Matrix database where similarities exist in the compounds and models tested. We believe the DrugMatrix database is the most suitable database for comparison with the Open TG-GATEs data, particularly in consideration of its scale, the wide variety of compounds, animal strains and type of GeneChip (Figure 2).

Since the data was made publicly available, a significant number of studies and reviews have been conducted by agencies/groups outside of the TGP. The first comprehensive review of a comparison of DrugMatrix and Open TG-GATEs was written by Chen *et al.* (19). Zhang *et al.* successfully identified a consensus gene network between *in vivo* and *in vitro* studies (20). The Open TG-GATEs and DrugMatrix data was used as reference data in a toxicogenomics analysis using RNA-seq with text-mining (21). A carcinogenicity prediction study was conduced using human *in vitro* data with a connectivity map (22). Recently, a web-based tool that assesses potential liver toxicity using specific gene signatures was also developed (23). It also should be noted that Open TG-GATEs data was utilized as trial data in CAMDA (Critical Assessment of Massive Data Analysis) 2012 (http://camda.bioinfo.cipf.es/camda2012/) and 2013 (http://dokuwiki.bioinf.jku.at/doku.php).

A new and prominent feature in this database is the massive amount of digital pathology images. We think the digital pathology images also have the potential to improve toxicity assessments by their use as phenotypic end points. One of the great challenges in this field is to create an automatic pathology annotation system for liver and kidney toxicity. This will greatly improve the efficiency of current pathology diagnosis. Even before this is accomplished, the extraction of further parameters, such as the degree, extent and location of injury, and their associated gene expression profiles and toxicological data, would enable more accurate toxicity predictions.

There are some important points to consider when analyzing Open TG-GATEs data, several of which are discussed here. The first point is concerned with the gene expression data obtained from *in vitro* experiments. When a test compound with direct cytotoxicity in hepatocytes is applied to cells, mRNA expression is drastically changed (1). In such cases, even after global normalization, the fold-change values (differential expression between treated and control samples) will not reflect actual fold-changes. Secondly, it is important to note that the concentration of some compounds (*in vitro* experiments) will not achieve the toxic doses because of limited solubility in the growth medium. In such cases, gene expression will not exhibit significant alterations. A third concern is that of potential variations in methodology. TGP hematology and biochemical parameters were generated over the course of 10 years among different contract research organizations. Thus, the instruments and reagents used to obtain the data are not likely to remain identical over a 10 year period, even within a single organization. Therefore, analyses comparing parameters across the entire database may exhibit certain systematic trends. Consequently, the use of individual experimental sets is recommended for conducting rigorous analyses.

As mentioned above, the Open TG-GATEs data has already been utilized by third-party groups. At the same time, we have started to receive questions about detailed aspects of the data. Although some of the answers to these inquiries have already been published, they may be dispersed in publications published over the previous 10 years, while other answers remain unpublished. In order to understand the characteristics and structure of the data and avoid misuse, we have attempted to provide here an overview of Open TG-GATEs as well as some general guidelines for use of the database.

FUTURE DIRECTIONS

Although toxicogenomics using transcriptomics data is a powerful approach to identify gene set signatures for toxicity, we recognize that a multi-omics approach, such as in combination with metabolomics data, can facilitate the interpretation of gene expression changes due to its complementary role to transcriptomics data (24). We plan to integrate metabolomics data and leverage its complementary relationship in the future. Currently, we are focusing on the efficacy and safety of adjuvants that are components of various vaccines. Similar protocols and methodologies are being applied to obtain gene expression profiles and toxicology data related to adjuvants. These data will be open to the public in a format that can be integrated with Open TG-GATEs data to assess the efficacy and safety of adjuvants.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We are grateful to all researchers, technicians and staffs who took part in the TGP.

FUNDING

Construction of Drug Safety Prediction System by Toxicogenomics Technology and Related Basic Research [H14-Toxico-001]; Drug Safety Assessment Based on Toxicity Mechanisms using Toxicogenomics Database [H19-Toxico-001]; Health and Labour Sciences Research Grant, Japanese Ministry of Health, Labour and Welfare (in part) [H25-souyaku-008, H24-souyaku_sougou-010, H25-jitsuyouka_saisei-016]. Funding for open access charge: Health and Labour Sciences Research Grant, Japanese Ministry of Health, Labour and Welfare [H25-Jitsuyouka(saisei)-016)].

Conflict of interest statement. None declared.

REFERENCES

1. Urushidani,T. (2007) Prediction of hepatotoxicity based on the toxicogenomics database. In: Sahu,SC (ed). *Hepatotoxicity: From Genomics to In Vitro and In Vivo Models.* John Wiley & Sons Ltd., Chichester, UK, pp. 507–529.
2. Kondo,C., Minowa,Y., Uehara,T., Okuno,Y., Nakatsu,N., Ono,A., Maruyama,T., Kato,I., Yamate,J., Yamada,H. *et al.* (2009) Identification of genomic biomarkers for concurrent diagnosis of drug-induced renal tubular injury using a large-scale toxicogenomics database. *Toxicology*, **265**, 15–26.

3. Gao,W., Mizukawa,Y., Nakatsu,N., Minowa,Y., Yamada,H., Ohno,Y. and Urushidani,T. (2010) Mechanism-based biomarker gene sets for glutathione depletion-related hepatotoxicity in rats. *Toxicol. Appl. Pharmacol.*, **247**, 211–221.
4. Uehara,T., Minowa,Y., Morikawa,Y., Kondo,C., Maruyama,T., Kato,I., Nakatsu,N., Igarashi,Y., Ono,A., Hayashi,H. *et al.* (2011) Prediction model of potential hepatocarcinogenicity of rat hepatocarcinogens using a large-scale toxicogenomics database. *Toxicol. Appl. Pharmacol.*, **255**, 297–306.
5. Minowa,Y., Kondo,C., Uehara,T., Morikawa,Y., Okuno,Y., Nakatsu,N., Ono,A., Maruyama,T., Kato,I., Yamate,J. *et al.* (2012) Toxicogenomic multigene biomarker for predicting the future onset of proximal tubular injury in rats. *Toxicology*, **297**, 47–56.
6. Yamada,F., Sumida,K., Uehara,T., Morikawa,Y., Yamada,H., Urushidani,T. and Ohno,Y. (2012) Toxicogenomics discrimination of potential hepatocarcinogenicity of non-genotoxic compounds in rat liver. *J. Appl. Toxicol.*, **33**, 1284–1293.
7. Hirode,M., Ono,A., Miyagishima,T., Nagao,T., Ohno,Y. and Urushidani,T. (2008) Gene expression profiling in rat liver treated with compounds inducing phospholipidosis. *Toxicol. Appl. Pharmacol.*, **229**, 290–299.
8. Uehara,T., Ono,A., Maruyama,T., Kato,I., Yamada,H., Ohno,Y. and Urushidani,T. (2010) The Japanese toxicogenomics project: application of toxicogenomics. *Mol. Nutr. Food Res.*, **54**, 218–227.
9. Sumida,K., Igarashi,Y., Toritsuka,N., Matsushita,T., Abe-Tomizawa,K., Aoki,M., Urushidani,T., Yamada,H. and Ohno,Y. (2011) Effects of DMSO on gene expression in human and rat hepatocytes. *Hum. Exp. Toxicol.*, **30**, 1701–1709.
10. Nuwaysir,E.F., Bittner,M., Trent,J., Barrett,J.C. and Afshari,C.A. (1999) Microarrays and toxicology: the advent of toxicogenomics. *Mol. Carcinog.*, **24**, 153–159.
11. Engelberg,A. (2004) Iconix Pharmaceuticals, Inc.–removing barriers to efficient drug discovery through chemogenomics. *Pharmacogenomics*, **5**, 741–744.
12. Waters,M., Stasiewicz,S., Merrick,B.A., Tomer,K., Bushel,P., Paules,R., Stegman,N., Nehls,G., Yost,K.J., Johnson,C.H. *et al.* (2008) CEBS-Chemical effects in biological systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. *Nucleic Acids Res.*, **36**, D892–D900.
13. Davis,A.P., Murphy,C.G., Johnson,R., Lay,J.M., Lennon-Hopkins,K., Saraceni-Richards,C., Sciaky,D., King,B.L., Rosenstein,M.C., Wiegers,T.C. *et al.* (2013) The comparative toxicogenomics database: update 2013. *Nucleic Acids Res.*, **41**, D1104–D1114.
14. Hubbell,E., Liu,W.-M. and Mei,R. (2002) Robust estimators for expression analysis. *Bioinformatics*, **18**, 1585–1592.
15. Irizarry,R.A., Bolstad,B.M., Collin,F., Cope,L.M., Hobbs,B. and Speed,T.P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.*, **31**, e15.
16. McCall,M.N., Bolstad,B.M. and Irizarry,R.A. (2010) Frozen robust multiarray analysis (fRMA). *Biostatistics*, **11**, 242–253.
17. Nyström-Persson,J., Igarashi,Y., Ito,M., Morita,M., Nakatsu,N., Yamada,H. and Mizuguchi,K. (2013) Toxygates: interactive toxicity analysis on a hybrid microarray and linked data platform. *Bioinformatics*, **29**, 3080–3086.
18. Cronin,M.T.D. (2013) Computational toxicology is now inseparable from experimental toxicology. *Altern. Lab. Anim.*, **41**, 1–4.
19. Chen,M., Zhang,M., Borlak,J. and Tong,W. (2012) A decade of toxicogenomic research and its contribution to toxicological science. *Toxicol. Sci.*, **130**, 217–228.
20. Zhang,J.D., Berntenis,N., Roth,A. and Ebeling,M. (2014) Data mining reveals a network of early-response genes as a consensus signature of drug-induced in vitro and in vivo toxicity. *Pharmacogenomics J.*, **14**, 208–216.
21. Yu,K., Gong,B., Lee,M., Liu,Z., Xu,J., Perkins,R. and Tong,W. (2014) Discovering functional modules by topic modeling RNA-Seq Based Toxicogenomic Data. *Chem. Res. Toxicol.*, **27**, 1528–1536.
22. Caiment,F., Tsamou,M., Jennen,D. and Kleinjans,J. (2014) Assessing compound carcinogenicity in vitro using connectivity mapping. *Carcinogenesis*, **35**, 201–207.
23. Xing,L., Wu,L., Liu,Y., Ai,N., Lu,X. and Fan,X. (2014) LTMap: a web server for assessing the potential liver toxicity by genome-wide transcriptional expression data. *J. Appl. Toxicol.*, **34**, 805–809.
24. Uehara,T., Horinouchi,A., Morikawa,Y., Tonomura,Y., Minami,K., Ono,A., Yamate,J., Yamada,H., Ohno,Y. and Urushidani,T. (2014) Identification of metabolomic biomarkers for drug-induced acute kidney injury in rats. *J. Appl. Toxicol.*, **34**, 1087–1095.

Original Article

Repeated dose and reproductive/developmental toxicity of long-chain perfluoroalkyl carboxylic acids in rats: perfluorohexadecanoic acid and perfluorotetradecanoic acid

Mutsuko Hirata-Koizumi[1], Sakiko Fujii[2], Kato Hina[1], Mariko Matsumoto[1], Mika Takahashi[1], Atsushi Ono[1] and Akihiko Hirose[1]

[1]*Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[2]*Safety Research Institute for Chemical Compounds Co., Ltd., 363-24 Sin-ei, Kiyota-ku, Sapporo, Hokkaido 004-0839, Japan*

(Received August 27, 2015; Accepted September 2, 2015)

ABSTRACT — Perfluoroalkyl carboxylic acids (PFCAs) are global environmental contaminants that are the cause of concern due to their possible effects on wildlife and human health. Since few studies have investigated the toxicity of long-chain PFCAs, we have performed combined repeated dose toxicity studies with the reproduction/developmental toxicity screening tests. We previously examined perfluoroundecanoic acid (C11), perfluorododecanoic acid (C12), and perfluorooctadecanoic acid (C18). We herein reported our results for perfluorotetradecanoic acid (PFTeDA; C14) and perfluorohexadecanoic acid (PFHxDA: C16). Male and female rats were administered PFTeDA at 1, 3 or 10 mg/kg/day or PFHxDA at 4, 20 or 100 mg/kg/day by gavage, and each female was then mated with a male in the same dose group after 14 days. Males were dosed for a total of 42 days and females were dosed throughout the gestation period until day 5 after parturition. PFTeDA and PFHxDA caused hepatocyte hypertrophy and/or fatty changes in the liver at the middle and high doses. PFTeDA also induced follicular cell hypertrophy in the thyroid at the middle and high doses. The only reproductive/developmental effect observed was an inhibited postnatal body weight gain in pups in the 10 mg/kg/day PFTeDA group. Based on these results, the NOAELs for the repeated dose and reproductive/developmental toxicity were concluded to be 1 and 3 mg/kg/day for PFTeDA and 4 and 100 mg/kg/day for PFHxDA, respectively. Our current and previous results indicate that the toxicity of PFCAs decreases with increases in the carbon chain length from 12 to 18.

Key words: Perfluoroalkyl carboxylic acids, Perfluorotetradecanoic acid, Perfluorohexadecanoic acid, Repeated dose toxicity, Reproductive and developmental toxicity, Rat

INTRODUCTION

A large number of chemicals are industrially produced and used without appropriate evaluations of their potential hazards to human health. The toxicity of these chemicals is continuously assessed in Japan by safety programmes for existing chemicals. These programmes have recently targeted perfluoroalkyl carboxylic acids (PFCAs) with carbon chain lengths of 11 to 18.

PFCAs are global environmental contaminants that are the cause of concern due to their possible effects on human health (Hekster *et al.*, 2003; Lau *et al.*, 2007; Post *et al.*, 2012). Although extensive toxicological research has been performed, especially on perfluorooctanoic acid (PFOA), which has a carbon chain length of 8, few studies have examined the toxicity of PFCAs with a carbon chain length of 11 and higher. Combined repeated dose toxicity studies with the reproduction/developmental toxicity screening tests (combined studies) have been conducted by Japanese safety programmes for existing chemicals in order to obtain initial toxicological information on such long-chain PFCAs.

We have reported our findings in combined studies on perfluoroundecanoic acid (PFUnA, C11), perfluorododecanoic acid (PFDoA, C12) and perfluorooctadecanoic acid (PFOcDA, C18) (Hirata-Koizumi *et al.*, 2012; Kato

Correspondence: Akihiko Hirose (E-mail: hirose@nihs.go.jp)

et al., in press; Takahashi *et al.*, 2014). We showed that the main toxic target of these long-chain PFCAs was the liver, but they also affected reproduction/development at the higher doses. Based on these findings, the NOAELs were concluded to be 0.1 mg/kg/day for PFUnA (C11) and PFDoA (C12) and 40 mg/kg/day for PFOcDA (C18). The value of NOAEL for repeated dose toxicity of PFOcDA (C18) was much higher than those of PFUnA (C11) and PFDoA (C12). The present study described the results obtained from combined studies on perfluorotetradecanoic acid (PFTeDA, C14, CAS No. 376-06-7) and perfluorohexadecanoic acid (PFHxDA, C16, CAS No. 67905-19-5), whose carbon lengths are in between previously reported substances. In this paper, we discuss the toxicity of PFCAs in terms of their carbon chain length.

MATERIALS AND METHODS

Combined repeated dose toxicity studies with the reproduction/developmental toxicity screening tests were performed on PFTeDA and PFHxDA at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan), according to the OECD guidelines for testing chemicals No. 422 under good laboratory practice (GLP) standards.

Chemicals and treatment

PFTeDA (lot No. 3728, purity: 96.5%) and PFHxDA (lot No. 1262, purity: 95.3%) were obtained from Exfluor Research Corporation (Round Rock, TX, USA). They were suspended in a 0.5% water solution of carboxymethylcellulose sodium, and administered by gavage. The homogeneity of test substances in the dosing solution and their stability until they were administered was confirmed before the start of the study. A separate control group was used for each chemical evaluation, and the control rats received vehicle only. The daily volume administered was 10 mL/kg, which was calculated based on the latest body weight. Dose levels were determined to be 1, 3, and 10 mg/kg/day or PFTeDA and 4, 20, and 100 mg/kg/day for PFHxDA based on the results of 14-day dose finding studies.

Animals and housing conditions

Eight-week-old male and female Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). This species and strain was selected because its reproductive performance is stable and sufficient historical data was available on this strain at the laboratory.

Following quarantine and acclimation periods, the animals were subjected to oral administration of PFTeDA or PFHxDA at 10 weeks of age. They were housed individually, except for the mating and lactation periods, in bracket-type metallic cages with a wire-mesh floor, and maintained in an air-conditioned room with controlled temperature (22 ± 3°C) and humidity (50 ± 20%). Light was provided on a 12-hr light/dark cycle (light: 8:00-20:00). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Pregnant females were reared using wood chips as bedding from day 17 of gestation to day 4 after delivery.

The present study protocols were approved by the Ethical Committee for animal experiments in the Safety Research Institute for Chemical Compounds Co., Ltd., and performed in accordance with the standard operational procedure contained in the Institutional Ethical Code for Animal Experiments. The use and care of animals complied with the Act on Welfare and Management of Animals (Japanese Animal Welfare Law, Act No. 105 of October 1, 1973. As amended up to Act No. 50 of June 2, 2006), Standards Relating to the Care, Management of Laboratory Animals and, Relief of Pain (Announcement No. 88 of Ministry of the Environment, Japan, dated April 28, 2006) and Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, dated May 22, 1987).

Study design

Male rats (12 animals/dose) were administered PFTeDA or PFHxDA for 14 days and then cohabited with females. This administration of PFTeDA or PFHxDA was continued during and after the mating period, and seven males in the control and high dose groups and all of animals in the low and middle dose groups were euthanized after a 42-day administration (main group). The remaining rats were maintained without the administration of PFTeDA or PFHxDA for 14 days after a 42-day administration and then euthanized for examination (recovery group).

Female rats were assigned to the main group or recovery group before PFTeDA and PFHxDA were administered. The number of females was 12 per dose in the main group, and PFTeDA or PFHxDA was administered for 14 days before mating, and continued throughout the mating, gestation, and lactation periods up to 5 days after parturition. In the recovery group, 5 females/dose (vehicle control and high dose only) were administered for 42 days without mating and euthanized after the 14-day recovery period.

Repeated dose toxicity evaluation

All animals were observed twice daily for general appearance and behavior. Detailed clinical observations, including evaluations in the home cage, during handling and outside the home cage in an open field, were also conducted using a standardized scoring system once a week. Body weight and food consumption was measured at regular intervals (at least once a week).

Males and females in the recovery group were subjected to urinalysis and functional observations in the sixth week of the administration period and second week of the recovery period. Functional observations were also performed for females in the main group on day 4 of lactation. The parameters examined were as follows:

- Functional observations: sensory reactivity to visual, tactile, auditory, pain, and proprioceptive stimuli, mid-air righting reflex, forelimb and hindlimb grip strength, and spontaneous motor activity
- Urinalysis: pH, protein, glucose, ketone body, urobilinogen, bilirubin, occult blood, color, urine volume, and specific gravity

The effects of the administration of PFTeDA and PFHxDA on hematology, blood biochemistry, organ weight, and histopathology were examined on the day after the final administration in the main group and after the completion of the recovery period in the recovery group. Serum thyroid-related hormone levels were also analyzed in the study on PFHxDA because changes were observed in thyroid weight.

The surviving rats were anesthetized deeply after 16- to 22-hr of starvation, and blood samples were collected from the abdominal aorta. The animals were then euthanized by exsanguination, and the organs and tissues of the entire body were examined macroscopically. The major organs were isolated and weighed, and organ weight per body weight (relative weight) was calculated. The eyeball and Harderian gland were fixed and preserved with Davidson's fixative solution. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol. The other organs were stored in 10% neutral-buffered formalin. All preserved organs in the control and high dose groups were sectioned, stained with hematoxylin-eosin, and examined under a light microscope. If treatment-related histopathological changes were found, the same tissues were examined in the low and middle dose groups. The parameters and organs examined were as follows:

- Hematology: red blood cell count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cell count, differential count of white blood cells, prothrombin time (PT), and activated partial thromboplastin time (APTT)
- Blood biochemistry: total protein, albumin, albumin/globulin ratio, protein fraction ratio, glucose, total cholesterol, triglyceride, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALP), γ-glutamyltranspeptidase, calcium, inorganic phosphorus (IP), sodium (Na), potassium, and chlorine (Cl)
- Hormonalysis (only in the study on PFHxDA): triiodothyronine (T_3), thyroxine (T_4), and thyroid-stimulating hormone (TSH)
- Organ weight: the brain, pituitary gland, thyroid, heart, liver, spleen, kidney, adrenal gland, thymus, testis, epididymis, prostate gland, seminal vesicle, and ovary
- Histopathology: the brain, spinal cord, pituitary gland, thymus, thyroid, adrenal gland, spleen, heart, esophagus, stomach, liver, pancreas, duodenum, jejunum, ileum, cecum, colon, rectum, trachea, lung, kidney, bladder, testis, epididymis, prostate, seminal vesicle, ovary, uterus, eyeball, Harderian gland, mammary gland, femur, mesenteric and mandibular lymph nodes, sciatic nerve, and grossly abnormal tissues

Reproductive/developmental toxicity evaluation

The estrous cyclicity was evaluated daily by vaginal lavage sampling from the first day of the administration period until evidence of copulation was detected in the main group and until the necropsy day in the recovery group. Females having repeated 4-6 day estrous cycles were judged to have normal estrous cycles.

During the mating period, males and females randomly selected from the same dose group were cohabited on a 1:1 basis until successful copulation occurred for a maximum of 14 days. The presence of sperm in the vaginal smear and/or a vaginal plug was considered to be evidence of successful mating. The day of successful mating was designated as day 0 of gestation. Successfully cohabited females were allowed to spontaneously deliver and nurse their pups until the end of the study. They were checked at least three times daily on days 21-25 of gestation, and the day on which dams held their pups under the abdomen in the nest by 9:00 was designated as day 0 of lactation or postnatal day (PND) 0. Gestational length was recorded, and the following indices were computed for each dose group.

$$\text{Copulation index (\%)} = \frac{\text{Number of animals with successful copulation}}{\text{Number of animals cohabited}} \times 100$$

$$\text{Fertility index (\%)} = \frac{\text{Number of pregnant females}}{\text{Number of pairs with successful copulation}} \times 100$$

$$\text{Gestation index (\%)} = \frac{\text{Number of females with live pups}}{\text{Number of pregnant females}} \times 100$$

All live and dead pups born were counted, and live pups were sexed and examined grossly on PND 0. They were observed daily for general appearance and behavior, and the body weight of live pups was recorded on PNDs 0, 1, and 4. On PND 4, the pups were euthanized and subjected to a gross external and internal observation. At necropsy of maternal animals, the numbers of corpora lutea in the ovary and implantation sites in the uterus were recorded.

Statistical analysis

Parametric data were evaluated by Bartlett's test for the homogeneity of variances. The neonatal sex ratio and body weights of male and female pups were analyzed using the litter as the experimental unit. When homogeneity was recognized, a one-way analysis of variance was applied. If a significant difference was found, Dunnett's test was used for pairwise comparisons between the control and individual treatment groups. Data without homogeneity were subjected to the Kruskal-Wallis test, and if significant differences were detected, the Mann-Whitney U test was used to compare PFTeDA- or PFHxDA-treated groups with the correspondent control group.

The results of the detailed clinical and functional observations, qualitative parameters of urinalysis, specific gravity of urine, and histopathological findings with multiple grades were evaluated for the trend in each group by the Kruskal-Wallis test. When significant differences were found, data were compared between the control and each dosage group using the Mann-Whitney U test. The incidence of females with normal estrous cycles, copulation, fertility, and gestation indices, and histopathological findings with a single grade were analyzed using the chi-square test or Fisher's exact test.

RESULTS

Perfluorotetradecanoic acid (PFTeDA; C14)

Repeated dose toxicity

No treatment-related abnormalities were observed in general appearance or behavior throughout the administration and recovery periods. In the 10 mg/kg/day group, the body weights of male rats were significantly lower than those in the control group on days 7 and 14 of the recovery period (Fig. 1). Although similar results were observed in the female recovery group, significant differences were not observed from the control. Body weights in the female main group were significantly lower on day 4 of the lactation period at 3 mg/kg/day and during the lactation period at 10 mg/kg/day. A significant decrease in food consumption was only found in females given

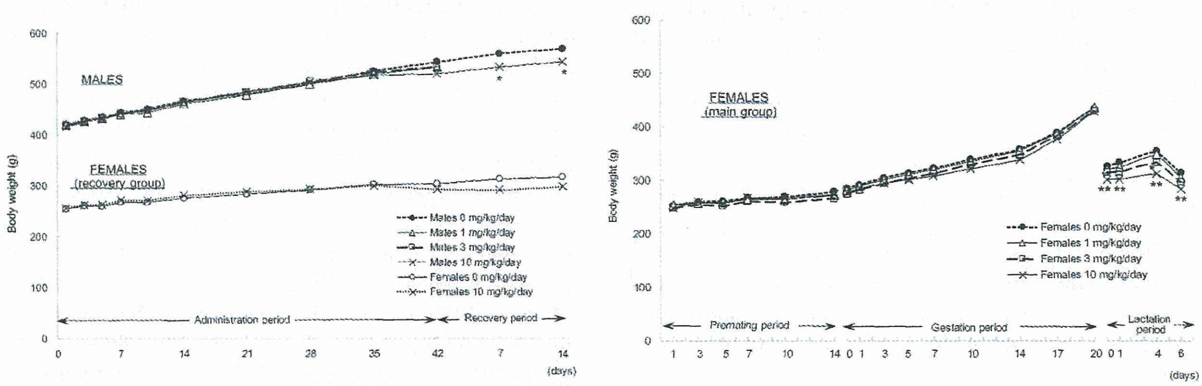

Fig. 1. Body weight changes in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFTeDA in rats. *: Significantly different from the control, $P \leq 0.05$. **: Significantly different from the control, $P \leq 0.01$.

10 mg PFTeDA/kg/day in the main group on days 5 and 10 of gestation and on day 4 of lactation (data not shown).

At the end of the administration period, the hindlimb grip strength of male rats decreased in a dose-dependent manner, and a significant difference from the control was found in the 3 and 10 mg/kg/day groups (Fig. 2). No significant changes were observed in grip strength in males of the recovery group or in females. Furthermore, no significant differences were observed in urinalysis parameters between the PFTeDA-treated and control groups, either at the end of the administration period or at the end of the recovery period (data not shown).

In the main group, the only significant effect observed on hematology was a shortening in APTT in males given 10 mg PFTeDA/kg/day (Table 1). Blood biochemical examinations showed significant decreases in total protein in males and the β-globulin fraction in both sexes at 10 mg/kg/day (Table 1). Significant increases were also observed in ALP and BUN in males and Cl in females in the 10 mg/kg/day group. Absolute and relative liver weights were significantly increased at 3 and 10 mg/kg/day in males (Table 1). A significant increase in the relative liver weight was also found in females at 10 mg/kg/day. In males, the absolute weight of the pituitary gland was significantly decreased at 3 and 10 mg/kg/day and the relative weight was also significantly decreased at 3 mg/kg/day. The absolute weight of the seminal vesicle was significantly decreased at all doses.

Histopathologically, centrilobular hepatocyte hypertrophy was observed in males at 3 and 10 mg/kg/day and in females at 10 mg/kg/day (Table 2). Microgranulomas were noted in the liver of both sexes in all groups containing the control; however, the extent of these was significantly higher in females given 10 mg PFTeDA/kg/day. Focal necrosis was detected in the liver of one female given 10 mg PFTeDA/kg/day. Follicular cell hypertrophy was observed in the thyroids of males at 3 and 10 mg/kg/day. In females, the incidences of decreases in extramedullary hematopoiesis in the spleen and cortex atrophy in the thymus were significantly increased at 10 mg/kg/day. No treatment-related changes were detected in histopathology in other organs, including the pituitary gland and seminal vesicle.

In the recovery group, the hemoglobin concentration and hematocrit value were significantly decreased, and PT was significantly shortened in females in the 10 mg/kg/day group (Table 1). Significant increases were also observed in ALP and IP and decreases in triglyceride levels in males, as well as a significant decrease in total cholesterol and increase in BUN in females in the 10 mg/kg/day group. In this group, the absolute and/or relative liver weights were significantly increased, and histopathologically, centrilobular hypertrophy of hepatocytes, diffuse hypertrophy of hepatocytes or diffuse fatty change was found in the liver (Table 2). Hypertrophy of follicular cells was observed in the thyroids of two males in the 10 mg/kg/day group.

Reproductive/developmental toxicity

Reproductive/developmental results are summarized in Table 3. No significant changes were found in reproduc-

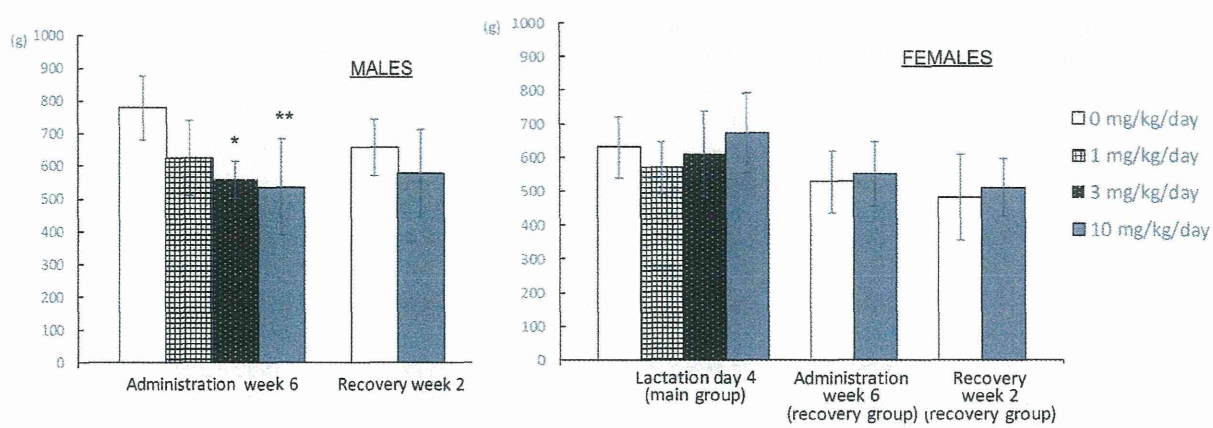

Fig. 2. The hindlimb grip strength of male and female rats in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFTeDA. *: Significantly different from the control, $P \leq 0.05$. **: Significantly different from the control, $P \leq 0.01$.

Table 1. Significant changes in hematological and blood biochemical parameters and organ weights in rats given PFTeDA.

Dose (mg/kg/day)	At the end of the administration period (Main group)				At the end of the recovery period (Recovery group)	
	0	1	3	10	0	10
MALES						
Hematology						
Hemoglobin (g/dL)	15.78 ± 0.61	16.00 ± 0.85	15.90 ± 0.55	15.98 ± 0.77	16.42 ± 0.38	15.88 ± 0.61
Hematocrit (%)	44.86 ± 2.02	45.26 ± 2.10	44.80 ± 1.81	45.16 ± 2.38	46.16 ± 1.04	45.50 ± 1.99
PT (sec)	21.56 ± 6.33	22.46 ± 0.78	22.74 ± 2.34	21.78 ± 4.27	20.10 ± 3.33	21.14 ± 1.62
APTT (sec)	27.42 ± 3.61	28.18 ± 1.36	26.42 ± 1.97	23.62 ± 1.28*	25.78 ± 2.34	24.68 ± 3.86
Blood biochemistry						
Total protein (g/dL)	5.74 ± 0.19	5.66 ± 0.21	5.78 ± 0.30	5.26 ± 0.11**	5.80 ± 0.12	5.52 ± 0.34
β-Globulin fraction of protein (%)	17.12 ± 0.91	16.14 ± 0.80	16.24 ± 0.57	15.68 ± 0.99*	16.40 ± 0.72	16.36 ± 0.51
ALP (IU/L)	363.2 ± 81.5	352.6 ± 113.1	355.0 ± 51.8	520.8 ± 75.6*	334.2 ± 51.7	470.4 ± 67.3**
Triglyceride (mg/dL)	36.0 ± 9.4	50.4 ± 22.1	46.4 ± 17.1	21.8 ± 6.3	59.2 ± 14.2	31.8 ± 8.9**
Total cholesterol (mg/dL)	60.0 ± 9.9	53.8 ± 6.0	44.0 ± 11.2	50.8 ± 14.7	63.8 ± 18.9	50.6 ± 10.7
BUN (mg/dL)	14.26 ± 1.43	14.02 ± 1.47	16.00 ± 1.71	19.88 ± 1.99**	14.82 ± 1.04	16.20 ± 2.11
Cl (mEq/L)	106.8 ± 1.3	107.4 ± 1.8	107.0 ± 0.7	108.4 ± 2.3	105.4 ± 1.1	106.6 ± 1.1
IP (mg/dL)	6.36 ± 0.47	6.04 ± 0.50	6.42 ± 0.60	6.98 ± 0.44	6.20 ± 0.33	6.68 ± 0.29*
Organ weight						
Liver (g)	11.95 ± 1.53	12.09 ± 0.73	14.52 ± 1.82*	15.21 ± 0.53**	13.09 ± 0.95	16.41 ± 0.48**
(%)	2.41 ± 0.11	2.49 ± 0.12	2.87 ± 0.23**	3.25 ± 0.07**	2.43 ± 0.15	3.18 ± 0.16**
Pituitary gland (mg)	13.20 ± 0.74	13.06 ± 1.17	11.18 ± 0.87*	11.48 ± 1.07*	13.10 ± 2.46	13.70 ± 1.04
(10^{-3}%)	2.69 ± 0.28	2.69 ± 0.26	2.22 ± 0.21*	2.46 ± 0.28	2.43 ± 0.44	2.65 ± 0.21
Seminal vesicle (g)	2.53 ± 0.51	2.00 ± 0.19*	2.01 ± 0.29*	1.91 ± 0.15*	2.18 ± 0.12	2.14 ± 0.42
(%)	0.512 ± 0.102	0.412 ± 0.035	0.402 ± 0.077	0.408 ± 0.023	0.402 ± 0.029	0.414 ± 0.074
FEMALES						
Hematology						
Hemoglobin (g/dL)	15.14 ± 0.40	14.68 ± 0.70	15.02 ± 0.87	15.50 ± 0.60	15.68 ± 0.62	14.46 ± 0.68*
Hematocrit (%)	43.94 ± 2.17	43.24 ± 2.58	44.26 ± 2.83	44.94 ± 1.48	44.34 ± 1.67	40.90 ± 1.95*
PT (sec)	18.78 ± 0.93	17.60 ± 1.04	17.76 ± 0.47	17.52 ± 1.32	17.26 ± 0.50	16.12 ± 0.87*
APTT (sec)	19.00 ± 0.46	19.54 ± 0.52	19.88 ± 0.60	19.44 ± 0.75	18.28 ± 1.30	19.48 ± 1.03
Blood biochemistry						
Total protein (g/dL)	6.30 ± 0.22	6.52 ± 0.25	6.40 ± 0.23	6.12 ± 0.44	6.46 ± 0.32	6.30 ± 0.28
β-Globulin fraction of protein (%)	18.32 ± 0.54	17.32 ± 1.15	17.56 ± 0.98	15.90 ± 0.92**	14.86 ± 0.98	14.54 ± 0.48
ALP (IU/L)	196.8 ± 54.0	174.0 ± 33.7	177.8 ± 51.3	236.0 ± 32.1	177.6 ± 51.8	234.2 ± 72.9
Triglyceride (mg/dL)	49.6 ± 29.2	43.0 ± 10.9	47.6 ± 13.9	29.2 ± 18.0	15.8 ± 7.7	18.8 ± 16.5
Total cholesterol (mg/dL)	68.8 ± 12.2	63.0 ± 14.5	57.2 ± 10.9	51.2 ± 15.4	75.8 ± 11.6	56.6 ± 10.6*
BUN (mg/dL)	25.58 ± 2.73	24.08 ± 2.27	23.80 ± 3.17	30.44 ± 4.18	15.00 ± 1.60	20.24 ± 4.60*
Cl (mEq/L)	102.6 ± 2.3	103.8 ± 0.8	105.0 ± 1.6	105.8 ± 0.8*	108.4 ± 2.2	107.4 ± 1.1
IP (mg/dL)	9.08 ± 0.91	8.86 ± 0.79	8.20 ± 0.45	8.30 ± 0.60	4.70 ± 0.85	5.64 ± 0.61
Organ weight						
Liver (g)	10.14 ± 0.75	10.96 ± 1.13	10.17 ± 0.19	10.44 ± 0.89	7.18 ± 0.69	7.90 ± 1.07
(%)	3.33 ± 0.17	3.49 ± 0.21	3.42 ± 0.16	3.70 ± 0.29*	2.40 ± 0.27	2.83 ± 0.27*
Pituitary gland (mg)	16.26 ± 2.43	17.44 ± 1.80	16.54 ± 0.93	15.82 ± 2.99	16.32 ± 2.29	18.06 ± 3.75
(10^{-3}%)	5.37 ± 0.97	5.58 ± 0.62	5.56 ± 0.24	5.67 ± 1.37	5.44 ± 0.63	6.42 ± 1.04

Data are shown as the mean ± S.D.

*: Significantly different from the control group at $P \leq 0.05$.

**: Significantly different from the control group at $P \leq 0.01$.

Table 2. Histopathological findings in the combined repeated dose toxicity study with reproduction/developmental toxicity screening test for PFTeDA in rats.

Dose (mg/kg/day)		At the end of the administration period (Main group)				At the end of the recovery period (Recovery group)	
		0	1	3	10	0	10
MALES							
Number of examined animals		7	12	12	7	5	5
Liver							
- Centrilobular hypertrophy of hepatocytes	+	0	0	6 ┐	0 ┐	0	2 ┐
	++	0	0	2 ┘*	7 ┘**	0	3 ┘**
- Microgranuloma	+	4	10	6	1	3	4
- Diffuse fatty change	+	0	0	0	0	0	2
Thyroid							
- Hypertrophy of follicular cells	+	0	0	4	4	0	2
FEMALES							
Number of examined animals		12	12	12	12	5	5
Liver							
- Centrilobular hypertrophy of hepatocytes	+	0	0	0	9**	0	2
- Diffuse hypertrophy of hepatocytes	+	0	0	0	0 ┐	0	2
- Microgranuloma	+	6	9	8	3 ┘**	4	1
	++	0	0	0	7	0	3
- Focal necrosis	+	0	0	0	1	0	0
Spleen							
- Decrease in extramedullary hematopoiesis	+	2	0	2	8*	0	0
Thymus							
- Cortex atrophy	+	1	2	1	8**	0	0

Values represent the number of animals with findings.
+: Slight change, ++: moderate change
*: Significantly different from the control group at $P \leq 0.05$.
**: Significantly different from the control group at $P \leq 0.01$.
Brackets in the data columns mean that statistical analysis was performed for a total number of animals with findings in consideration of grades.

tive parameters, including estrous cyclicity, the copulation index, fertility index, gestation index or gestation length. No significant differences were observed in the number of corpora lutea, implantation sites, delivered pups, or live pups on PNDs 0 and 4, or in the sex ratio of live pups between the PFTeDA-treated and control groups. In the 10 mg/kg/day group, the body weights of male and female pups were significantly lower on PNDs 1 and 4. There were no abnormalities in the general appearance or necropsy findings of neonates.

Perfluorohexadecanoic acid (PFHxDA: C16)

Repeated dose toxicity

No treatment-related clinical signs of toxicity were observed throughout the study. The body weights of males in the 100 mg/kg/day group were significantly lower than those of the control on days 35 and 42 of the administration period (Fig. 3). Such effects on body weight were not detected in the females. Food consumption was significantly reduced on day 14 of the recovery period in males given 100 mg PFHxDA/kg/day, on days 5-14 of the gestation period, and on day 4 of the lactation period in females given 100 mg PFHxDA/kg/day in the main group (data not shown).

A functional observation at the end of the administration period revealed no significant changes in any of the PFHxDA-treated groups, but a significant decrease in hindlimb grip strength at the end of recovery period in both sexes given 100 mg PFHxDA/kg/day (Fig. 4). No significant difference was seen in any urinalysis parameters between the control and PFHxDA-treated groups either at the end of the administration period or at the end of the recovery period (data not shown).

At the end of the administration period, no significant differences were observed in any hematological parameters between the control and PFHxDA-treated groups

Table 3. Reproductive/developmental findings in the combined repeated dose toxicity study with the reproduction/developmental screening test for PFTeDA in rats.

Dose (mg/kg/day)		0	1	3	10
Incidence of females with normal estrous cycle[a] (%)		91.7	91.7	91.7	100
Estrous cycle length[a, b] (days)		4.06 ± 0.21	4.00 ± 0.00	3.98 ± 0.17	4.06 ± 0.20
Number of cohabited pairs		12	12	12	12
Copulation index (%)	Males	91.7	91.7	100	100
	Females	100	100	100	100
Fertility index (%)		100	100	91.7	100
Gestation index (%)		100	100	100	100
Gestation length[b] (days)		22.3 ± 0.7	22.3 ± 0.5	22.2 ± 0.4	22.0 ± 0.0
Number of pregnant females		12	12	11	12
Number of corpora lutea[b]		16.7 ± 1.9	16.4 ± 1.8	16.1 ± 1.6	17.0 ± 2.2
Number of implantation sites[b]		16.0 ± 1.7	16.2 ± 1.6	15.9 ± 1.8	16.4 ± 2.0
Number of pups delivered[b]		14.5 ± 3.8	15.3 ± 2.0	15.3 ± 2.1	15.8 ± 1.8
Sex ratio of pups (male pups / all pups)[b]		0.470 ± 0.113	0.532 ± 0.101	0.481 ± 0.132	0.547 ± 0.116
Number of live pups[b]	on PND 0	14.5 ± 3.8	15.3 ± 2.0	15.2 ± 2.0	15.8 ± 1.8
	on PND 4	14.1 ± 3.6	15.0 ± 1.9	15.1 ± 1.8	15.2 ± 1.3
Body weight of male pups[b] (g)					
	on PND 0	6.58 ± 0.93	6.62 ± 0.76	6.43 ± 0.41	6.01 ± 0.34
	on PND 1	7.32 ± 1.14	7.19 ± 0.89	6.97 ± 0.52	6.31 ± 0.46**
	on PND 4	10.66 ± 2.03	10.53 ± 1.31	9.93 ± 0.76	8.77 ± 0.85**
Body weight of female pups[b] (g)					
	on PND 0	6.29 ± 0.81	6.28 ± 0.68	6.05 ± 0.34	5.78 ± 0.36
	on PND 1	6.99 ± 1.03	6.83 ± 0.78	6.53 ± 0.49	6.05 ± 0.45**
	on PND 4	10.18 ± 1.72	9.98 ± 1.21	9.35 ± 0.68	8.41 ± 0.85**

a: Data of the main group are shown. No significant changes in estrous cycle normality were found in the recovery group, either.
b: Data are shown as the mean ± S.D.
*: Significantly different from the control group at P ≤ 0.05.
**: Significantly different from the control group at P ≤ 0.01.

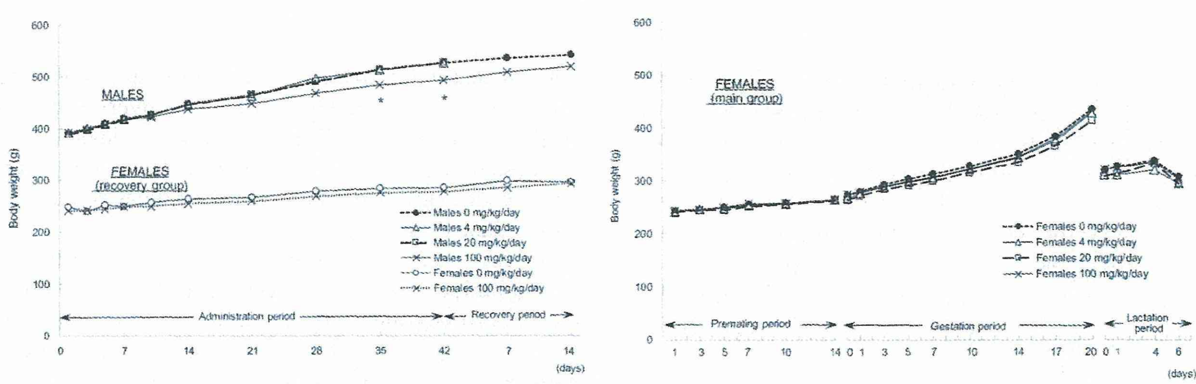

Fig. 3. Body weight changes in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for PFHxDA in rats. *: Significantly different from the control, P ≤ 0.05.