vitro genotoxicity is equivocal.

These branched alkylphenols are widely used as antioxidants in rubbers, plastics, foods and oils to inhibit or slow oxidative process, and they are also used as intermediates for synthesis of resins, plasticizers, surface-active agents, perfumes and other products[8]. Consumer exposure to these branched alkylphenols can occur through the use of products containing these chemicals. The similarities in structure, use and *in vitro* genotoxicity seem to support grouping these chemicals into one category. To ascertain robustness of the chemical category of these branched alkylphenols on genotoxicity, additional *in vivo* micronucleus tests were assessed on 2-isopropyl-5-methylphenol (CAS : 89-83-8) as a target of isopropyl substitutions and *o-sec*-butylphenol (CAS : 89-72-5) as a target of *sec*-butyl substitutions. The present paper reports the results of *in vivo* micronucleus tests of 2-isopropyl-5-methylphenol and *o-sec*-butylphenol and discusses genotoxic potential of these chemicals by the category approach.

Materials and Methods

In vivo micronucleus test in mouse bone marrow

The test was performed according to the Guideline for Screening Mutagenicity Testing of Chemicals, Japan and OECD TG 474[9] and in compliance with GLP requirements[5]. 2-Isopropyl-5-methylphenol (Purity>98% ; Lot No.CAN1119) was obtained from Wako Pure Chemical Industries, Ltd., Japan and cyclophosphamide (CAS No.50-18-0 ; Lot No.73H0846) obtained from Sigma Chemical Co. was used as a positive control. Crj : BDF1 mice, 8-weeks of age, from Charles River Laboratories, Japan were used after more than 1 week acclimatization. Mice were housed in a temperature-and humidity-controlled room (23±1℃ ; 55±5%) with a light-dark (12 h-12 h) cycle. In a dose finding study, 5 male and 5 female mice were singly given 2-isopropyl-5-methylphenol by gavage at 500, 750, 1000, 1250, 1750 or 2000 mg/kg bw, in which deaths were observed at 1500 and 1750 mg/kg bw in females while no death was observed in males for four days (data not shown). Subsequently, a single dose of 2-isopropyl-5-methylphenol at 1500-2000 mg/kg bw caused deaths in males in a preliminary study (data not shown) ; therefore, 1250 mg/kg bw was set as the highest dose in the main test. Sampling time was set at 24 h after administration according to the preliminary study, in which no differences were observed in a sampling time of 24, 48, or 72 h.

Mice (5/sex/dose) were received single oral gavage administration of 2-isopropyl-5-methylphenol at 0 (control : olive oil), 156.3, 312.5, 625, or 1250 mg/kg bw. Positive control mice (5/sex) received single oral doses of cyclophosphamide at 50 mg/kg bw. All groups of mice were killed 24 h after treatment. Bone marrow samples were prepared according to the method of Schmid[10,11] for the control, 312.5, 625, or 1250 mg/kg bw groups. Samples were stained with 0.04 mg/mL acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand polychromatic erythrocytes (PCE) for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 500 total erythrocytes for each animal.

Data were analyzed using the Fisher's exact test with Bonferroni correction for group mean comparisons[13]. Dose-dependent increases of the number of micronucleated polychromatic erythrocyte per total number of PCE (MNPCE) was detected using the Cochran-Armitage test[14]. Proportion of PCE among the total erythrocyte population was analyzed by the t-test with Bonferroni correction.

In vivo micronucleus test in rat bone marrow

The test was performed according to OECD TG 474[9] and Guideline for Genotoxicity Tests on Drugs[15], and in compliance with GLP requirements[5]. *o-sec*-Butylphenol (Purity 99.15%) was obtained from Honshu Chemical Industry, Japan and cyclophosphamide monohydrate (CAS No.6055-199-2 ; Lot No.036K1225) obtained from Sigma-Aldrich Co. was used as a positive control. Crl : CD (SD) rats, 7-weeks of age, from Charles River Laboratories, Japan were used after one week of acclimatization. Rats were housed in a temperature-and humidity-controlled room (21.8-22.9℃ ; 46.6-62.2%) with a light-dark (12 h-12 h) cycle. The animals were given commercial food and water *ad libitum*. In a dose finding study, 3 male and 3 female rats were given *o-sec*-butylphenol by gavage at 150, 300, 600, 1200 mg/kg bw once a day for two days (24 h interval), in which deaths were observed at 1200 mg/kg bw/day and clinical changes were observed at 600 mg/kg bw/day in both sex (data not shown). Therefore, 600 mg/kg bw was set as the highest dose.

Table 2 Results of the micronucleus test in mice after gavage dose of 2-isopropyl-5-methylphenol (CAS: 89-83-8)

Dose	Number of mice	MNPCE (%)[a]	PCE/ (PCE+NCE) (%)[b]
Male			
0 mg/kg (Solvent control: olive oil)	5	0.12 ± 0.08[c]	48.6 ± 8.6
312.5 mg/kg	5	0.20 ± 0.10	55.7 ± 5.4
625 mg/kg	5	0.19 ± 0.16	48.2 ± 12.3
1250 mg/kg	5	0.15 ± 0.12	53.6 ± 10.5
50 mg/kg (Positive control: CP)	5	1.57 ± 0.70*	45.6 ± 13.1
Female			
0 mg/kg (Solvent control: olive oil)	5	0.17 ± 0.14	63.8 ± 4.8
312.5 mg/kg	5	0.14 ± 0.07	60.6 ± 8.0
625 mg/kg	5	0.15 ± 0.09	62.8 ± 4.8
1250 mg/kg	5	0.11 ± 0.04	64.2 ± 8.2
50 mg/kg (Positive control: CP)	5	1.43 ± 0.35*	54.9 ± 6.2

PCE: Polychromatic erythrocyte, MNPCE: Micronucleated PCE, NCE: Normochromatic erythrocyte, CP: Cyclophosphamide
*: Significantly different from the solvent control ($P<0.01$)
[a]: Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
[b]: Number of polychromatic erythrocytes/ total number of erythrocytes observed.
[c]: Values are given as mean ± S. D.

All rats were weighed prior to dosing and preparation of bone marrow samples. Clinical signs of toxicity were observed at 1 and 3 h after treatment, and prior to dosing and preparation of bone marrow samples. Rats (5/sex/dose) were received oral gavage administration of *o-sec*-butylphenol twice with 24 h intervals at 0 (control: corn oil), 75, 150, 300 or 600 mg/kg bw. Positive control rats (5/sex) received two oral doses of cyclophosphamide (24 h intervals) at 20 mg/kg bw/day.

All groups of rats were killed 24 h after last treatment. One femur was removed from each rat, and bone marrow cells were flushed out with 10% neutral buffer formalin. Excess serum was removed by centrifugation. Bone marrow samples were stained with 0.05 w/v% acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand PCE for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 1000 total erythrocytes for each animal.

Data were analyzed using the Kastenbaum and Bowman's method[16] for group mean comparisons. Dose-dependent increases of the MNPCE were detected using the Cochran-Armitage test[17]. Body weight and proportion of PCE among the total erythrocyte population were analyzed by the MiTOX® (Mitsui Engineering & Shipbuilding Co., Ltd).

Results

In vivo micronucleus test in mouse bone marrow after gavage dose

Table 2 shows a result of the micronucleus test in mice after gavage doses of 2-isopropyl-5-methylphenol. There were no deaths at any doses of 2-isopropyl-5-methylphenol although signs of toxicity were observed at 1250 mg/kg bw. A frequency of MNPCE was not significantly increased in males and females up to the dose of 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed in any dosing groups.

In vivo micronucleus test in rat bone marrow after gavage dose

Table 3 shows a result of the micronucleus test in rats after gavage doses of *o-sec*-butylphenol. One male showed diarrhea, and two males showed ataxic gait and a decrease in locomotor activity at 600 mg/kg bw/day. Four females showed ataxic gait and three of them also showed a decrease in locomotor activity at 600 mg/kg bw/day. One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration. Body weights were not statistically changed in both sexes at any doses. A frequency of MNPCE was not changed in females at any doses. On the other hand, gavage dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control

Table 3 Results of the micronucleus test in rats after gavage dose of *o-sec*-butylphenol (CAS: 89-72-5)

Dose	Number of mice	MNPCE (%)[a]	PCE/(PCE+NCE) (%)[b]
Male			
0 mg/kg (Solvent control: corn oil	5	0.06 ± 0.08[c]	51.0 ± 5.2
150 mg/kg	5	0.10 ± 0.05	54.7 ± 3.4
300 mg/kg	5	0.14 ± 0.09	52.9 ± 5.1
600 mg/kg	5	0.20 ± 0.05*[d]	56.6 ± 1.4
20 mg/kg (Positive control: CP)	5	5.45 ± 1.25*	44.7 ± 5.1
Female			
0 mg/kg (Solvent control: corn oil)	5	0.11 ± 0.05	55.0 ± 5.7
150 mg/kg	5	0.13 ± 0.08	57.0 ± 3.5
300 mg/kg	4[e]	0.10 ± 0.07	53.8 ± 5.8
600 mg/kg	5	0.11 ± 0.04	52.7 ± 3.0
20 mg/kg (Positive control: CP)	5	3.19 ± 1.30*	25.8 ± 4.0*

PCE: Polychromatic erythrocytes, MNPCE: Micronucleated PCE, NCE: Normochromatic erythrocyte, CP: Cyclophosphamide
*: Significantly different from the solvent control ($P<0.05$)
a): Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
b): Number of polychromatic erythrocytes/ total number of erythrocytes observed.
c): Values are given as mean ± S. D.
d): The frequency of MNPCE (0.20 ± 0.05%) was within background control data from 2001 to 2007 of the laboratory (Mean ± 3SD=0.13 ± 0.24%; n=449).
e): One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration.

at 600 mg/kg bw/day in males. Proportion of PCE among the total erythrocyte populations was not changed.

Discussion

Equivocal results on *in vitro* genotoxicity of branched alkylphenols were obtained in the previous studies. Müller and Sofuni[18] indicated that some chemicals produce chromosome aberration *in vitro* but do not produce positive results in Ames assays. The clastogenic response of such chemicals is often associated with high cytotoxicity[19], high osmolality and pH extremes[20]. There are also chemicals that show positive results in the *in vitro* chromosome aberration tests but negative in the rodent micronucleus tests. The numerical proportions of positive results in the Ames assays, *in vitro* chromosome aberration assays and *in vivo* micronucleus assays were reported to be 7.7% (23/298), 28.9% (77/266) and 6.7% (19/283), respectively in pharmaceutical chemicals[18]. To ascertain if genotoxic potential of branched alkylphenols can be expressed in animals, additional *in vivo* micronucleus tests were performed on 2-isopropyl-5-methylphenol and *o-sec*-butylphenol.

After gavage doses of 2-isopropyl-5-methylphenol, a frequency of MNPCE was not significantly increased in males and females up to 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed; indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that 2-isopropyl-5-methylphenol does not induce genotoxic effects *in vivo*.

After gavage doses of *o-sec*-butylphenol, a frequency of MNPCE was not changed in females at any doses. In contrast, dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control at 600 mg/kg bw/day in males. However, the frequency of MNPCE (0.20 ± 0.05%) was within background control data from 2001 to 2007 of the laboratory (Mean ± 3SD=0.13 ± 0.24%; n=449). Therefore, the increase in MNPCE was considered to be due to low MNPCE in the control group. Proportion of PCE among the total erythrocyte populations was not changed; indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that *o-sec*-butylphenol does not induce genotoxic effects *in vivo*.

The previous assessments under the HPV programme also showed that gavage doses of 6-*tert*-butyl-m-cresole up to 125 mg/kg bw, the maximum tolerated dose, did not induce micronucleus in bone marrow cells nor suppress their proliferation in ICR mice[6], and 4-*tert*-butylphenol did not induce micronucleus in bone

Table 4 The category approach on genotoxicity of alkylphenols

Substance name	Molecular weight	Log Kow[a]	Ames	In vitro Chromosome Aberration	In vivo micronucleus
o-sec-Butylphenol	150.22	3.27	negative	positive	negative (current study)
p-sec-Butylphenol	150.22	3.08	negative	equivocal	negative (read across)
2-*tert*-Butylphenol	150.22	3.31	negative	positive	negative[b]
2, 4-di-*tert*-Butylphenol	206.32	5.19	negative	positive	negative (read across)
4-*tert*-Butylphenol	150.22	2.4-3.4	negative	positive	negative
6-*tert*-Butyl-*m*-cresole	164.24	4.11	negative	positive	negative[c]
2-Isopropyl-5-methylphenol	150.22	3.3	negative	positive	negative (current study)

[a] Data from NITE (2010)[31] and OECD (2010)[6].
[b] Data from BUA (2003)[21].
[c] Data from OECD (2010)[6].

marrow cells nor suppress their proliferation at up to the maximum tolerated dose of 50 mg/kg bw in ICR mice (not publically available). In addition, the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe: BUA) also stated that 2-*tert*-butylphenol does not induce any micronuclei in the bone marrow of mice at toxic dosages *in vivo*, while it is non-mutagenic in bacteria but is clastogenic *in vitro* in mammalian cells[21].

There are numerous reasons why activity shown *in vitro* may not be observed *in vivo*; for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination[22]. There are only a few data available on toxicokinetics for the whole body of these branched alkylphenols, but no direct information in the target cells of bone marrow. 4-*tert*-Butylphenol was rapidly excreted as glucouronide and sulfate conjugates in urine and feces in rats[23,24]. In workers handling 4-*tert*-butylphenol, most of the chemical was excreted within 24 hours, and metabolites in the urine was correlated with exposure levels of the chemical[25]. 2-Isopropyl-5-methylphenol is readily absorbed from the intestine and excreted rapidly as glucouronide and sulfate conjugates in humans, dogs, rabbits and rats[26-28]. After a single dose of 2-isopropyl-5-methylphenol, peak plasma concentrations were reached after 2 hours and eliminations half-life was 10.2 hours in humans. Sulphate and glucronide conjugates of 2-isopropyl-5-methylphenol, but not free 2-isopropyl-5-methylphenol, were corrected in urine.

The physicochemical properties and chemical structure can be used to make some predictions regarding the ADME of substances. A range of Log Kow of these category chemicals is 2.4-5.19 (Table 4), which suggests that the substances could readily absorbed and distributed in physiological fluids[29]. The alkylphenols are expected to have slightly higher acid dissociation constants (pKa) than phenol (pKa 10.0 at 25℃); therefore, will not be ionized significantly at physiological pH's[30]. Alkylphenols which contains phenol moieties are likely to undergo Phase II conjugation and systemic exposure to unchanged substance may be limited[29]. Based on available data, the rapid conjugation and excretion of these chemicals may explain why genotoxicity was not observed *in vivo* although *in vitro* clastogenicity was increased with S9 mix. However, there is a possibility that active metabolites did not reach the target cells of born marrow at high concentration and could react to chromosomes in hepatic cells. An *in vivo* genotoxic assay for hepatic cells may be useful for further evaluation.

In the present paper, we showed that 2-isopropyl-5-methylphenol and *o-sec*-butylphenol were not clastogenic *in vivo* under the test conditions, and existing data also showed that 6-*tert*-butyl-*m*-cresole, 4-*tert*-butylphenol and 2-*tert*-butylphenol were not clastogenic *in vivo*[6,21]. Based on the weight of evidence, it can be concluded that these branched alkylphenols are not genotoxic *in vivo* (Table 4). The use of the category approach is useful to identify common or trend properties of members of the category and to use measured data to similar untested chemicals without further testing to fill data gap. In conclusion, further *in vivo* micronucleus assays on *p-sec*-butylphenol and 2,4-di-*tert*-butylphenol may not be required by using the category approach, but further supporting information such as physicochemical profiles and (Q)

SAR predictions may be necessary to strengthen the rationale for the category approach.

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Original Article

Repeated dose and reproductive/developmental toxicity of perfluorooctadecanoic acid in rats

Mutsuko Hirata-Koizumi[1], Sakiko Fujii[2], Masatoshi Furukawa[2], 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*

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ABSTRACT — Male and female rats were given perfluorooctadecanoic acid (PFOdA) by gavage at 40, 200 or 1,000 mg/kg/day, and each female was mated with a male in the same dose group after 14-day administration. Males were dosed for 42 days and females were dosed throughout the gestation period until day 5 of lactation. One female given 1,000 mg/kg/day was euthanized on day 18 of gestation due to a moribund condition; however, no other treatment-related clinical signs of toxicity were observed. Body weights fell at 1,000 mg/kg/day from day 28 through the administration period in males and throughout gestation and lactation in females. Red blood cell count, hemoglobin level and hematocrit were decreased at 200 and 1,000 mg/kg/day in males and activated partial thromboplastin time was prolonged at 1,000 mg/kg/day in females. Histopathological examination revealed hepatic changes, such as centrilobular hypertrophy and necrosis, in males given 200 and 1,000 mg/kg/day and in females given 1,000 mg/kg/day. Pancreatic zymogen granule was decreased in both sexes at 1,000 mg/kg/day. As for reproductive and developmental toxicity, there were decreases in the number of corpora lutea, implantation, total number of pups born and the number of live pups on postnatal days 0 and 4 at 1,000 mg/kg/day. At this dose, birth weights of pups were decreased and postnatal body weight gain was inhibited. Based on these findings, the NOAEL of PFOdA was considered to be 40 mg/kg/day for repeated dose toxicity and 200 mg/kg/day for reproductive/developmental toxicity.

Key words: Perfluorooctadecanoic acid, Repeated dose toxicity, Reproductive and developmental toxicity, Screening test, Rat

INTRODUCTION

Perfluoroalkyl acids (PFAAs) consist of a carbon chain surrounded by fluorine atoms and a charged functional moiety (primarily carboxylate, sulfonate or phosphonate) at the end of the carbon chain. Since PFAAs have extremely low surface tension and show a unique hydrophobic and oleophobic nature (Lau *et al.*, 2007), they have been widely used as a surface protectant for paper and packaging products, carpets, leather products and textiles (Hekster *et al.*, 2003; Schultz *et al.*, 2003). They have been also used in industrial surfactants, additives and coatings and in firefighting foam. PFAAs are stable in air at high temperatures, nonflammable, not readily degraded by strong acids, alkalis or oxidizing agents and not subject to photolysis (Lau *et al.*, 2007). Such stability of PFAAs must be favorable for their use, but as they are also considered to be stable in the environment (non-biodegradable and persistent) (OECD, 2002, 2007; Schultz *et al.*, 2003), there is growing concern regarding the impact on the environment and on human health via the environment.

The most well-known PFAAs are the 8-carbon chemicals: perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), which are most effective surfactants among PFAAs (Lau *et al.*, 2007). Although the global production volume of PFOS was as high as 3,500 metric tons in 2000 (Lau *et al.*, 2007), the major manufacturer of PFOS, 3M, expressed its intention to discontinue the production of PFOS and related chem-

Correspondence: Mutsuko Hirata-Koizumi (E-mail: mkoizumi@nihs.go.jp)

icals in 2000 and completed its phase-out in 2002 (3M year not specified; US EPA, 2000). PFOS and related chemicals were listed as a persistent organic pollutant under the Stockholm Convention in 2009 (Stockholm Convention, 2010), and are now regulated in the United States, European Union, Canada and Japan (Canada, 2010; US EPA, 2008b; EU, 2006; MHLW, ME and NITE year not specified). The phase-out of PFOS and related chemicals increased the production volume of PFOA to 1,200 metric tons per year by 2004 (Lau *et al.*, 2007); however, in 2006, the United States Environmental Protection Agency and the eight major producers of fluoropolymer/telomer launched the PFOA Stewardship Program, in which manufacturers gave their pledge to reduce global facility emissions and product content of PFOA and related chemicals by 95% by 2010, and to work toward eliminating emissions and product content by 2015 (US EPA, 2008a). Although PFOS and PFOA have been reported to be detected in the environmental media, wildlife and human tissues in many different geographic locations throughout the world (Lau *et al.*, 2007), these industrial and regulatory activities must reduce their concentrations in the future. Of current concern is the anticipated growth of the production of other PFAAs as alternative products and the subsequent increase in their environmental concentrations.

Many toxicological researches on PFOS or PFOA have been carried out, and their hepatotoxicity, including carcinogenic effects, and reproductive/developmental toxicity have been shown in laboratory animals (ATSDR, 2009; US EPA, 2005; Lau *et al.*, 2007; OECD, 2002). Data on the toxic properties of other PFAAs are limited, but they have been studied in recent years (Chengelis *et al.*, 2009; Das *et al.*, 2008; Fang *et al.*, 2008; Lieder *et al.*, 2009a, 2009b; Mertens *et al.*, 2010; Shi *et al.*, 2007; Stump *et al.*, 2008; van Otterdijk 2007a, 2007b; Zhang *et al.*, 2008). Available data indicate that PFAAs with a longer carbon chain are eliminated more slowly from the body (Chang *et al.*, 2008; Ohmori *et al.*, 2003), and their toxic potency increases by lengthening the carbon chain (Kudo *et al.*, 2000, 2006; Permadi *et al.*, 1992). Since the bioaccumulation potential of PFAAs also increases depending on their carbon number (Martin *et al.*, 2003), long-chain PFAAs may cause serious environmental and/or human health concerns in the future. As no data are available for the toxicity of PFAAs with a carbon chain length 13 and above, toxicological researches are needed urgently. In order to obtain initial risk information on the toxicity of such long-chain PFAAs, the Ministry of Health, Labour and Welfare, Japan, conducted repeated dose and reproductive/developmental toxicity screening tests for several long-chain perflurocarboxylic acids (carbon chain length C12 to C18) under the Japanese existing chemical safety programme in 2009-2011. The present article reports the result of the study of perfluorooctadecanoic acid [PFOdA (C18); CAS No. 16517-11-6] concluded recently.

MATERIALS AND METHODS

This study was conducted in accordance with OECD guideline 422 "Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test" (OECD, 1996) at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). All procedures involving the use and care of animals complied with the principles for Good Laboratory Practice (ME, METI and MHLW, 2008) and applicable animal welfare regulations ["Act on Welfare and Management of Animals" (Japanese Animal Welfare Law, 2005) and "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" (ME 2006)].

Animals and housing conditions

Crl:CD(SD) rats (8 weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were acclimated to the laboratory for 13 days, and then rats found to be in good health were divided into each dose group by stratified random sampling based on body weight. For female animals, vaginal smears were prepared daily to examine the estrous cycle for 9 days before assignment to each group, but no abnormalities were found in any animal.

Throughout the study, animals were 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). The animals were housed individually, except during acclimation, mating and nursing periods, in metal bracket-type cages with wire-mesh floors. From day 17 of gestation to day 4 after delivery, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), and had free access to tap water (Sapporo, Japan).

Chemicals and dosing

PFOdA was purchased from Exfluor Research Corporation (Round Rock, TX, USA). The PFOdA (Lot No. 3654) used in this study was 98.9% pure, and was kept at room temperature in a dark place. The test article was suspended in a 0.5% aqueous solution of carboxymethylcel-

lulose sodium (CMC-Na; Maruishi Pharmaceutical Co., Ltd., Osaka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. The daily application volume (10 ml/kg body weight) was calculated according to the latest body weight.

Before the start of the study, the stability of PFOdA in a 0.5% CMC-Na aqueous solution at concentrations of 1 and 100 mg/ml was confirmed after 8-hr storage at room temperature following 15-day refrigerated storage; therefore, dosing solutions were prepared at least once per 15 days and kept in a cool (2.0-6.7°C) and dark place under airtight conditions until dosing. The concentrations of PFOdA in the formulations were analyzed at the first and last preparation using high-performance liquid chromatography-tandem mass spectrometry, and were confirmed to be 85.0 to 115% of the target.

Experimental design

Twelve male rats and twelve female rats were daily administered PFOdA at 0 (vehicle control), 40, 200 or 1,000 mg per kilogram of body weight per day (mg/kg/day). The dose levels were determined based on the previous dose-finding study in which male and female rats were given PFOdA by gavage at 0, 30, 100, 300 or 1,000 mg/kg/day for 14 days. In that study, inhibition of body weight gain, grossly enlarged liver with an increase in the organ weight and various changes in blood biochemical parameters were observed in the 1,000 mg/kg/day group. Increased liver weight was also found in males given 300 mg/kg/day, but there were no treatment-related changes in any examined parameter at 30 and 100 mg/kg/day.

After 14-day administration, each female was mated with a male rat in the same dose group, and pregnant females were allowed to deliver spontaneously and nurse their pups. Males were dosed for 42 days, including the mating period, and seven males in the control and 1,000 mg/kg/day groups and all males in the 40 and 200 mg/kg/day groups were necropsied on the day after the last treatment (main group). The remaining 5 males in the control and 1,000 mg/kg/day groups were further maintained for 2 weeks without PFOdA treatment and then necropsied (recovery group). For females, administration of PFOdA was continued throughout the mating and gestation periods until day 5 of lactation (total administration period: 42-56 days), and all twelve females were necropsied on the day after the last treatment (main group). Aside from these animals, 5 females were given 0 or 1,000 mg/kg/day PFOdA for 42 days without mating and were necropsied after the 14-day recovery period (recovery group). The first day of dosing was designated as day 1 of administration and the day after the final dose as day 1 of the recovery period.

Observation and examinations

Repeated dose toxicity data

Throughout the study, all animals were observed twice daily for general appearance and behavior during the administration period (before and after the administration) and during the recovery period (morning and afternoon). In addition, detailed clinical observations were conducted using a standardized scoring system for all of the animals once a week throughout the administration and recovery periods. Evaluations included observations in the home cage, during handling and outside the home cage in an open field.

Body weight was measured on days 1, 3, 5, 7, 10, 14, 21, 28, 35 and 42 of the dosing period and on days 7 and 14 of the recovery period. For females exhibiting evidence of successful mating (main group), body weight was recorded on days 0, 1, 3, 5, 7, 10, 14, 17 and 20 of gestation and on days 0, 1 and 4 of lactation. Food consumption was measured at a similar interval throughout the study, except during cohabitation.

At the end of the administration and recovery period, five males and five females per dose were subjected to the following functional observations: (i) sensory reactivity to visual, tactile, auditory, pain and proprioceptive stimuli was assessed on an examination table, (ii) forelimb and hindlimb grip strength was measured three times with a CPU gauge (Aikoh Engineering Co., Ltd., Osaka, Japan), and (iii) spontaneous motor activity was recorded for 1 hr at intervals of 10 min using an automated activity monitor system [SUPERMEX and CompAct AMS (Muromachi Kikai Co., Ltd., Tokyo, Japan)].

Urinalysis was performed at the end of the administration and recovery periods for five males per dose selected for functional observations and for five females per dose in the recovery group. Urine was collected for 3 hr in the metabolism cage, and examined for color and dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin and urobilinogen. A 21-hr urine sample was also collected to measure the volume of the urine and the specific gravity.

For hematology and blood biochemistry, blood samples were collected from the abdominal aorta of five males and females per dose each in the main and recovery groups at necropsy under ether anesthesia after overnight starvation. One portion of the blood was treated with ethylenediaminetetraacetic acid dipotassium (EDTA-2K) and examined for the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin

concentration (MCHC), white blood cell count, platelet count, reticulocyte count and differential count of white blood cells. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using plasma separated from another blood sample treated with 3.8% sodium citrate. Further, serum or plasma prepared from the remaining portions of blood was analyzed for total protein, albumin, protein fraction ratio, glucose, total cholesterol, triglyceride, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) γ-glutamyltranspeptidase (γ-GTP), calcium, inorganic phosphorus, sodium, potassium and chloride.

All surviving animals were euthanized by exsanguination under deep ether anesthesia on the day after the final administration (main group) or after completion of the recovery period (recovery group). The external surfaces and cavity of the body and the organs and tissues of the entire body were examined macroscopically. The brain, thymus, heart, liver, spleen, kidney, adrenal gland, testis, epididymis and ovary were isolated and weighed. Further, the spinal cord, pituitary gland, thyroid, esophagus, stomach, pancreas, duodenum, jejunum, ileum, cecum, colon, rectum, trachea, lung (after tracheal infusion fixation), urinary bladder, prostate, seminal vesicle, uterus, eyeball, Harderian gland, mammary gland, femur (including bone marrow), lymph nodes (mesenteric and mandibular), sciatic nerve as well as grossly abnormal tissues were removed. The eyeball and Harderian gland were fixed and preserved with Davidson' 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. Histopathological evaluations were performed for five males and females in the control and 1,000 mg/kg/day group. All preserved organs were sectioned, stained with hematoxylin–eosin, and examined histopathologically under a light microscope. Since histopathological changes suggestive of fatty changes of the liver and hemosiderin deposit in the liver and spleen were observed, additional specimens of the liver stained with Berlin blue or with oil red O and specimens of the spleen stained with Berlin blue were prepared and examined microscopically to confirm the observed findings. If treatment-related histopathological changes were found, the same tissues from the remaining animals were then examined.

Reproductive/developmental toxicity data

For each female, daily vaginal lavage samples were evaluated for estrous cyclicity from the first day of administration 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.

In the main group, each female was transferred to the home cage of a randomly chosen male from the same exposure group on day 14 of administration, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating, and the day of successful mating was designated as day 0 of gestation. Females that did not mate successfully during the 2-week mating period were cohabited with another male from the same group who had been proven to copulate. Following confirmation of mating, females were returned to their home cages and allowed to deliver spontaneously and nurse their pups. 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. Precoital interval and gestational length were recorded, and the copulation index, fertility index and gestation index were computed for each dose group.

On PND 0, all live and dead pups born were counted, and live pups were sexed and examined grossly. 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 by inhalation of carbon dioxide and subjected to a gross external and internal observation. For the pups with gross abnormalities, the whole body was stored in 10% neutral-buffered formalin. At necropsy of maternal animals, the numbers of corpora lutea and implantation in the uterus were recorded, and the implantation index, delivery index, live birth index and viability index were calculated for each group.

Statistical analysis

Body weight, food consumption, grip strength, spontaneous motor activity, urine volume, hematological and blood biochemical findings, organ weights, length of estrous cycle, precoital interval, the number of corpora lutea and implantation, implantation index, gestational length, the number of pups born and live pups on PNDs 0 and 4 and delivery index were evaluated by Bartlett's test for homogeneity of variances ($p \leq 0.05$). The live birth index, neonatal sex ratio, viability index and body weight of male and female pups were similarly analyzed using the litter as the experimental unit. When homogeneity was recognized, one-way analysis of variance was applied ($p \leq 0.10$). If a significant difference was found, Dunnett's

test was used for pairwise comparisons between control and individual treatment groups ($p \leq 0.01$ or 0.05). Data without homogeneity were subjected to the Kruskal-Wallis test ($p \leq 0.10$), and if significant differences were detected, the Mann-Whitney U test was used to compare PFOdA-treated groups with the control group ($p \leq 0.01$ or 0.05).

For detailed clinical and functional observations, qualitative parameters of urinalysis, specific gravity of urine and histopathological findings with multiple grades, the trend for each group was evaluated by the Kruskal-Wallis test ($p \leq 0.10$). If significant differences were found, the data were compared between the control and each dosage group using Mann-Whitney U test ($p \leq 0.01$ or 0.05). The incidence of females with normal estrous cycles, copulation, fertility and gestation index and histopathological findings with single grade were analyzed using chi-square test or Fisher's exact test ($p \leq 0.01$ or 0.05).

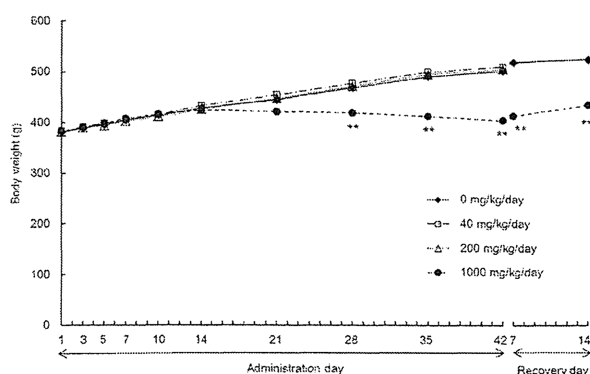


Fig. 1. Body weight changes of male rats dosed orally with PFOdA. **: Significantly different from the control, $p \leq 0.01$.

RESULTS

Clinical and functional observations

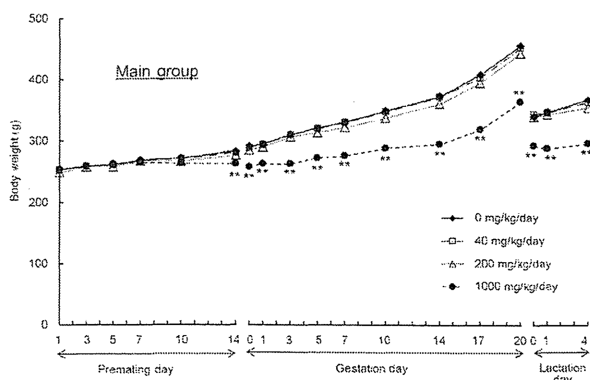
One female given 1,000 mg/kg/day in the main group was euthanized on day 18 of gestation due to a moribund condition with bleeding from the vagina, hypothermia, decreased locomotor activity and pale skin. Histopathological examination did not identify the cause of the moribund condition. No treatment-related clinical signs of toxicity were observed in other animals. Detailed clinical observation also revealed no abnormalities during the administration and recovery periods.

In the functional observation, a significant decrease in forelimb grip strength was found at 1,000 mg/kg/day at the end of the administration period (day 4 of lactation) in the female main group (860.4 ± 74.8 g, compared with 1065.2 ± 104.7 g in control, $p \leq 0.05$). Such effects were not observed in males and in the female recovery group at the end of either the administration or recovery period (data not shown). No other treatment-related changes were found in functional observations at the end of the administration and recovery period.

Body weight and food consumption

In males, body weight was significantly lower at 1,000 mg/kg/day than in the control from day 28 through the administration and recovery periods (Fig. 1). Body weight of females showed a significantly lower value at 1,000 mg/kg/day from day 14 of administration through the gestation and lactation periods in the main group and from day 14 through the administration and recovery periods in the recovery group (Fig. 2).

Food consumption was significantly decreased on days 35 and 42 of administration in males given 1,000



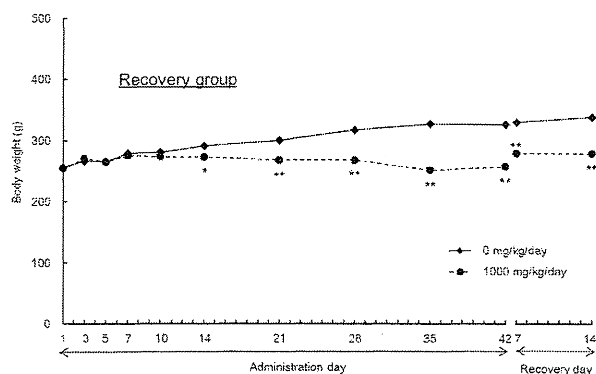


Fig. 2. Body weight changes of female rats dosed orally with PFOdA. *: Significantly different from the control, $p \leq 0.05$, **: Significantly different from the control, $p \leq 0.01$.

mg/kg/day (Fig. 3). In females, a significant decrease in food consumption was found on day 5 of gestation and on day 4 of lactation at 200 mg/kg/day and from day 7 through the administration period, except for days 1 and 20 of gestation at 1,000 mg/kg/day in the main group, and from days 14 to 35 of administration at 1,000 mg/kg/day in the recovery group (Fig. 4). There were no significant changes in food consumption during the recovery period in either sex.

Urinalysis

No significant difference was seen in any urinalysis parameters between the control and PFOdA-treated groups either at the end of the administration period or at the end of the recovery period (data not shown).

Hematology

Red blood cell count, hemoglobin level and hematocrit were significantly decreased in males given 200 mg/kg/day and above at the end of the administration period (Table 1). These changes remained significant at the end of the recovery group. In males given 1,000 mg/kg/day, the reticulocyte ratio was significantly lower at the end of the administration period, but it was significantly higher than that of the control after the recovery period. At the end of the recovery period, APTT was significantly prolonged at 1,000 mg/kg/day in males.

In the female main group, the differential leukocyte count revealed a significant increase in basophils in the 1,000 mg/kg/day group (Table 1). There was also a significant reduction of PT at 200 and 1,000 mg/kg/day and a significant prolongation of APTT at 1,000 mg/kg/day in females in the main group. In the female recovery group, a significant reduction of PT, a significant prolongation of APTT, significant increases in MCHC, total white blood cell count and differential lymphocyte count, and a significant decrease in MCV were found at 1,000 mg/kg/day.

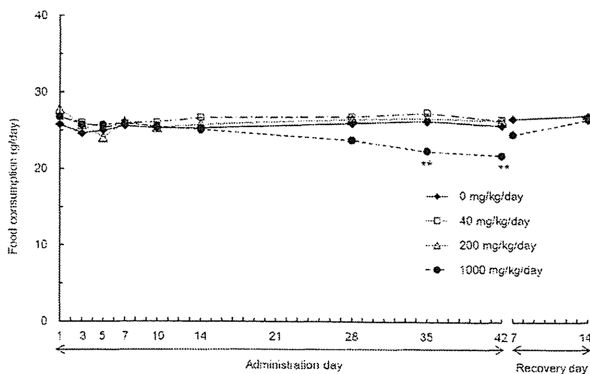
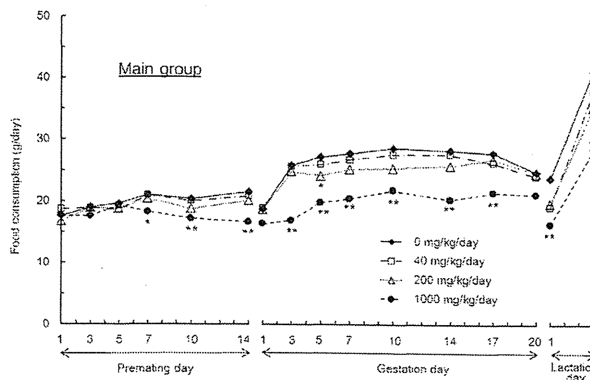


Fig. 3. Food consumption of male rats dosed orally with PFOdA. *: Significantly different from the control, $p \leq 0.05$, **: Significantly different from the control, $p \leq 0.01$.

Blood biochemistry

At the completion of the administration period, total protein was significantly lower at 1,000 mg/kg/day than in the control in males (Table 2). In the protein fraction, the albumin ratio was significantly increased at 200 mg/kg/day and above, and there was also a significant decrease in the α_1-globulin ratio and increase in the γ-globulin ratio at 1,000 mg/kg/day in males. Further, significantly higher values in ALT, ALP, total bilirubin and BUN were found at 1,000 mg/kg/day in the male main group. Significant increases in the albumin fraction and ALP and significant decreases in total protein and the α_1-globulin fraction remained after the recovery period in males given 1,000 mg/kg/day. There was also a significant decrease in the



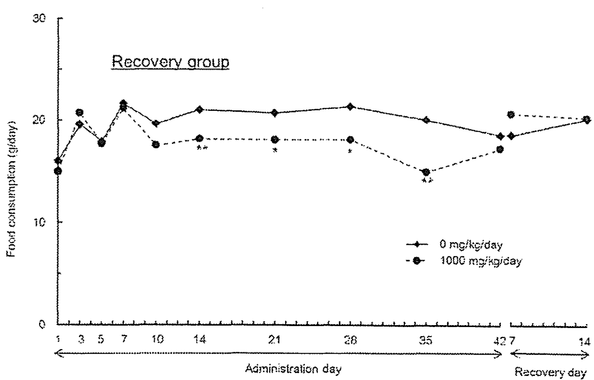


Fig. 4. Food consumption of female rats dosed orally with PFOdA. *: Significantly different from the control, $p \leq 0.05$, **: Significantly different from the control, $p \leq 0.01$.

Table 1. Hematological findings in male and female rats given PFOdA

Dose (mg/kg/day)	Main Group				Recovery Group	
	0 (control)	40	200	1000	0 (control)	1000
MALE						
Number of animals examined	5	5	5	5	5	5
Red blood cells (10^4/μl)	941 ± 26	907 ± 25	865 ± 30**	829 ± 51**	927 ± 27	804 ± 28**
Hemoglobin (g/dl)	16.3 ± 0.6	16.3 ± 0.5	15.2 ± 0.5*	14.7 ± 0.9**	16.1 ± 0.4	13.9 ± 0.7**
Hematocrit (%)	46.0 ± 1.7	46.0 ± 1.6	42.6 ± 1.0*	40.6 ± 2.0**	44.8 ± 1.3	39.4 ± 2.2**
MCV (fl)	48.9 ± 2.0	50.8 ± 2.0	49.3 ± 0.7	49.3 ± 0.7	48.4 ± 1.5	49.0 ± 1.7
MCH (pg)	17.3 ± 0.6	18.0 ± 0.7	17.6 ± 0.3	17.7 ± 0.7	17.4 ± 0.3	17.2 ± 0.4
MCHC (g/dl)	35.5 ± 0.3	35.5 ± 0.4	35.7 ± 0.4	36.1 ± 0.5	36.0 ± 0.6	35.2 ± 0.6
Reticulolocyte (%)	3.29 ± 0.39	2.71 ± 0.51	3.09 ± 0.66	2.24 ± 0.46*	3.35 ± 0.38	4.98 ± 0.80**
Platelet (10^4/μl)	117.3 ± 20.7	99.3 ± 17.3	114.1 ± 14.1	123.8 ± 8.5	120.8 ± 20.4	111.7 ± 6.1
White blood cells (10^2/μl)	94.6 ± 20.4	89.2 ± 16.0	92.8 ± 25.5	120.5 ± 33.6	118.5 ± 25.3	136.0 ± 28.9
Neutrophil (10^2/μl)	15.8 ± 5.2	18.9 ± 8.3	13.6 ± 6.6	20.2 ± 11.6	16.3 ± 4.3	13.9 ± 2.2
Lymphocyte (10^2/μl)	74.0 ± 15.5	66.1 ± 9.9	74.9 ± 18.1	95.3 ± 32.5	95.1 ± 24.3	116.6 ± 27.6
Monocyte (10^2/μl)	3.66 ± 1.84	2.84 ± 0.83	3.00 ± 1.23	3.32 ± 1.85	5.10 ± 0.99	4.22 ± 0.90
Eosinophil (10^2/μl)	1.14 ± 0.71	1.42 ± 0.74	1.22 ± 0.67	1.64 ± 1.21	1.98 ± 1.12	1.24 ± 0.31
Basophil (10^2/μl)	0.02 ± 0.04	0.02 ± 0.04	0.00 ± 0.00	0.06 ± 0.05	0.04 ± 0.05	0.02 ± 0.04
PT (sec)	20.2 ± 3.3	19.9 ± 2.2	20.5 ± 2.6	21.0 ± 2.7	17.8 ± 0.8	19.3 ± 1.8
APTT (sec)	27.7 ± 3.4	26.8 ± 2.7	26.0 ± 2.9	29.2 ± 1.7	25.6 ± 2.0	28.8 ± 1.0*
FEMALE						
Number of animals examined	5	5	5	5	5	5
Red blood cells (10^4/μl)	809 ± 18	815 ± 30	820 ± 25	814 ± 24	846 ± 20	819 ± 60
Hemoglobin (g/dl)	15.0 ± 0.3	15.7 ± 0.5*	15.5 ± 0.4	14.8 ± 0.4	15.4 ± 0.5	14.4 ± 1.0
Hematocrit (%)	42.6 ± 0.8	45.1 ± 1.4*	44.5 ± 1.5	42.0 ± 1.6	43.3 ± 1.7	40.0 ± 2.7
MCV (fl)	52.6 ± 1.7	55.5 ± 2.8	54.4 ± 1.5	51.6 ± 1.5	51.1 ± 1.4	48.9 ± 1.5*
MCH (pg)	18.5 ± 0.6	19.3 ± 0.7	18.9 ± 0.4	18.2 ± 0.4	18.1 ± 0.4	17.6 ± 0.6
MCHC (g/dl)	35.1 ± 0.4	34.9 ± 1.0	34.8 ± 0.4	35.4 ± 0.5	35.5 ± 0.2	35.9 ± 0.2*
Reticulocyte (%)	8.63 ± 1.15	9.35 ± 3.39	8.24 ± 2.08	6.23 ± 0.48	3.23 ± 0.20	3.06 ± 1.52
Platelet (10^4/μl)	141.5 ± 34.1	111.0 ± 13.9	137.6 ± 12.3	135.5 ± 12.7	119.7 ± 11.4	125.4 ± 28.2
White blood cells (10^2/μl)	102.8 ± 17.6	106.6 ± 25.1	99.6 ± 24.9	144.7 ± 53.6	68.0 ± 12.2	91.9 ± 16.1*
Neutrophil (10^2/μl)	23.2 ± 6.6	25.8 ± 12.1	14.1 ± 4.0	27.9 ± 16.3	11.9 ± 2.9	11.2 ± 7.1
Lymphocyte (10^2/μl)	72.0 ± 19.2	71.6 ± 15.2	79.4 ± 21.8	107.5 ± 37.6	52.2 ± 9.0	75.1 ± 12.3*
Monocyte (10^2/μl)	6.42 ± 1.45	7.26 ± 1.09	4.70 ± 1.56	7.10 ± 1.58	2.78 ± 1.01	4.38 ± 3.11
Eosinophil (10^2/μl)	1.22 ± 0.22	1.90 ± 0.73	1.40 ± 0.66	2.02 ± 0.88	1.10 ± 0.44	1.22 ± 0.45
Basophil (10^2/μl)	0.02 ± 0.04	0.02 ± 0.04	0.00 ± 0.00	0.14 ± 0.05*	0.00 ± 0.00	0.06 ± 0.05
PT (sec)	18.2 ± 0.3	17.4 ± 0.8	16.3 ± 0.4**	16.1 ± 0.9**	16.9 ± 0.4	15.1 ± 1.3*
APTT (sec)	19.6 ± 1.0	20.8 ± 1.5	20.1 ± 0.9	22.2 ± 2.20*	20.4 ± 0.9	27.0 ± 4.4**

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leq 0.05$
**: Significantly different from the control, $p \leq 0.01$

Table 2. Blood biochemical findings in male and female rats given PFOdA

Dose (mg/kg/day)	Main Group				Recovery Group	
	0 (control)	40	200	1000	0 (control)	1000
MALES						
Number of animals examined	5	5	5	5	5	5
Total protein (g/dl)	5.68 ± 0.22	5.34 ± 0.29	5.50 ± 0.19	5.10 ± 0.19**	5.68 ± 0.15	5.40 ± 0.21*
Albumin (g/dl)	2.79 ± 0.07	2.75 ± 0.14	2.90 ± 0.08	2.88 ± 0.07	2.81 ± 0.05	3.14 ± 0.16**
Protein fraction (%)						
Albumin	49.2 ± 0.7	51.6 ± 1.6	52.8 ± 2.2*	56.7 ± 2.3**	49.4 ± 2.2	58.1 ± 1.5**
Globulin α_1	23.1 ± 2.5	19.8 ± 2.3	19.6 ± 2.2	13.7 ± 1.3**	21.6 ± 2.7	15.7 ± 1.5**
Globulin α_2	7.24 ± 0.94	7.94 ± 0.98	7.24 ± 0.43	7.24 ± 1.01	7.14 ± 0.46	7.20 ± 0.70
Globulin β	15.8 ± 0.9	16.0 ± 1.5	15.5 ± 1.2	14.5 ± 1.1	17.0 ± 0.6	14.1 ± 0.7**
Globulin γ	4.72 ± 0.58	4.76 ± 0.56	4.84 ± 0.39	7.92 ± 1.85**	4.82 ± 0.59	4.88 ± 0.75
AST (IU/l)	67.4 ± 8.0	73.6 ± 7.3	72.8 ± 6.9	83.4 ± 16.8	67.4 ± 6.9	74.0 ± 6.5
ALT (IU/l)	30.4 ± 3.2	35.4 ± 8.4	35.0 ± 7.1	46.4 ± 9.7*	34.6 ± 3.1	43.0 ± 9.4
ALP (IU/l)	373 ± 46	387 ± 32	475 ± 74	703 ± 90**	325 ± 93	539 ± 112*
γ-GTP (IU/l)	0.70 ± 0.12	0.76 ± 0.17	0.48 ± 0.04**	0.50 ± 0.30	0.56 ± 0.21	0.50 ± 0.19
Total bilirubin (mg/dl)	0.048 ± 0.016	0.042 ± 0.004	0.030 ± 0.007	0.128 ± 0.027**	0.076 ± 0.015	0.070 ± 0.007
Glucose (mg/dl)	153 ± 16	159 ± 26	155 ± 14	139 ± 10	169 ± 11	163 ± 26
Total cholesterol (mg/dl)	48.4 ± 10.7	44.4 ± 15.4	42.2 ± 16.8	55.2 ± 12.2	57.4 ± 9.8	75.8 ± 23.8
Triglyceride (mg/dl)	33.4 ± 23.7	28.2 ± 30.3	26.4 ± 9.8	17.0 ± 5.8	49.6 ± 9.8	16.2 ± 4.3**
BUN (mg/dl)	16.0 ± 1.2	15.7 ± 0.6	15.3 ± 1.2	22.6 ± 1.9**	16.7 ± 1.8	18.7 ± 2.0
FEMALES						
Number of animals examined	5	5	5	5	5	5
Total protein (g/dl)	6.26 ± 0.21	6.02 ± 0.24	6.34 ± 0.35	6.04 ± 0.24	6.92 ± 0.44	5.98 ± 0.79*
Albumin (g/dl)	2.99 ± 0.17	2.97 ± 0.20	3.28 ± 0.29	3.12 ± 0.30	3.87 ± 0.30	3.64 ± 0.38
Protein fraction (%)						
Albumin	47.8 ± 1.6	49.3 ± 1.6	51.6 ± 2.9	51.6 ± 3.9	55.9 ± 1.5	61.1 ± 3.2*
Globulin α_1	21.2 ± 2.0	18.2 ± 2.5	17.7 ± 1.8*	16.4 ± 1.7**	17.4 ± 1.3	12.8 ± 2.5**
Globulin α_2	8.12 ± 0.96	8.32 ± 0.86	7.58 ± 0.71	8.32 ± 1.43	5.96 ± 0.30	6.68 ± 0.86
Globulin β	18.5 ± 0.7	18.9 ± 1.5	18.2 ± 2.3	16.6 ± 1.5	14.3 ± 0.6	12.9 ± 0.7**
Globulin γ	4.40 ± 1.24	5.22 ± 0.83	4.84 ± 1.00	6.98 ± 0.93**	6.42 ± 0.90	6.52 ± 1.69
AST (IU/l)	82.6 ± 23.7	74.4 ± 11.1	81.6 ± 16.3	98.8 ± 36.9	120.2 ± 97.0	124.0 ± 134.3
ALT (IU/l)	29.8 ± 5.3	29.0 ± 2.1	31.2 ± 5.8	35.8 ± 5.8	58.2 ± 52.9	72.4 ± 87.2
ALP (IU/l)	176 ± 24	228 ± 63	209 ± 38	303 ± 97*	151 ± 23	225 ± 116
γ-GTP (IU/l)	0.76 ± 0.19	0.68 ± 0.08	0.64 ± 0.15	0.98 ± 0.22	0.78 ± 0.16	3.28 ± 5.33
Total bilirubin (mg/dl)	0.068 ± 0.011	0.062 ± 0.008	0.058 ± 0.011	0.058 ± 0.008	0.104 ± 0.021	0.340 ± 0.649
Glucose (mg/dl)	158 ± 10	154 ± 19	153 ± 7	144 ± 10	155 ± 11	150 ± 22
Total cholesterol (mg/dl)	65.2 ± 6.5	58.4 ± 10.5	62.4 ± 11.5	49.2 ± 4.8*	72.4 ± 14.6	66.4 ± 14.8
Triglyceride (mg/dl)	29.2 ± 14.6	39.8 ± 17.6	45.6 ± 10.5	21.6 ± 7.4	26.0 ± 21.1	22.8 ± 15.2
BUN (mg/dl)	22.2 ± 2.5	23.2 ± 2.9	21.6 ± 2.3	31.4 ± 3.4**	15.9 ± 2.0	15.5 ± 3.3

Values are given as the mean ± S.D.

*: Significantly different from the control, $p \leq 0.05$

**: Significantly different from the control, $p \leq 0.01$

β-globulin fraction and triglyceride, and an increase in the absolute albumin level at 1,000 mg/kg/day in the male recovery group.

In the female main group, no significant change was found in total protein, but in the protein fraction, the α_1-globulin ratio was significantly lower at 200 and 1,000 mg/kg/day, and the γ-globulin ratio was significantly higher at 1,000 mg/kg/day (Table 2). There were also significant increases in ALP and BUN, and a significant decrease in total cholesterol at 1,000 mg/kg/day in the female main group. In the female recovery group, total protein and α_1- and β-globulin fractions were significantly lower and the albumin fraction was significantly higher at 1,000 mg/kg/day than in the control.

Organ weight

In males, the absolute and relative liver weight was significantly higher at 200 mg/kg/day and above at the end of the administration period (Table 3). The absolute weight of the heart, spleen and thymus was decreased significantly in males given 1,000 mg/kg/day, but the relative weights of these organs was not changed significantly. In this 1,000 mg/kg/day group, the relative weights of the brain, kidney and testis were significantly increased without significant changes in the absolute weights. At the end of the recovery period, increased liver weight remained significant at 1,000 mg/kg/day. There was also a significant decrease in the absolute heart weight and increase in the relative brain and kidney weight in males given 1,000 mg/kg/day in the recovery group.

In the female main group, there were significant increases in the absolute liver weight at 1,000 mg/kg/day and in the relative liver weight at 200 mg/kg/day and above (Table 3). In the 1,000 mg/kg/day group, the absolute heart weight was decreased and the relative brain weight was increased significantly. In the female recovery group, significant increases in the absolute and relative liver weight and in the relative kidney weight, and a decrease in the absolute heart weight were found at 1,000 mg/kg/day.

Histopathological findings

Histopathological findings of scheduled-sacrifice animals in each group are shown in Table 4. Centrilobular hypertrophy of hepatocytes was observed in males given 200 mg/kg/day and above and centrilobular degeneration/necrosis of hepatocytes in males given 1,000 mg/kg/day at the end of the administration period. For these hepatic changes, the increase in the incidence was statistically significant. In the male main group, there was also focal necrosis at 200 mg/kg/day and above and hemosiderin deposit (mainly in Kupffer cells) at 1,000 mg/kg/day in the liver, although the incidence was not significantly different from that in the control group. Further, centrilobular fatty change was observed in two males at 200 mg/kg/day, but there was no dose-dependency in the incidence. In the pancreas, the incidence of decreased zymogen granules showed an increasing tendency (not significant) in males given 1,000 mg/kg/day. In the thymus, cortex atrophy was observed in one male in the main group. Centrilobular hypertrophy of hepatocytes, focal necrosis and hemosiderin deposit in the liver and decreased zymogen granules in the pancreas remained after the recovery period in males given 1,000 mg/kg/day. The incidence of centrilobular hepatocyte hypertrophy in the liver and decreased zymogen granules in the pancreas was significantly higher than in the control. A significant increase in the incidence of microgranuloma was also found in the liver in the male recovery group at 1,000 mg/kg/day.

In the female main group, similar histopathological changes were observed in the liver, pancreas and thymus at 1,000 mg/kg/day. The incidence of centrilobular hepatocyte hypertrophy, focal necrosis and microgranuloma in the liver and decreased pancreatic zymogen granules was significantly higher than in the control. In the main group, hemosiderin deposit was observed in the spleen in all females in all dose groups, but the grade was significantly increased at 1,000 mg/kg/day. In addition, periportal fatty change was observed in the control, 40 mg/kg/day and 1,000 mg/kg/day groups, but the incidence did not increase dose-dependently. In the female recovery group, the incidence of centrilobular hypertrophy of hepatocytes and hemosiderin deposit in the liver was significantly higher in the 1,000 mg/kg bw/group.

Reproductive and developmental findings

In the main group, persistent diestrus was found in one female each in the control and 1,000 mg/kg/day group; however, there were no significant deviations in the mean estrous cycle and in the incidence of females with a normal estrous cycle between the control and PFOdA groups either in the main group or in the recovery group (data not shown).

Reproductive performance and developmental findings are summarized in Table 5. During the cohabitation period, copulation was not observed in two males given 1,000 mg/kg/day. In the 1,000 mg/kg/day group, one female with successful copulation was not impregnated, and one pregnant female did not deliver live pups (all pups stillborn). There were, however, no significant differences in the copulation, fertility or gestation index, and the precoital interval or gestation length between the control and

Table 3. Organ weights of male and female rats given PFOdA

Dose (mg/kg/day)		Main Group				Recovery Group	
		0 (control)	40	200	1000	0 (control)	1000
MALES							
Number of animals examined		5	5	5	5	5	5
Brain	(g)	2.15 ± 0.08	2.17 ± 0.09	2.18 ± 0.11	2.14 ± 0.06	2.16 ± 0.11	2.16 ± 0.10
	(%)[a]	0.470 ± 0.024	0.478 ± 0.031	0.466 ± 0.050	0.586 ± 0.038**	0.436 ± 0.030	0.528 ± 0.026**
Thymus	(mg)	297 ± 85	271 ± 84	335 ± 53	178 ± 51*	253 ± 33	274 ± 94
	(10^{-3}%)[a]	64.5 ± 15.1	59.6 ± 17.6	71.6 ± 11.8	48.4 ± 10.0	50.8 ± 6.8	67.2 ± 23.3
Heart	(g)	1.376 ± 0.084	1.398 ± 0.162	1.408 ± 0.187	1.056 ± 0.100**	1.460 ± 0.070	1.164 ± 0.139**
	(%)[a]	0.300 ± 0.019	0.306 ± 0.021	0.298 ± 0.016	0.288 ± 0.018	0.294 ± 0.017	0.284 ± 0.030
Liver	(g)	10.9 ± 1.8	11.3 ± 1.6	15.8 ± 1.8**	18.2 ± 1.2**	11.9 ± 0.3	18.3 ± 2.1**
	(%)[a]	2.36 ± 0.28	2.48 ± 0.25	3.35 ± 0.14**	5.00 ± 0.13**	2.40 ± 0.06	4.46 ± 0.42**
Spleen	(g)	0.738 ± 0.091	0.704 ± 0.059	0.656 ± 0.087	0.580 ± 0.103*	0.698 ± 0.055	0.682 ± 0.094
	(%)[a]	0.160 ± 0.020	0.156 ± 0.005	0.140 ± 0.012	0.160 ± 0.022	0.140 ± 0.010	0.168 ± 0.027
Kidney[b]	(g)	2.96 ± 0.15	2.90 ± 0.22	3.08 ± 0.28	2.94 ± 0.26	2.93 ± 0.10	2.94 ± 0.19
	(%)[a]	0.648 ± 0.047	0.636 ± 0.044	0.660 ± 0.076	0.806 ± 0.043**	0.590 ± 0.019	0.718 ± 0.030**
Testis[b, c]	(g)	3.28 ± 0.38	3.38 ± 0.470	3.320 ± 0.227	3.421 ± 0.251	3.468 ± 0.190	3.270 ± 0.362
	(%)[a]	0.697 ± 0.124	0.702 ± 0.097	0.697 ± 0.071	0.906 ± 0.066**	0.698 ± 0.041	0.802 ± 0.096
FEMALES							
Number of animals examined		5	5	5	5	5	5
Brain	(g)	2.05 ± 0.06	2.08 ± 0.11	2.09 ± 0.06	2.01 ± 0.07	2.04 ± 0.06	2.04 ± 0.11
	(%)[a]	0.652 ± 0.019	0.638 ± 0.039	0.680 ± 0.061	0.738 ± 0.066*	0.640 ± 0.034	0.788 ± 0.106
Thymus	(mg)	268 ± 29	322 ± 31*	305 ± 27	245 ± 34	274 ± 24	250 ± 86
	(10^{-3}%)[a]	85.1 ± 6.3	98.9 ± 14.4	99.3 ± 11.1	90.6 ± 17.1	86.2 ± 11.5	94.5 ± 27.4
Heart	(g)	1.076 ± 0.048	1.104 ± 0.065	1.048 ± 0.094	0.844 ± 0.056**	1.056 ± 0.054	0.890 ± 0.071**
	(%)[a]	0.342 ± 0.016	0.340 ± 0.028	0.340 ± 0.024	0.310 ± 0.016	0.330 ± 0.012	0.344 ± 0.040
Liver	(g)	10.2 ± 0.5	10.6 ± 0.8	11.7 ± 1.0	13.2 ± 1.6**	7.8 ± 0.2	10.3 ± 1.2**
	(%)[a]	3.23 ± 0.16	3.23 ± 0.11	3.80 ± 0.10**	4.82 ± 0.49**	2.4 ± 0.1	3.96 ± 0.53**
Spleen	(g)	0.710 ± 0.056	0.766 ± 0.161	0.666 ± 0.098	0.570 ± 0.045	0.556 ± 0.098	0.494 ± 0.070
	(%)[a]	0.224 ± 0.015	0.232 ± 0.046	0.218 ± 0.024	0.212 ± 0.015	0.174 ± 0.030	0.186 ± 0.018
Kidney[b]	(g)	2.05 ± 0.20	2.04 ± 0.25	2.01 ± 0.28	1.97 ± 0.10	2.00 ± 0.05	1.94 ± 0.10
	(%)[a]	0.650 ± 0.062	0.624 ± 0.061	0.648 ± 0.054	0.724 ± 0.049	0.628 ± 0.030	0.752 ± 0.120**
Ovary[b]	(mg)	113.8 ± 8.0	112.4 ± 9.4	102.0 ± 20.4	105.2 ± 7.9	106.2 ± 12.5	81.0 ± 21.7
	(10^{-3}%)[a]	35.8 ± 1.5	34.3 ± 2.2	32.8 ± 4.4	38.7 ± 4.7	33.3 ± 4.5	30.5 ± 5.0

Values are given as the mean ± S.D.

[a]: ratio of organ weight to body weight (relative organ weight). [b]: values are represented as the total weights of the organs on both sides. [c]: organ weight was measured for all animals (number of examined animals: 7 at 0 and 1,000 mg/kg/day and 12 at 40 and 200 mg/kg/day in the main group and 5 at 0 and 1000 mg/kg/day in the recovery group)

*: Significantly different from the control, $p \leq 0.05$. **: Significantly different from the control, $p \leq 0.01$

Table 4. Histopathological findings in male and female rats given PFOdA

	Grade	Main Group				Recovery Group	
		0 [a]	40 [a]	200 [a]	1000 [a]	0 [a]	1000 [a]
MALES							
Number of animals examined		7	12	12	7	5	5
Liver							
Centrilobular hypertrophy of hepatocytes	+	0	0	12]**	0]**	0	4]**
	++	0	0	0	7	0	1
Centrilobular degeneration/necrosis of hepatocytes	+	0	0	0	4]**	0	0
	++	0	0	0	2	0	0
Focal necrosis	+	0	0	1	2	0	2
Microgranuloma	+	5	9	7	6	2	2]*
	++	0	0	0	0	0	3
Hemosiderin deposit	+	0	0	0	4	0	2
Centrilobular fatty change	+	0	0	2	0	0	0
Pancreas							
Decrease in zymogen granule	+	1	0	0	4	0	4]*
	++	0	0	0	0	0	1
Spleen							
Hemosiderin deposit	+	5	8	8	2	5	5
	++	2	4	4	5	0	0
Thymus							
Atrophy of cortex	+	0	0	0	1	0	0
FEMALES							
Number of animals examined		12	12	12	9[b]	5	5
Liver							
Centrilobular hypertrophy of hepatocytes	+	0	0	0	8]**	0	4 *
	++	0	0	0	1	0	0
Focal necrosis	+	0	0	0	4*	0	0
Microgranuloma	+	4	4	5	2]**	1	3
	++	0	0	0	7	0	1
Hemosiderin deposit	+	0	0	0	1	0	5 **
Periportal fatty change	+	3	1	0	2	0	0
	++	0	0	0	0	0	1
Pancreas							
Decrease in zymogen granule	+	0	0	0	8]**	0	1
	++	0	0	0	1	0	2
Spleen							
Hemosiderin deposit	+	11	12	10	3]*	0	0
	++	1	0	2	6	5	5
Thymus							
Atrophy of cortex	+	0	0	0	1	0	1

Values are the number of animals with findings.
Grade: +: slight change, ++: moderate change.
*: Significantly different from the control at $p \leq 0.05$.
**: Significantly different from the control at $p \leq 0.01$.
[a]: Dose (mg/kg/day).
[b]: Among 12 females in the main group, three females each were euthanized on day 18 of gestation because of a moribund condition, on day 26 of gestation because of non-pregnancy and on day 1 of lactation because of abnormal delivery (stillbirth).

PFOdA-treated groups.

The number of corpora lutea and implantation sites was significantly lower at 1,000 mg/kg/day than in the control. No significant changes were found in the implantation index. The total number of pups born and the number of live pups on PNDs 0 and 4 were significantly lower at 1,000 mg/kg/day. There was no significant difference in the delivery, live birth and viability indices

Table 5. Reproductive performance and developmental findings in rats given PFOdA

Dose (mg/kg/day)	0 (control)	40	200	1000
Number of pairs	12	12	12	12
Copulation index (%) [a] Male	100	100	100	83.3
Female	100	100	100	100
Precoital interval (day)[b]	2.8 ± 0.9	2.5 ± 1.2	2.8 ± 0.9	1.9 ± 1.1
Fertility index (%) [c]	100	100	100	91.7
Gestation index (%) [d]	100	100	100	90
Gestation length (days) [b]	22.4 ± 0.5	22.2 ± 0.4	22.2 ± 0.4	22.1 ± 0.3
Number of pregnant females	12	12	12	10
Number of corpora lutea [b]	16.6 ± 1.4	17.2 ± 1.8	15.3 ± 2.0	14.0 ± 2.4**
Number of implantation [b]	16.4 ± 1.3	16.6 ± 1.7	15.0 ± 2.0	14.0 ± 2.4*
Implantation index (%) [b, e]	99.1 ± 2.2	96.8 ± 5.5	98.4 ± 2.8	100.0 ± 0.0
Number of litters	12	12	12	9
Total number of pups born [b]	15.5 ± 1.2	15.3 ± 1.6	14.3 ± 2.1	11.2 ± 3.6**
Delivery index (%) [b, f]	94.6 ± 5.8	92.7 ± 8.2	95.4 ± 4.5	80.4 ± 22.0
Sex ratio of pups born [b, g]	0.524 ± 0.135	0.450 ± 0.092	0.438 ± 0.136	0.410 ± 0.105
Number of live pups on PND 0 [b]	15.5 ± 1.2	15.3 ± 1.6	14.3 ± 2.1	10.0 ± 5.0**
Live birth index (%) [b, h]	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	84.2 ± 32.6
Number of live pups on PND 4 [b]	15.4 ± 1.2	14.8 ± 1.5	13.8 ± 2.3	10.6 ± 3.5**
Viability index (%) [b, i]	99.4 ± 1.9	96.9 ± 5.0	96.1 ± 9.2	94.8 ± 8.7
Male pups				
Body weight (g) on PND 0 [b]	7.03 ± 0.70	6.63 ± 0.57	6.66 ± 0.51	5.39 ± 0.87**
on PND 1 [b]	7.72 ± 0.80	7.22 ± 0.65	7.32 ± 0.61	5.60 ± 0.86**
on PND 4 [b]	11.08 ± 1.24	10.53 ± 0.99	10.73 ± 1.02	7.96 ± 1.19**
Body weight gain (g) on PNDs 0-4 [b]	4.05 ± 0.62	3.90 ± 0.58	4.08 ± 0.88	2.57 ± 0.68**
Female pups				
Body weight (g) on PND 0 [b]	6.63 ± 0.76	6.18 ± 0.48	6.38 ± 0.51	5.11 ± 0.86**
on PND 1 [b]	7.26 ± 0.92	6.78 ± 0.62	6.94 ± 0.58	5.34 ± 0.84**
on PND 4 [b]	10.50 ± 1.31	9.88 ± 1.06	10.12 ± 0.86	7.76 ± 1.17**
Body weight gain (g) on PNDs 0-4 [b]	3.88 ± 0.67	3.70 ± 0.71	3.74 ± 0.63	2.64 ± 0.75**

[a] Copulation index (%) = (no. of animals with successful copulation/no. of animals mated) × 100
[b] Values are given as the mean ± S.D.
[c] Fertility index (%) = (no. of pregnant females/no. of females with successful copulation) × 100
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100
[e] Implantation index = (no. of implantation sites/no. of corpora lutea) × 100
[f] Delivery index = (no. of pups born/no. of implantation sites) × 100
[g] Sex ratio of pups born = (no. of male pups born/no. of female pups born)
[h] Live birth index = (no. of live pups on PND 0/no. of pups born) × 100
[i] Viability index = (no. of live pups on PND 4/no. of live pups on PND 0) × 100
*: Significantly different from the control at $p \leq 0.05$
**: Significantly different from the control at $p \leq 0.01$

between the control and PFOdA-treated groups, but these indices indicated a decreasing tendency at 1,000 mg/kg/day. In the 1,000 mg/kg/day group, the body weight of male and female pups was significantly lower than in the control on PNDs 0, 1 and 4. Body weight gain for the 4 days after birth was significantly decreased in males and females given 1,000 mg/kg/day. Gross external and internal observation did not reveal any treatment-related alterations either in pups that survived until PND 4 or pups found dead during the postnatal period (data not shown).

DISCUSSION

PFAAs are known to affect the liver, causing hypertrophy and in some cases necrosis, in rodents (Chengelis *et al.*, 2009; Goldenthal, 1978; Griffith and Long, 1980; Kawashima *et al.*, 1995; Kennedy, 1987; Lieder *et al.*, 2009b; Mertens *et al.*, 2010; van Otterdijk, 2007a, 2007b; Zhang *et al.*, 2008). Hepatocyte hypertrophy was observed at as low as 0.064~0.23 mg/kg/day in a 2-year dietary study of PFOS (Thomford, 2002) and 0.64 mg/kg/day in a 13-week dietary study of PFOA (Perkins *et al.*, 2004) in rats. Such hepatic effects of PFAAs are considered to be exerted via the peroxisome proliferator-activated receptor alpha (PPARα), but other mechanisms have also been suggested (Lau *et al.*, 2007). Kudo *et al.* (2006) reported that intraperitoneal administration of PFAAs with six- to nine-carbon length increased the liver weight and hepatic peroxisomal β-oxidation activity in mice, and the potency was in the order of perfluorononanonic acid (PFNA; C9) $\approx$ PFOA (C8) > perfluoroheptanoic acid (PFHpA; C7) > perfluorohexanoic acid (PFHxA; C6). In the other study, PFOA (C8) and perfluorodecanoic acid (PFDeA; C10) considerably increased the liver weight, and hepatic peroxisomal palmitoyl- and lauroyl-CoA oxidase activities, but the responses to perfluorobutanoic acid (PFBA; C4) were lower and perfluoroacetic acid (C2) was inactive in male mice fed a diet containing PFAAs (Permadi *et al.*, 1993). These data suggest that the toxic potency of PFAAs increased with lengthening of the carbon chain, at least up to C9. Such carbon chain length dependency is considered to be associated with the difference in the elimination rate of PFAAs: the elimination half-life was reported to be 6.38 hr for PFBA (C4), 2.4 hr for PFHpA (C7), 135-185 hr for PFOA (C8), 710 hr for PFNA (C9) and 958 hr for PFDeA (C10) in male rats administered intravenously with PFAAs (Chang *et al.*, 2008; Kemper 2003; Kudo *et al.*, 2002; Ohmori *et al.*, 2003).

The present study demonstrated that the major target of the toxicity of PFOdA (C18) was the liver as with the other PFAAs. Centrilobular hepatocyte hypertrophy and necrosis were observed in males given 200 and 1,000 mg/kg/day and in females given 1,000 mg/kg/day. Increased incidence of microgranuloma was considered to be a reparative response to the hepatic injury. These histopathological changes were accompanied with increased liver weight and leaking out of hepatic enzymes. Such hepatic changes were not observed in the 40 mg/kg/day group, indicating that the toxic potency of PFOdA was unexpectedly low. Differences in the toxic potency of PFOdA and the other PFAAs with shorter carbon chain might come from differences in the elimination rate from the body, as suggested for the other PFAAs. However, since hepatic effects of PFOdA as well as other toxic effects observed at the end of the administration period were detected even after the 14-day recovery period in the present study, the elimination rate of PFOdA was considered to be slow as with PFOA, PFNA and PFDeA (Kemper, 2003; Kudo *et al.*, 2002; Ohmori *et al.*, 2003). Intestinal absorption or distribution in the body might differ between PFOdA and other PFAAs. Other possible factors included toxicodynamic determinants, such as PPARα agonistic activity. In the present study, hepatocyte hypertrophy was not accompanied with eosinophilic granular cytoplasm, which is a characteristic change observed in rodents administered with peroxisome proliferators. In addition, no clear effects on lipid metabolism were found in blood biochemical examination, except for decreased serum cholesterol observed in females given 1,000 mg/kg/day. Therefore, the peroxisome proliferative activity of PFOdA was considered weak, if any, and there is the possibility that a different mechanism from peroxisome proliferation is involved in the hepatotoxicity of PFOdA. Further study is needed to clarify the cause of the difference in the toxic potency of PFOdA and the other PFAAs.

In the present study, one female given 1,000 mg/kg/day was found moribund on day 18 of gestation. General appearance and behavior suggest that the moribund state might be associated with anemia caused by bleeding from vagina. Although hematological examination was not performed for this animal, the effect of PFOdA on blood coagulation (discussed below) might be involved in the vaginal bleeding during late pregnancy. In other animals, no clinical signs of toxicity were found. In the female main group, forelimb grip strength was reduced on day 4 of lactation at 1,000 mg/kg/day. No such effect was detected at the end of the 42-day administration in males and in the female recovery group. This result might be due to the higher maternal body burden of PFOdA during pregnancy in comparison to males and non-pregnant females in the same dose group, because the dose level for pregnant females was determined based on the weight

of the pregnant body containing fetuses. Further, altered physical condition during the gestation and lactation periods could make their toxic susceptibility higher. However, considering that no other dose-related changes were found in detailed clinical and functional observations, the toxicological significance of this forelimb grip weakness is unclear. Effects on grip strength were examined in 90-day repeated oral dose toxicity studies of PFBA, perfluorohexanoic acid (PFHxA) and perfluorobutane sulfonic acid (PFBS) in rats, but no dose-related changes were reported (Chengelis *et al.*, 2009; Lieder *et al.*, 2009a; van Otterdijk, 2007b).

Body weight gain of male and female rats was markedly inhibited at 1,000 mg/kg/day in the present study. Although decreases in body weight coincided with the reduction of food consumption during PFOdA administration, they were hardly recovered during the 14-day recovery period when food consumption was not different between the control and 1,000 mg/kg/day group. The effects on body weight are typically observed in rodents given PFAAs at relatively high doses (ATSDR, 2009). Interestingly, these effects are not associated with reduced food intake in many cases. For example, 7-day or 10-day dietary administration of PFOA at 0.02% caused more than 10% reduction of body weight, compared with the control (Xie *et al.*, 2003; Yang *et al.*, 2000). In these studies of PFOA, food intake was not reduced significantly. Yang *et al.* (2002) reported that body weight gain was not inhibited in PPARα-null mice given PFOA with the same dose regimen, suggesting that PPARα activation is involved in the body weight effects of PFAAs. In the present study, however, no clear effects indicating the alteration of lipid metabolism were found as mentioned above. The other mechanisms may contribute to the effects of PFOdA on body weights.

PFOdA affected various hematological parameters at 200 mg/kg/day and above. Anemic effects observed in males (i.e. reduced red blood cell count, hemoglobin level and hematocrit) were previously reported in repeated oral dose studies of PFBA, PFHxA, PFOA, PFBS and PFOS in rats (Chengelis *et al.*, 2009; Goldenthal *et al.*, 1978; Lieder *et al.*, 2009a; Sibinski, 1987 ; van Otterdijk, 2007b); however, the mechanism has yet to be revealed. In the present study, increased serum total bilirubin and hepatic hemosiderin deposition indicated a hemolytic effect of PFOdA. A decrease in reticulocyte ratio also suggests a decline in hematopoietic function, but histopathological examination of bone marrow did not reveal any abnormalities. At the end of the recovery period, the reticulocyte ratio was considerably increased, but reduced red blood cells, hemoglobin and hematocrit did not indicate recovery. In females, these anemic changes were not found, but the number of basophils was increased at 1,000 mg/kg/day. Its etiology and toxicological significance is unknown. Blood clotting parameters were also affected in females. Prolongation of APTT suggests the inhibition of an intrinsic and/or common pathway in blood coagulation. Shortening of PT was considered to be toxicologically insignificant, but showed that PFOdA does not inhibit the extrinsic pathway of coagulation. At the end of the recovery period, this effect on APTT was also observed in males. Elevation of basophils and prolongation of APTT were not reported in previous studies of other PFAAs (ATSDR, 2009). In order to gain a better understanding of the etiology of the hematological effects occurring in male and female rats given PFOdA, additional investigations are needed.

In the 1,000 mg/kg/day group, other dose-related changes were found in blood biochemical variables, organ weights and histopathological findings. In males, serum total protein was reduced, and this change seemed to result primarily from a decrease in the globulin α_1 fraction, which was also found in females given 200 and 1,000 mg/kg/day. Since most serum proteins, with the exception of γ-globulin, are synthesized, and also degraded in the liver (Ove *et al.*, 1972), observed effects on serum protein may be attributable to hepatic effects of PFOdA, although other etiologies could be responsible. An elevated serum level of BUN, observed in both sexes, increases the likelihood that the hepatic protein catabolism was increased because urinalysis parameters and the gross and microscopic appearance of the kidneys were not affected by PFOdA treatment. In the pancreas, zymogen granules were decreased in both sexes. This change might result from increased pancreatic secretion of digestive juice. Lipids are known to stimulate pancreatic secretion, and long chain fatty acids stimulate this more intensively than medium chain fatty acids (Ioannidis *et al.*, 2008), which might explain why such effects on the pancreas were not reported for the other PFAAs (ATSDR, 2009).

As for the reproductive/developmental effects, all pups were stillborn in one pregnant female given 1,000 mg/kg/day. Although the incidence was low, the possibility that this change was treatment-related could not be ruled out because of the very low incidence of such effect (all pups stillborn) in the laboratory's historical control data (approximately 0.5~1.0%). Another pregnant female given 1,000 mg/kg/day was found moribund during late pregnancy as described above, suggesting that PFOdA might have a certain effect on the maintenance of pregnancy. In the 1,000 mg/kg/day group, the number of corpora lutea was slightly decreased. Such effects on the