ myeloperoxidase (MPO) activity

MPO activity was measured as an index of neutrophil infiltration (5). In the fasted and non-fasted OCD groups, rats were exsanguinated under ether anesthesia 6 and 15 h after ligation to remove the heart, lungs, liver, pancreas, kidneys, and spleen. Specimens of these tissues were washed in physiological saline, and homogenized in 50 mM potassium phosphate buffer (pH 6.0) using a polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenate was refrigerated and ultracentrifuged at 12,000 × g for 10 min. Pellets were solubilized in 3 mL of the above buffer containing 0.5% hexadecytrimethylammonium bromide. After ultrasonication, the solution was refrigerated and ultracentrifuged at 12,000 × g for 10 min. A 5-20 μ L aliquot of supernatant was added to 100 mM potassium phosphate buffer (480-495 μ L, pH 6.0) containing 1.6 mM 3,3',5,5'-tetramethylbenzidine and 0.3 mM hydrogen peroxide. The enzyme reaction was stopped after incubation at 37℃ for 3 min by addition of 1 M acetic acid containing 0.1% triton X-100 (1.75 mL). Absorbance at 655 nm was measured using a photometer. Furthermore, in the fasted OCD group, ascites was collected 6 h after ligation, supernatant was prepared by centrifugion (1,000 × g, 10min) and supernatant (5mL) administered intraperitoneally to normal animals that had been fasted. Three hours after administration, pulmonary MPO activity was measured, as described above. In the control group, 5 mL of physiological saline was administered intraperitoneally.

Mortality rate

In the fasted and non-fasted OCD groups, cumulative mortality rates were calculated 6 days after ligation. To regulate the duration of fasting, food was given immediately after ligation in the fasted OCD group, while rats were fasted for 24 h after ligation in the non-fasted OCD group.

Statistical analysis

All data are expressed as means ± standard error. Super ANOVA v1.11 software (Abacus Concepts Inc., Berkeley, CA, U.S.A.) was used for statistical analysis. The significantly differences was tested by use of Student's *t*-test for comparison between two groups. $P<0.05$ were regarded as significant.

RESULTS

Changes in serum and ascites amylase activity and the volume of ascites

In the fasted OCD group, serum amylase activity reached a maximum 1 h after ligation. The value was 4-fold that in the surgical control group (Fig. 1-A). Furthermore, the volume of ascites increased steadily, but reached a plateau 6 h or more after ligation (Fig. 1-B). Amylase activity in ascites increased compared to serum amylase activity, but the increase reached a plateau 6 h or more after ligation (Fig. 1-C). In the non-fasted OCD group, serum amylase activity reached a maximum (at about 17.9 times that in the surgical control group) 1 h after ligation (Fig. 1-A). Thereafter, serum amylase activity gradually decreased. Furthermore, there was no development of ascites.

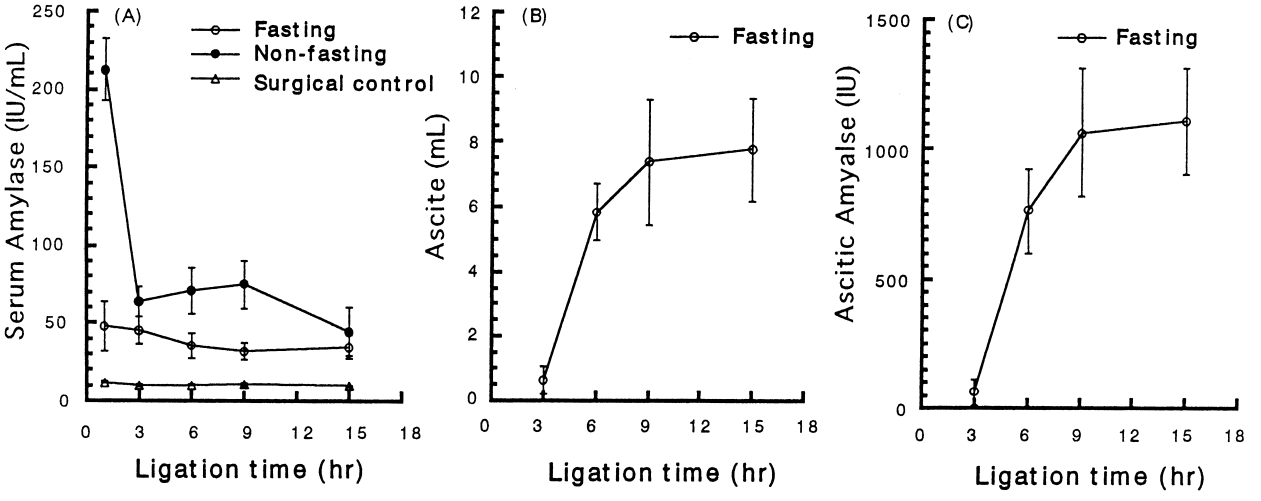

Figure 1. Changes in S-Amylase activity(A), ascites volume(B) and output of ascitic amylase activity(C) after OCD treatment under non-fasting or fasting conditions. The data represent the mean ± S.E.M. of five rats.

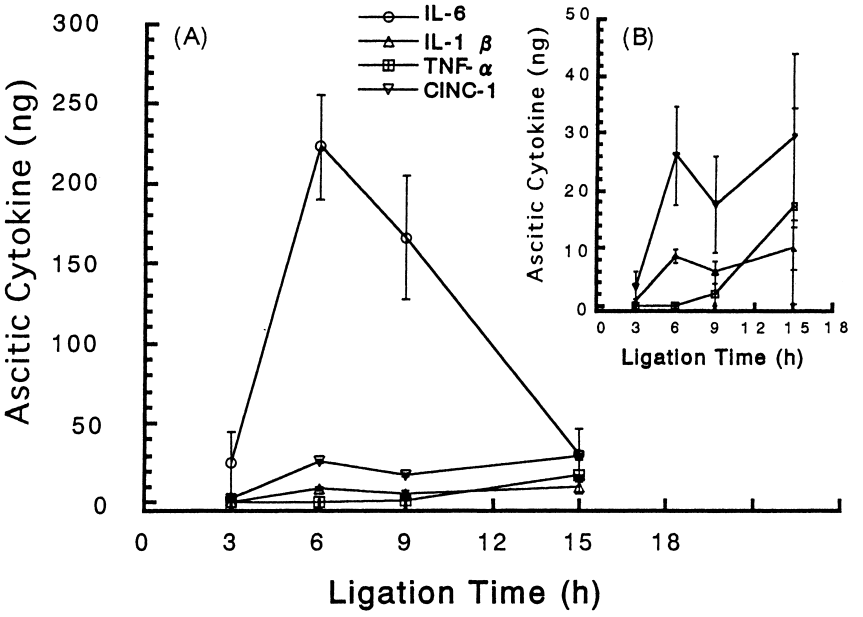

Figure 2. Changes in inflammatory cytokines (IL-6, IL-1 β, TNF- α) and chemokine (CINC-1) contents in ascites after OCD treatment under fasting conditions. Panel B shows the same data, except for IL-6, by an expanded vertical scale. The data represent the mean $\pm$ S.E.M. of 4-5 rats.

Moreover, CCK-8 loading to the fasted OCD group decreased ascites volume 6 h after ligation (n=6-8, fasted OCD group vs CCK-8 loading fasted OCD group: 3.78 $\pm$ 0.98 mL vs 0.76 $\pm$ 0.68 mL, $P<0.05$).

Changes in levels of inflammatory cytokines and chemokines in ascites

In the fasted OCD group, the presence of IL-1 β, IL-6, CINC-1, and TNF- α in ascites was confirmed (Fig. 2-A,B). The level of IL-6 reached a maximum 6 h after ligation, but then decreased gradually (Fig.2-A). Furthermore, IL-1 β and CINC-1 levels increased to a plateau by 6 h and TNF- α was detected 15 h or more after ligation (Fig. 2-B). In the fasted OCD group 6 h after ligation, IL-1 β, CINC-1 and IL-6 contents in ascites were 14.33 $\pm$ 6.52, 67.77 $\pm$ 13.63 and 110.25 $\pm$ 25.06 ng, respectively. On the other hand, IL-1 β, CINC-1 and IL-6 contents in peritoneal cavity of the non-fasted OCD group were 3.16 $\pm$ 2.63, 3.13 $\pm$ 1.30 and 2.67 $\pm$ 1.20 ng, respectively.

Pancreatic pathology in the OCD-induced pancreatitis model

Six hours or more after ligation, cellular infiltration and vacuolation of the pancreatic acinar cells and interstitial edema were observed regardless of the presence or absence of fasting, although the incidences of these findings were low (Table 1). In the fasted OCD group, acinar cells necrosis and hemorrhage were observed more frequently compared to the non-fasted OCD group (Table 1).

Measurement of organ MPO activity

In the fasted OCD group, pulmonary MPO activity was 3.4 times that in the surgical control group 6 h after ligation, and was 1.6 times that in the surgical control group 15 h after ligation. In the non-fasted OCD group, pulmonary MPO activities were 1.8 and 1.3 times those in the surgical control group at 6 and 15 h after ligation, respectively (Fig. 3). Furthermore, ascites (5 mL) from the fasted OCD group at 6 h after ligation was intraperitoneally administered to normal animals. As a result, pulmonary MPO activity was increased 1.6 times 3 h after administration (n=6, control group vs ascites-treated group: 14.3 $\pm$ 2.0 vs 23.2 $\pm$ 1.5, $P<0.01$).

Mortality rate

In the fasted OCD group, some animals died 1 day or more after ligation. The cumulative mortality rate was 50% 6 days after ligation (Fig. 4). In the non-fasted OCD group, none of the animals died until 6 days after ligation.

DISCUSSION

In this study, we examined the factors involved in gallstone-related pancreatitis using a rat OCD-induced pancreatitis model, and clarified factors related to the severity and exacerbation of the disease.

Findings in rats with OCD-induced pancreatitis under fasting conditions reflected the clinical features of multiple organ failure associated with gallstone-related acute pancreatitis, suggesting factors related to severity. In the OCD-induced pancreatitis model, the regurgitation of bile in pancreatic duct may be a trigger for the development of ascites or severe conditions (increased inflammatory cytokine and chemokine levels, pulmonary infiltration of neutrophils, fatal outcome) in rats with OCD-induced pancreatitis. It has been reported that bile

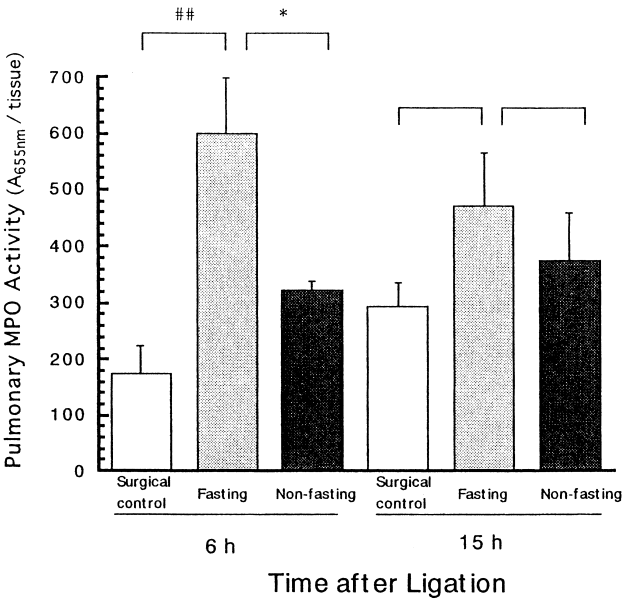

Figure 3. Changes in pulmonary MPO activity after OCD treatment under fasting conditions. The data represent the mean ± S.E.M. of 4-5 rats. ##: Significantly different from the control group at P <0.01 (Student's t-test). * : Significantly different from Non-fasting group at P <0.05 (Student's t-test).

Table 1. Histopathological findings of pancreas on OCD induced-pancreatitis model under fasting or non-fasting conditions..

Cellular infiltration

Time after ligation (h)	Fasting	Non-fasting
1	0/5	0/5
3	4/5	2/5
6	4/5	3/5
9	3/5 + 2/5 ++ 1/5	4/5
15	5/5	5/5

Acinar cells vacuolization

Time after ligation (h)	Fasting	Non-fasting
1	0/5	0/5
3	0/5	1/5
6	2/5	4/5 + 3/5 ++ 1/5
9	3/5 + 2/5 ++ 1/5	2/5 + 1/5 ++ 1/5
15	5/5	3/5 + 2/5 ++ 1/5

Interstitial edema

Time after ligation (h)	Fasting	Non-fasting
1	0/5	0/5
3	3/5 + 2/5 ++ 1/5	1/5
6	4/5 + 1/5 ++ 3/5	3/5 + 1/5 ++ 2/5
9	5/5 + 1/5 ++ 4/5	3/5
15	5/5 + 1/5 ++ 1/5 +++3/5	3/5 + 2/5 ++ 1/5

Acinar cells necrosis

Time after ligation (h)	Fasting	Non-fasting
1	0/5	0/5
3	2/5	0/5
6	4/5 + 2/5 ++ 2/5	0/5
9	3/5 + 1/5 ++ 2/5	1/5
15	4/5 + 2/5 ++ 1/5 +++1/5	2/5

Hemorrhage

Time after ligation (h)	Fasting	Non-fasting
1	0/5	0/5
3	1/5	0/5
6	0/5	2/5
9	1/5	1/5
15	5/5 + 2/5 ++ 3/5	0/5

Histopathological findings were examined at each time after ligation. Pancreatic head site was examined under a light microscope. Each parameter was evaluated using 4 grades ; no change (-), slight (+), moderate (++), severe(+++). Figures showed the number of histopathological findings per total number of animals examined.

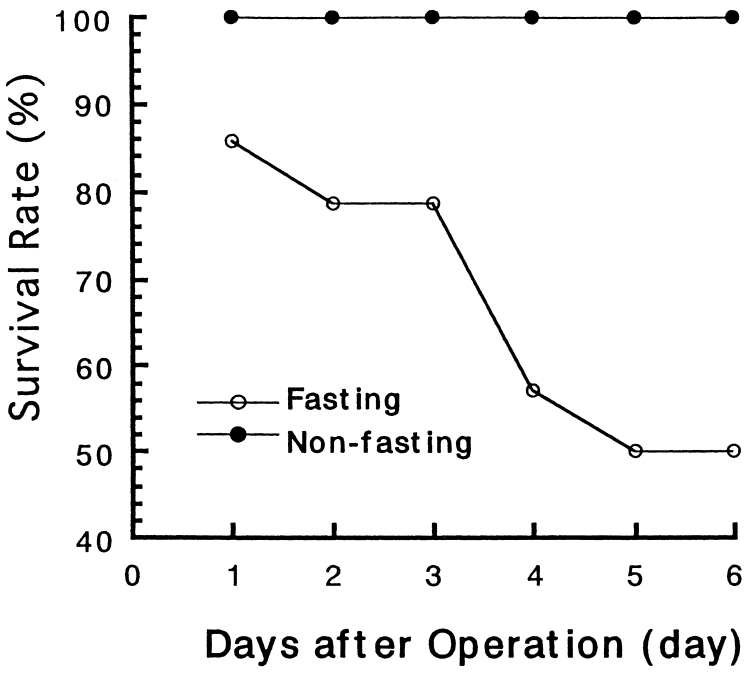

Figure 4. The survival rate after OCD treatment under non-fasting or fasting conditions. The data represent the mean ± S.E.M. of 14-15 rats.

components such as bile acid and lysolecithin show cytotoxic actions via interaction with pancreatic juice, and that pancreatic enzymes are activated by bile acid (19).

It was speculated that the components of ascites induced by regurgitation of bile in pancreatic duct contributed to multiple organ failure related to pancreatitis. In the fasted and non-fasted OCD groups, there were no increases in MPO activity in the liver, spleen, heart, kidneys, or pancreas at 6 and 15 h after ligation (Data is unpublished observation). Pulmonary MPO activity was increased in the OCD-induced pancreatitis model and in animals treated with ascites. This suggests that ascites components are directly involved in organ failure related to severe acute pancreatitis.

Large concentrations of IL-1 β and CINC-1 (20,21), which are involved in activation, chemotaxis, and infiltration of neutrophils, are also detected in ascites of the fasted OCD group (Fig. 2). These components may be involved in pulmonary infiltration of neutrophils in this pancreatitis model. Furthermore, IL-6 may have been induced by IL-1 β and CINC-1 (22).

It is known that infection, trauma, and surgical invasion induce production of inflammatory cytokines and chemokines and activate vascular endothelial cells and neutrophils, causing neutrophil infiltration in the main organs (23). Neutrophils cause organ disorders by releasing neutral proteases including elastase and producing active oxygen, phospholipid metabolites (leukotriene, thromboxane, PAF), and NO. Therefore, it is speculated that neutrophils are involved in the pathogenesis of multiple organ failure related to severe acute pancreatitis (8,13,24,25). In this OCD-induced pancreatitis model, organ failure in the pulmonary system may have occurred via a similar mechanism. The direct association between the development of ascites or pulmonary infiltration of neutrophils soon after onset and fatal outcome is unclear. However, these findings were consistent with the clinical features of severe acute pancreatitis from multiple organ failure to fatal outcome, suggesting the usefulness of this OCD-induced pancreatitis model.

The difference in the severity of OCD-induced pancreatitis between fasting conditions and non-fasting conditions suggests that structural/functional changes in the pancreas influence the prognosis of gallstone-related pancreatitis. In the fasted OCD group, the development of ascites, appearance of hemorrhagic necrosis in pancreas, pulmonary infiltration of neutrophils, and a high mortality rate were noted (Table 1, Fig. 1,3,4) although serum amylase activity was low, suggesting that fasting contributed markedly to inducing severe pancreatitis. Ascites, which was observed only in the fasted OCD group, contained a high level of amylase and total bile acid (corresponding to 90% and 10% of the total 6 h output of pancreatic juice and bile, respectively), suggesting that pancreatic tissue easily exudes pancreatic juice and bile (Fig. 1). Furthermore, pancreatic hemorrhage and necrosis in the fasted OCD group suggests that the pancreas under fasting conditions is easily affected by the regurgitation of pancreatic juice and bile in pancreatic duct(Table 1). It has been reported that the fasting load significantly reduces constituents of the pancreas (total protein, amylase, ribonucleic acid, water content) and protein synthesis, causing structural/functional

changes in the pancreas (26,27). These differences may have resulted in differences in the severity of OCD-induced pancreatitis between fasting conditions and non-fasting conditions.

There was no ascites development in the non-fasted OCD group. Physiological CCK-8 loading inhibited the development of ascites in the fasted OCD group in this study, suggesting that the effects of non-fasting conditions (for example, continuous food stimulation) can be replaced by CCK. CCK is a nutrient factor involved in the growth and proliferation of the pancreas (28,29). Structural/functional changes in the pancreas, which contributed to the above difference in severity, may have occurred because fasting blocked the actions of CCK.

In clinical practice, it is known that enhancement of pancreatic exocrine secretion related to food stimuli causes exacerbation after the development of acute pancreatitis. In various experimental pancreatitis models including the OCD-induced pancreatitis model, it has also been reported that food stimuli or CCK-8 loading induces exacerbation of pancreatitis (18,30,31). Furthermore, in cerulein-induced pancreatitis model, it has been reported that fasting inhibits exacerbation of pancreatitis (32). However, the results of this study demonstrated that fasting increased the disease severity in rats with OCD-induced pancreatitis, and those food stimuli (or CCK-8) loading prevented severe pancreatitis. These contrary findings may reflect the two actions of food stimuli (or CCK): as a nutrient factor and as an enhancer of pancreatic exocrine secretion. The nutrient impairment of pancreas would become a trigger for severe pancreatitis in a period after the development of pancreatitis.

In conclusion, these results showed that the regurgitation of bile in pancreatic duct promoted disease severity in rats with gallstone-related pancreatitis, and the inflammatory cytokines and chemokines in ascites were involved in pulmonary failure. It suggested that fasting-related structure /functional changes in the pancreatic tissue may be involved in the progress of gallstone-related acute pancreatitis.

ACKNOWLEDGMENTS

We are grateful to Ms. M. Sawata and Ms. A. Iguchi for their competent technical assistance.

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APPENDIX

Appendix

ABSTRACTS OF ORIGINALS

Diet Restriction in Rat Toxicity Studies: Automated Gravimetric Dispensing Equipment for Allocating Daily Rations of Powdered Rodent Diet into Pouches and 7-Day Feeders

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The American Association for Laboratory Animal Science, 40 (5), 37-43, 2001

Abstract: Survival of ad libitum-fed rats (Crl:CD(SD)IGS, Kingston, NY) has declined in the 2-year carcinogenicity bioassay. Restriction of the number of calories, without compromise of the overall nutrition offered to animals including rats, results in increased lifespan of animals. Diet restriction in rat is best achieved through offering of rations of feed daily instead of weekly, as is routinely done in ad libitum studies. The objective of this project was to develop an accurate and precise method of dispensing daily rations of feed. A gravimetric vibratory-type dispenser was used to dispense target weights of 15 and 20g of powdered certified rodent diet into either labeled pouches or seven-well carousel feeders. The tolerance of the dispenser was the target weight ± 3%. The amount of food offered to the diet-restricted rats was approximately 25% lower than that consumed by rats offered diet ad libitum. After 2 years, male rats offered 20g and female rats offered 15g of powered rodent diet daily had remarkably lower body weights than did animals offered the diet ad libitum. Generally, the rats ate the entire ration of food offered to them each day. Survival of the diet-restricted rats was 70% to 82% at the end of a 2-year study. This investigation demonstrates that modest reduction of food intake, resulting in increased survival of Sprague Dawley rats in 2-year carcinogenicity bioassays, can be achieved reliably and efficiently through use of an accurate and precise automated method of dispensing powdered diet for use in multiple rat studies. In addition, this method of food dispensing provides a practical way to administer test compound in the diet under the conditions of diet restriction.

Appendix

This report is published as a reference in long-term examination of rats.

UNEXPECTED SUDDEN DEATH OF F344 RATS IN LONG-TERM TOXICITY STUDIES

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Abstract. We have experienced a high incidence of unexpected sudden deaths in F344 rats in the long-term toxicity studies by oral gavage administration conducted in our laboratory since 1997. There were some common characteristics in the sudden deaths: 1) sudden death occurred from week 20 after the start of administration, more frequently from week 27, 2) females had more susceptability than males, 3) no abnormal clinical signs had been observed in-life, 4) the animals had almost the lowest body weight or body weight gain in the respective group; however, no sudden decrease in the body weight was recorded, 5) necropsy revealed packed food in the oral, pharyngeal and laryngeal cavities in every case, 6) histopahtology revealed no severe lesions leading to death, although histopathological examination had not extended to the pharynx or larynx. The unexpected sudden death seen in F344 rats have not been observed in other strain of rats, including SD(CD)IGS.
－ Key words: F344 rats, Sudden death, Oral gavage administration, Long-term toxicity study

INTRODUCTION

F344 rat is one of the strains predominantly used for the carcinogenicity study in rodents. However, we have frequently experienced the sudden death of F344 rats, in which cause of death could not been clarified, in oral gavage long-term toxicity studies conducted since 1997 in our laboratory. In the present paper, some characteristics in the F344 rats that died suddenly are reported.

MATERIALS AND METHODS

Animals and Husbandry: Data were collected from five carcinogenicity studies and two 26-week studies using F344 rats conducted in our laboratories since 1993. In all studies, F344 rats were obtained from Charles River Japan Inc. (Hino Breeding Center, Japan) and administration route was oral by gavage using a ball-tipped stainless-steel stomach tube. 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 ad libitum throughout the course of the study.

Observations and examinations:

General observations: The general condition of the animals was observed daily. The animals found dead were necropsied soon after discovery.

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.

Necropsy and histopathology: All animals found dead were subjected to necropsy as soon as discovery. HE stained specimens were made for major organs/tissues in the cephalic, thoracic and abdominal cavities and examined histopathologically. However, pharynx and larynx were not subjected to histopathology as they are not the standard tissues for examination in the routine toxicity study.

Table 1. Occurrence of sudden death of F344 rats in oral gavage long-term toxicity studies [a].

Experiment	A		B		C		D		E		F [b]		G [c]	
Start of study	1993.3		1994.4		1997.9		1998.2		1999.10		2000.1		2000.7	
Study period	104 w		104w		104w		26w		26w+ R13w		104w		104w	
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No. of animals	275	275	275	275	220	220	243	243	93	93	235	235	55	55
Week of study														
1-26 w	0	0	0	0	0	0	1	1	0	0	1	0	0	2
27-52w	0	0	1	0	1	6	—	—	1	1	2	8	0	1
53-78w	0	0	0	1	6	10	—	—	—	—	2	7		
79-104w	0	0	0	0	0	6	—	—	—	—				
Total	0	0	1	1	7	22	1	1	1	1	5	15	0	3
Incidence (%)	0	0	0.4	0.4	3	10	1	1	0.4	0.4	2	6	0	5

a): The data were obtained from all test groups including dose groups. However, in study G, only the data from the control group are shown.
b): On-going study (as of week 72 of administration).
c): On-going study (as of week 45 of administration).
—: No data

RESULTS

Incidence of sudden death: The incidence of sudden death is summarized in the following Table 1.

Before 1997, the sudden death had occurred very rarely. That is, no sudden death was found in a study A that was started in 1993 and sudden death was observed in only one male and one female in a study B that started in 1994. On the other hand, the incidence of the sudden death increased remarkably in the studies started since 1997. The sudden death began to occur around 20 weeks after the start of administration. The incidence increased over the time, and the highest incidence was observed between weeks 27 and 78 of administration. It was also apparent that the incidence was predominantly higher in females than in males.

Clinical signs: There were no remarkable changes in any of the animals that died suddenly.

Body weight: Characteristic change was observed in most of the animals that died suddenly. A representative pattern of body weight change is shown in the Fig. 1. In almost of all cases, body weight began to decrease gradually from several weeks before death or the body weight gain was suppressed remarkably in-life, and the body weight became nearly the lowest among the respective test group before death. However, decrease in the body weight or suppression of the body weight gain did not occur rapidly, but rather slowly. These changes in the body weight were different from those in the animals that died due to spontaneous lesions including lethal tumors or non-tumors (Fig. 2). In the latter cases, the body weight decreased rapidly due to moribundity or increased rapidly due to rapid growth of the tumor before death.

Food consumption: Although the body weight in the animals that died suddenly was nearly the lowest among the test group as described above, the values for food consumption was comparable to that of other animals.

Necropsy: Packed feed in the oral, pharyngeal and laryngeal cavities was observed in all cases, and no other remarkable lesions were found.

Histophathology: Although examination was not extended to the pharynx and larynx, no severe lesions were noted in any of the organs/tissues including the esophagus, trachea or lungs.

DISCUSSION

There are some common characteristics in the animals that died suddenly. They were: 1) death begins to occur from week 20 of administration and the incidence is highest between weeks 27 and 78 of administration, 2) females predominantly suffer, 3) no apparent clinical signs are noted in-life, 4) the body weight becomes nearly the lowest among the respective test group, 5) no changes are observed in food consumption, 6) packed feed in the oral, pharyngeal and laryngeal cavities is observed at necropsy and 7) there are no severe lesions in histopathology.

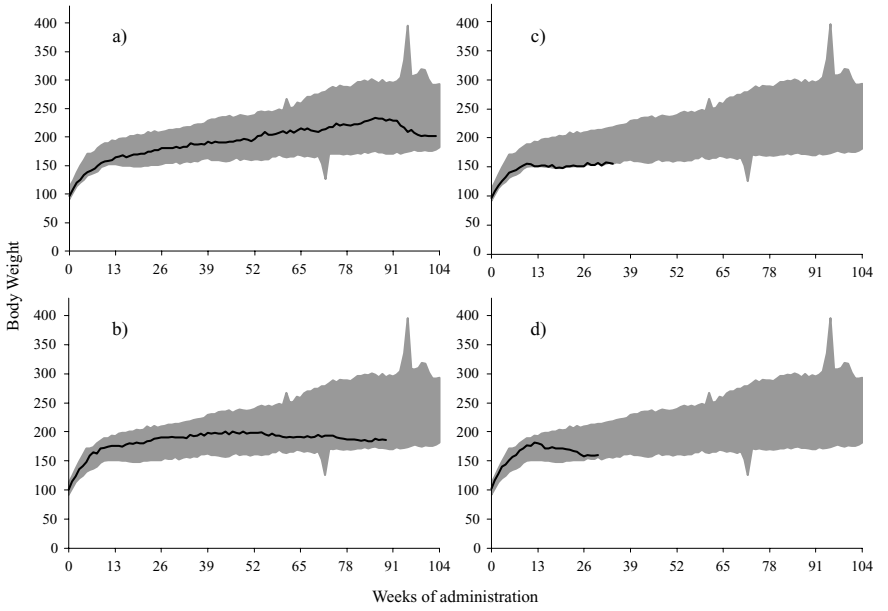

Figure 1. Body weight changes in the representative 4 control females (a to d) that died suddenly in a carcinogenicity study (study C in the table 1). Solid line indicates body weight change in a female that died suddenly and the shadow area indicates the range of individual body weights in the control group. The body weight began to decrease gradually from several weeks before death or the body weight gain was suppressed remarkably in-life, and the body weight became nearly the lowest among the control group before death.

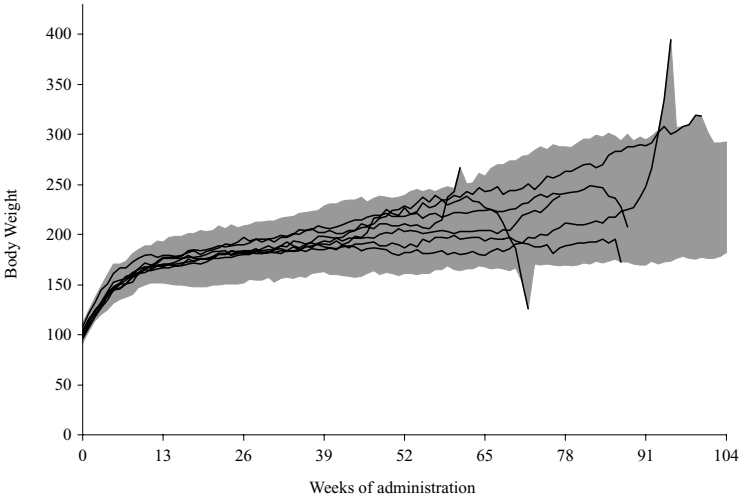

Figure 2. Body weight changes in each female that died suddenly in a carcinogenicity study (study C in the table 1). Solid line indicates body weight change in control females that died of spontaneous lesions and the shadow area indicates the range of individual body weights in the control group. Some animals that died exhibited rapid loss of body weight due to moribundity or other animals exhibited rapid increase in the body weight due to tumor growth.

In 1994, unexpected high mortality has been reported in a carcinogenicity study using F344 rats by gavage administration using a stainless-steel stomach tube and granulomatous inflammation in the oropharyngeal cavity was suggested as a possible cause for death [1]. In the above paper, it was reported that the deaths had occurred from week 16 of administration and reached 13/200 males and 64/200 females as of week 45 of administration, and were predominantly in females. Most of these animals (53%) had impacted food or bedding bolus in the orthopharyngeal cavity. Histopathology revealed a calcified, granulomatous inflammation of the sero-mucinous glands (60%), frequently accompanied by papillary projections (50%) into the lumen. In the paper, partial blockage of the orthopharyngeal lumina due to the above lesions is suggested as the cause of death and 3 major factors are indicated, 1) predisposition of the Fisher rat strain, 2) an unphysiological pH of the solvent (pH 10) and 3) chronic irritation due to an inflexible, metallic gavage tube.

Although some similarities are found in the sudden death between our laboratory and the above report, we could not identify the factors leading to sudden death. Further, it also remains to be solved whether packed feed in the oral, pharyngeal and laryngeal cavities was the cause of death or the result of death occurring during intake of the feed. At any rate, we could not rule out the possibility that metallic gavage tube (inflexible tube) plays an important role in the sudden death, and therefore it is considered that the use of inflerible tube should be avoided in the long-term oral gavage study using F344 rats. Actually, we changed the stomach tube from stainless-steel to a flexible one.

Some carcinogenicity studies using F344 and SD(CD)IGS rats are in the active phase in our laboratory, further information will be presented in future.

REFERENCES

1. Germann P.G. and Ockert D. 1994. Granulomatous inflammation of the oropharyngeal cavity as a possible cause for unexpected high mortality in a Fischer 344 rat carcinogenicity study. Lab. Anim. Sci., 44: 338-343.