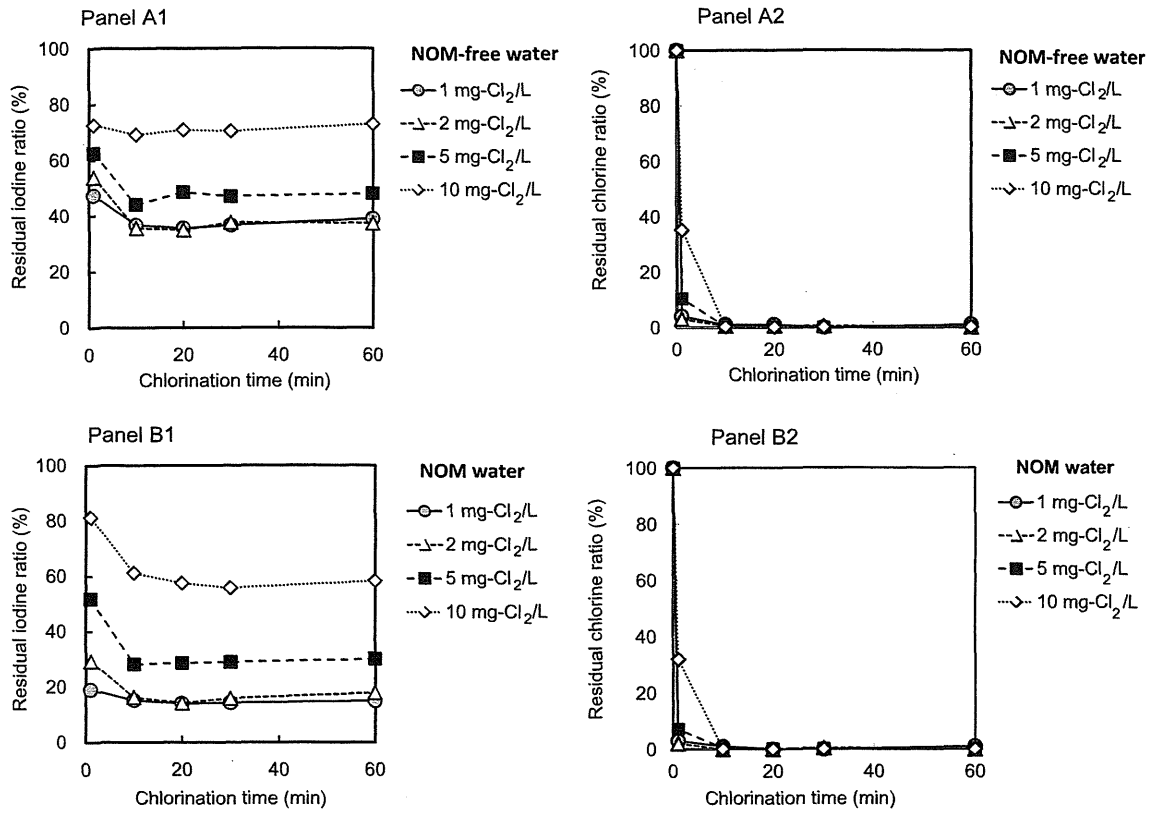

Fig. 7 – Effects of chlorination time and chlorine dose on residual iodine ratio (left) and residual chlorine ratio (right) in NOM-free water (top) and NOM water (bottom). After SPAC was added to the sample water, chlorine was added. SPAC dose, 100 mg/L; initial I^- concentration in sample water, 10 μg-I/L; initial DOC concentration, 1.5 mg-C/L.

of both adsorbable iodine (HOI and/or organic-I) and non-adsorbable iodine (I^- and IO_3^-). At a given water-phase iodine concentration (x-axis of Fig. 8), which was attained by one removal percentage, the adsorbable iodine (HOI and/or organic-I) concentrations were the same between SPAC and PAC because the prechlorination conditions were the same for the water samples. Therefore, the adsorption capacities of PAC and SPAC can be compared at a given water-phase concentration in Fig. 8. The adsorption isotherms for SPAC and PAC were the same for the NOM-free water (Fig. 8, Panel A), whereas the isotherms were different for the NOM water (Fig. 8, Panel B). In the presence of NOM, the adsorption capacity of PAC was roughly half that of SPAC. Such a trend of the lower adsorption capacity of PAC than of SPAC is reported for NOM adsorption (Ando et al., 2010), and the low adsorption capacity of PAC compared with that of SPAC is due to the difference in carbon particle size and to external adsorption: adsorbates do not diffuse into the interior of the PAC particles and instead are preferentially adsorbed near the particle surface (Ando et al., 2011; Matsui et al., 2011). Therefore, not all of the internal pore surface of the PAC particles is used for adsorption. In contrast, adsorbates can completely penetrate SPAC particles because the radial diffusion distance is short, and thus the entire internal pore surface of the SPAC particles is used for adsorption. The fact that there was no difference in adsorption capacity between PAC and SPAC for the NOM-free water indicates that internal adsorption occurred for both PAC and SPAC. Therefore, the experimental results for the NOM-free water indicate that HOI was adsorbed on the internal pore surface of the PAC particles. The experimental results for the NOM water indicate that organic-I was adsorbed on the external surface of the PAC particles. This is reasonable if the adsorption characteristics of organic-I (or iodinated NOM) are similar to those of the parent NOM, which exhibits external adsorption (Ando et al., 2010).

3.6. Visualization of solid-phase iodine concentration profiles

To verify the internal adsorption of HOI and the external adsorption of organic-I on PAC particles, we visualized the solid-phase iodine concentration profiles by using FE-EPMA. Panels A3 and A4 of Fig. 9 are iodine intensity maps of a cut PAC particle containing adsorbed HOI formed by chlorination in the NOM-free water. The intensity from iodine was slightly lower outside the particle (~230 cps) than inside the particle (~270 cps). However, this difference may have been due to the edge effect: the intensity from carbon was also slightly lower outside than inside (Fig. 9, Panel A2). An area of low intensity was also observed at the center right of the sample. However,

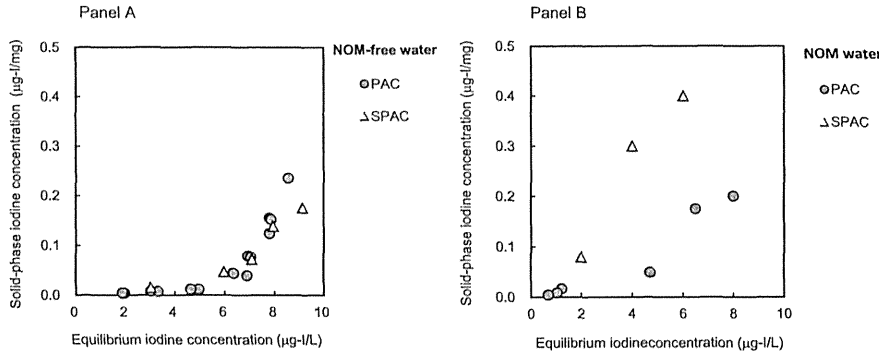

Fig. 8 – Comparison of iodine adsorption isotherms for PAC and SPAC in NOM-free water (left) and NOM water (right). After chlorination of sample water for 10 min, PAC or SPAC was added. Initial I^- concentration in sample water (NOM water and NOM-free water), 10 μg-I/L; chlorine doses, 0.5 mg-Cl_2/L for NOM-free water and 1.0 mg-Cl_2/L for NOM water; initial DOC concentration in NOM water, 1.5 mg-C/L; carbon contact time, 30 min.

there was a large open pore at the center right (Fig. 9, Pane A1), and thus the low intensity at the center right does not indicate low iodine concentration. Therefore, this result suggests that HOI was adsorbed uniformly in the PAC particle.

Iodine intensity maps of a cut sample of a PAC particle containing adsorbed organic-I formed by chlorination in the NOM water are shown in panels B3 and B4 of Fig. 9 (both HOI and organic-I were formed in the NOM water, but the HOI was eluted from the carbon particle, as described in the Supplementary Material, Section 3). For this particle, the iodine intensity was not higher inside than outside; instead the intensity was lower inside (~110 cps) than outside (~160 cps). Four open pores were observed in the sample (Fig. 9, panel B1). Therefore, the low intensity in the area close to these pores may have been due to the pores. However, there was a large area of low intensity (the area surrounded by the dotted line in Fig. 9) away from these pores. This result indicates that the concentration of solid-phase organic-I was lower inside the carbon particle than outside and that organic-I was adsorbed more on the outside than on the inside.

Overall, the visualization verified the hypothesis described in the previous section (external adsorption of organic-I and internal adsorption of HOI). The organic-I was adsorbed on the surface region of the PAC particles, but the total external surface area per unit weight of carbon was increased by the pulverization of PAC to SPAC. Accordingly, the increase in the surface area gave an advantage to SPAC compared to PAC with respect to organic-I adsorption capacity.

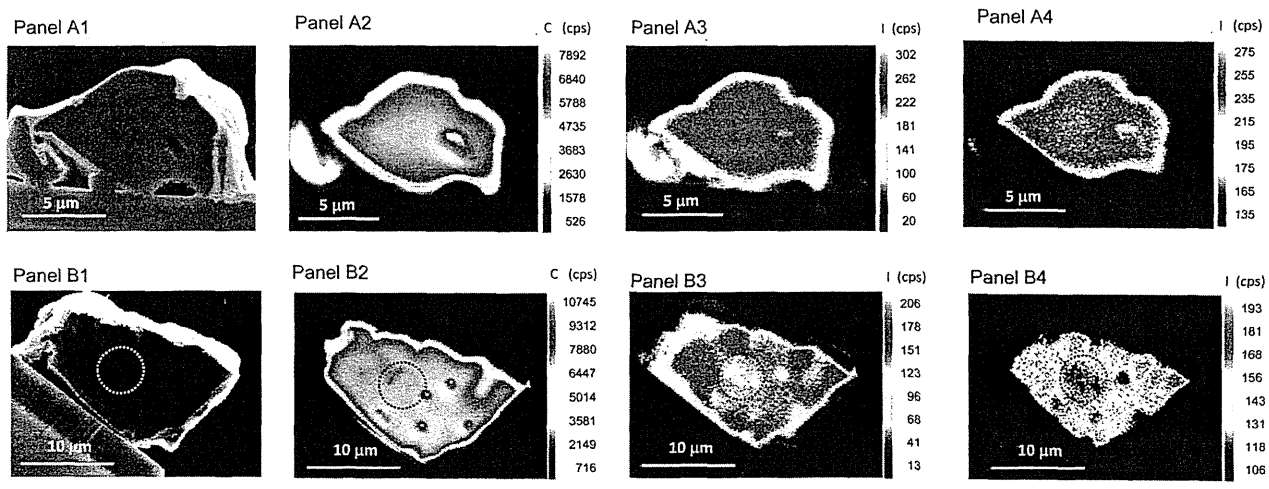

Fig. 9 – Surface elemental analysis of cut PAC particles with adsorbed HOI (A1–A4) and adsorbed organic-I (B1–B4) by means of field emission electron probe microanalysis (FE-EPMA). Initial I^- concentration in sample water, 10,000 μg-I/L; initial DOC concentrations, 0 mg-C/L for panels A1–A4 and 1.5 mg-C/L for panels B1–B4. After chlorination for 10 min at 5 mg-Cl_2/L, PAC was added. After a carbon contact time of 30 min, PAC particles were removed and cut with a focused ion beam. (A1) Scanning electron micrograph of a cut PAC particle, (A2) elemental mapping of carbon (C) (526–7892 cps), (A3) elemental mapping of iodine (I) (0–323 cps), and (A4) elemental mapping of iodine (I) (125–285 cps). (B1) Scanning electron micrograph of a cut PAC particle, (B2) elemental mapping of carbon (C) (716–10,745 cps), (B3) elemental mapping of iodine (I) (0–220 cps), (B4) elemental mapping of iodine (I) (100–200 cps).

4. Conclusions

(1) HOI and organic-I were adsorbed by activated carbon. In the presence of NOM, HOI was the major adsorbed species at a low chlorine dose, whereas organic-I was the major species at a high chlorine dose.
(2) There was an optimum chlorine dose for transformation of iodine to its adsorbable forms. The optimum dose was relatively low: 0.5–1.0 mg-Cl_2/L for 10 μg/L of iodine in the presence of NOM (1.5 mg-DOC/L). A long chlorination time reduced iodine removal because of the transformation of HOI to nonadsorbable IO_3^-. The optimum chlorination time was ~1 min or less.
(3) In the presence of Br^- in the NOM-free water, I^- was easily transformed into IO_3^- by chlorination even at low chlorine doses (0.1–0.2 mg-Cl_2/L), owing to a Br^-/HOBr-catalyzed process; and this process resulted in lower iodine removal in the presence of Br^- than in its absence. In the NOM water, however, co-existing Br^- did not decrease iodine removal greatly, owing to the high production of organic-I.
(4) In the NOM-free water, chlorination in the presence of PAC (Case 2) could enhance iodine removal compared with prechlorination followed by PAC adsorption (Case 1). This difference was due to the fact that in Case 2, the formation of IO_3^- was minimized by the consumption of chlorine by carbon. After HOI formed, it was readily adsorbed by the activated carbon before it could be oxidized to IO_3^-. In the NOM water, however, iodine removal was similar in Cases 1 and 2.
(5) The HOI adsorption capacity of SPAC was similar to that of PAC. For organic-I, however, SPAC showed higher adsorption capacity than PAC, because of the external adsorption (organic-I was adsorbed close to the PAC particle surface).
(6) FE-EPMA of PAC particles containing adsorbed HOI and organic-I revealed that the organic-I was adsorbed mostly close to the external surface of the PAC particles, whereas HOI was adsorbed uniformly in the PAC particles.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research S (no. 24226012) from the Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.10.021.

REFERENCES

Ando, N., Matsui, Y., Kurotobi, R., Nakano, Y., Matsushita, T., Ohno, K., 2010. Comparison of natural organic matter adsorption capacities of super-powdered activated carbon and powdered activated carbon. Water Res. 44 (14), 4127–4136.

Ando, N., Matsui, Y., Matsushita, T., Ohno, K., 2011. Direct observation of solid-phase adsorbate concentration profile in powdered activated carbon particle to elucidate mechanism of high adsorption capacity on super-powdered activated carbon. Water Res. 45 (2), 761–767.

AWWA, 1974. AWWA standard for granular activated carbon. J. Am. Water Works Assoc. 66 (11), 672–681.

Bichsel, Y., von Gunten, U., 1999. Oxidation of iodide and hypoiodous acid in the disinfection of natural waters. Environ. Sci. Technol. 33 (22), 4040–4045.

Bichsel, Y., von Gunten, U., 2000a. Hypoiodous acid: kinetics of the buffer-catalyzed disproportionation. Water Res. 34 (12), 3197–3203.

Bichsel, Y., von Gunten, U., 2000b. Formation of iodo-trihalomethanes during disinfection and oxidation of iodide-containing waters. Environ. Sci. Technol. 34 (13), 2784–2791.

Brown, J., Hammond, D., Wilkins, B.T., 2008. Handbook for Assessing the Impact of a Radiological Incident on Levels of Radioactivity in Drinking Water and Risks to Operatives at Water Treatment Works. Public Health England. http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1215762210590 **(accessed 07.23.13.).**

Criquet, J., Allard, S., Salhi, E., Joll, C.A., Heitz, A., von Gunten, U., 2012. Iodate and iodo-trihalomethane formation during chlorination of iodide-containing waters: role of bromide. Environ. Sci. Technol. 46 (13), 7350–7357.

Davis, J.A., Fox, P.M., Fuhrmann, M., 2009. Redox and Sorption Reactions of Iodine and Cesium during Transport through Aquifer Sediments (NUREG/CR-6977). Unites States Nuclear Regulatory Commission. http://www.nrc.gov/reading-rm/doc-collections/nuregs/contract/cr6977/ **(accessed 04.12.14.).**

Ding, G., Zhang, X., 2009. A picture of polar iodinated disinfection byproducts in drinking water by (UPLC/)ESI-tqMS. Environ. Sci. Technol. 43 (24), 9287–9293.

Gong, T., Zhang, X., 2013. Determination of iodide, iodate and organo-iodine in waters with a new total organic iodine measurement approach. Water Res. 47 (17), 6660–6669.

Hansen, V., Yi, P., Hou, X., Aldahan, A., Roos, P., Possnert, G., 2011. Iodide and iodate (129I and 127I) in surface water of the Baltic Sea, Kattegat and Skagerrak. Sci. Total Environ. 412–413 (0), 296–303.

Hua, G., Reckhow, D.A., Kim, J., 2006. Effect of bromide and iodide ions on the formation and speciation of disinfection byproducts during chlorination. Environ. Sci. Technol. 40 (9), 3050–3056.

Ikeda, N., Tanaka, K., 1975. Column chromatographic separation of radioactive tellurate, tellurite, iodide and iodate by active charcoal. J. Chromatogr. A 114 (2), 389–395.

Ikemoto, T., Magara, Y., 2011. Measures against impacts of nuclear disaster on drinking water supply systems in Japan. Water Pract. Technol. 6 http://dx.doi.org/10.2166/wpt.2011078.

Jones, D.B., Saglam, A., Triger, A., Song, H., Karanfil, T., 2011. I-THM formation and speciation: preformed monochloramine versus prechlorination followed by ammonia addition. Environ. Sci. Technol. 45 (24), 10429–10437.

Jones, D.B., Saglam, A., Song, H., Karanfil, T., 2012. The impact of bromide/iodide concentration and ratio on iodinated trihalomethane formation and speciation. Water Res. 46 (1), 11–20.

Kosaka, K., Asami, M., Kobashigawa, N., Ohkubo, K., Terada, H., Kishida, N., Akiba, M., 2012. Removal of radioactive iodine and cesium in water purification processes after an explosion at a nuclear power plant due to the Great East Japan Earthquake. Water Res. 46 (14), 4397–4404.

Kumar, K., Day, R.A., Margerum, D.W., 1986. Atom-transfer redox kinetics: general-acid-assisted oxidation of iodide by

chloramines and hypochlorite. Inorg. Chem. 25 (24), 4344–4350.
Lettinga, G., 1972. Radioactivity and Water Supplies. Delft, the Netherlands.
Matsui, Y., Ando, N., Yoshida, T., Kurotobi, R., Matsushita, T., Ohno, K., 2011. Modeling high adsorption capacity and kinetics of organic macromolecules on super-powdered activated carbon. Water Res. 45 (4), 1720–1728.
Plewa, M.J., Wagner, E.D., Richardson, S.D., Thruston, A.D., Woo, Y.-T., McKague, A.B., 2004. Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. Environ. Sci. Technol. 38 (18), 4713–4722.
Richardson, S.D., Fasano, F., Ellington, J.J., Crumley, F.G., Buettner, K.M., Evans, J.J., Blount, B.C., Silva, L.K., Waite, T.J., Luther, G.W., McKague, A.B., Miltner, R.J., Wagner, E.D., Plewa, M.J., 2008. Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. Environ. Sci. Technol. 42 (22), 8330–8338.
Siddiqui, M., Zhai, W., Amy, G., Mysore, C., 1996. Bromate ion removal by activated carbon. Water Res. 30 (7), 1651–1660.
Summers, R.S., Fuchs, F., Sontheimer, H., 1989. The fate and removal of radioactive iodine in the aquatic environment. In: MacCarthy, I.H.S.a.P. (Ed.), Aquatic Humic Substances: Influence on Fate and Treatment of Pollutants, Advances in Chemistry Series, Number 219. American Chemical Society, Washington DC.
Tate, C.H., Chow, B.M., Clark, R.R., Grams, N.E., Hashimoto, L.K., 1986. EPA Method Study 32: Method 450.1-Total Organic Halide (TOX). Environmental Monitoring and System Laboratory, USEPA, Cincinnati, Ohio, USA. http://www.epa.gov/nscep/index.html (accessed 05.18.14.).

Repeated Dose and Reproductive/Developmental Toxicity of Perfluorododecanoic Acid in Rats

Hina Kato,[1] Sakiko Fujii,[2] Mika Takahashi,[1] Mariko Matsumoto,[1] Mutsuko Hirata-Koizumi,[1] Atsushi Ono,[1] 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 19 November 2013; revised 25 March 2014; accepted 4 April 2014

ABSTRACT: Perfluoroalkyl carboxylic acids (PFCAs) are a series of environmental contaminants that have received attention because of their possible adverse effects on wildlife and human health. Although many toxicological studies have been performed on perfluorooctanoic acid with carbon chain length C8, available toxicity data on PFCAs with longer chains are still insufficient to evaluate their hazard. A combined repeated dose and reproductive/developmental toxicity screening study for perfluorododecanoic acid (PFDoA; C12) was conducted in accordance with OECD guideline 422 to fill these toxicity data gaps. PFDoA was administered by gavage to male and female rats at 0.1, 0.5, or 2.5 mg/kg/day. The administration of PFDoA at 0.5 and 2.5 mg/kg/day for 42–47 days mainly affected the liver, in which hypertrophy, necrosis, and inflammatory cholestasis were noted. Body weight gain was markedly inhibited in the 2.5 mg/kg/day group, and a decrease in hematopoiesis in the bone marrow and atrophic changes in the spleen, thymus, and adrenal gland were also observed. Regarding reproductive/developmental toxicity, various histopathological changes, including decreased spermatid and spermatozoa counts, were observed in the male reproductive organs, while continuous diestrous was observed in the females of the 2.5 mg/kg/day group. Seven of twelve females receiving 2.5 mg/kg/day died during late pregnancy while four other females in this group did not deliver live pups. No reproductive or developmental parameters changed at 0.1 or 0.5 mg/kg/day. Based on these results, the NOAELs of PFDoA were concluded to be 0.1 mg/kg/day for repeated dose toxicity and 0.5 mg/kg/day for reproductive/developmental toxicity.
© 2014 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2014.

Keywords: perfluorododecanoic acid; repeated dose toxicity; reproductive toxicity; developmental toxicity; screening test; rat; perfluoroalkyl carboxylic acid

INTRODUCTION

Perfluoroalkyl carboxylic acids (PFCAs) are a series of environmental contaminants that have recently received attention because of their possible effects on wild life and human health. They have been widely used as processing aids in the manufacture of fluoropolymers and as additives and components in consumer and industrial products (Prevedouros et al., 2006). Major sources of environmental pollution are considered to be fluoropolymer manufactures (Prevedouros et al., 2006). The stability and nonbiodegradability of PFCAs

Correspondence to: A. Hirose; e-mail: hirose@nihs.go.jp

Contract grant sponsor: Health and Labour Sciences Research Grant, Ministry of Health, Labour and Welfare, Japan.

Contract grant numbers: H22-Kenki-Ippan-006, H25-Kenki-Ippan-007

Contract grant sponsor: Ministry of Health, Labour and Welfare, Japan

Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.21996

© 2014 Wiley Periodicals, Inc.

allow them to be persistent in the environment (Lau et al., 2007).

Many toxicological studies have been performed on the 8-carbon chain length PFCA, perfluorooctanoic acid (PFOA), which was the most widely used PFCA at least until the PFOA Stewardship Program was launched by the United States Environmental Protection Agency and eight major industrial companies in 2006 (US EPA, 2013). Repeat-dose toxicity studies of PFOA in rodents revealed a steep dose-response curve for mortality, reduced body weight, and hepatocellular hypertrophy and necrosis (Griffith et al., 1980; Perkins et al., 2004; UK COT, 2006). The incidences of hepatocellular adenomas, Leydig cell tumors, and pancreatic acinar cell tumors were shown to be increased in a 2-year bioassay of PFOA in rats (Biegel et al., 2001). The developmental and hormonal effects and immunotoxic potential of PFOA have also been established in rodents (Lau et al., 2007). The lowest NOAEL of PFOA was 0.06 mg/kg/day based on its effects on the liver in a 13-week feeding study in rats (Perkins et al., 2004).

Recent studies have been extended to other PFCAs, which can be used as alternatives to PFOA. Findings have indicated that the toxic potency of PFCAs increases with lengthening of the carbon chain, at least up to C9 (Permadi et al., 1993; Kudo et al., 2006). Since the bioaccumulation potential of PFCAs has also been reported to increase depending on their carbon number (Martin et al., 2003), long-chain PFCAs may cause serious environmental and/or human health concerns in the future; however, available toxicity data on such long-chain PFCAs are still insufficient to evaluate the hazard.

To evaluate longer chain PFCAs, the Ministry of Health, Labour and Welfare, Japan, conducted combined repeat dose and reproductive/developmental toxicity screening tests on several long-chain PFCAs (carbon chain lengths C11 to C18) under the Japanese safety programmes for existing chemicals between 2007 and 2011. We previously reported the results for perfluorooctadecanoic acid [PFOdA (C18)], which demonstrated that the toxic potency of PFOdA was relatively low, compared to the other PFCAs; the NOAELs were 40 mg/kg/day for repeated dose toxicity and 200 mg/kg/day for reproductive/developmental toxicity (Hirata-Koizumi et al., 2012). This study reported the recently obtained results for perfluorododecanoic acid [PFDoA (C12); CAS No. 16517-11-6].

PFDoA is a white powder with a melting point of 107–109°C. It has been reported that PFDoA was detected in the influent, effluent, or sludge in sewage and also in industrial wastewater treatment plants in Japan, Thailand, and Australia (Clara et al., 2008; Murakami et al., 2009; Kunacheva et al., 2011), in the water, sediment, or soil of rivers in Japan, China, and Australia (Nishikoori et al., 2011; Thompson et al., 2011; Wang et al., 2012), and in house dust and indoor air in Norway (Haug et al., 2011). PFDoA has been detected in various wildlife including the albatross, harbor seals, and porpoises in many different geographic locations throughout the world (Hoff et al., 2004; Tao et al., 2006; Van de Vijver et al., 2007; Ahrens et al., 2009; Thompson et al., 2011). PFDoA was found at the levels of a few pg/mL to hundreds of pg/mL in the blood of humans in various parts of the world (Guruge et al., 2005; Falandysz et al., 2006; Harada et al., 2011; Haug et al., 2009; Olsen et al., 2012) and at <10–41 pg/mL in breast milk in East Asia (Fujii et al., 2012).

The concentration of PFDoA in the river sediment or soil and in wildlife exceeded that of PFOA, which may reflect the lower water solubility and higher bioaccumulative properties of PFDoA (Martin et al., 2003; Inoue et al., 2012). Although there is no data available on the production volume and application, PFDoA sources may not only be from the manufacture and use of PFDoA, but also from where PFDoA is present as an impurity or where substances may degrade to form PFDoA (Prevedouros et al., 2006).

PFDoA was recently reported to affect the liver, leading to lipidosis and widespread disintegrated cell systems, and inhibited steroidogenesis in the testis and ovary in rats (Shi et al., 2007, 2009a,b, 2010a,b; Zhang et al., 2008; Ding et al., 2009); however, no data are available on how PFDoA affects other organs, reproductive performance, and development. The results of the combined repeated dose and reproductive/developmental toxicity screening test described here may provide a more comprehensive toxicity profile of PFDoA than has been reported previously.

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 (Sapporo, Japan). All procedures involving the use and care of animals complied with the principles for Good Laboratory Practice (MOE et al., 2008) and applicable animal welfare regulations ["Act on Welfare and Management of Animals" (Japanese Animal Welfare Law, 2006), "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" (MOE, 2006), and "Guidelines for Animal Experimentation" (JALAS, 1987)].

Animals and Housing Conditions

Crl:CD(SD) rats (8-week-old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were maintained in an air-conditioned room with controlled temperature (22 ± 3°C) and humidity (50 ± 20%). Light was provided on a 12-h light/dark cycle (light: 8:00–20:00). Animals were housed in groups of two during the quarantine and acclimation periods, and after being assigned to each dose group, were reared individually, except for mating and nursing periods, in metal

bracket-type cages with wire-mesh floors.. Regarding pregnant animals, individual dams and litters were reared from day 17 of gestation to day 4 of nursing using wood chips as bedding (White Flake; Charles River Laboratories Japan). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast, Tokyo, Japan), and had free access to tap water (Sapporo, Japan).

Rats were acclimated to the laboratory for 14 days, during which general conditions were observed once a day and body weights were measured on the day of receipt, the 8th day of acclimation, and the end of acclimation. No abnormality was seen in the general state or weight in either animal. Vaginal smears were prepared daily for female animals in order to examine the estrous cycle for 9 days before animals were assigned to each group. Abnormalities were identified in two females. Animals found to be in good health and showing normal estrous cycles were divided into each dose group by stratified random sampling to equalize the mean body weight. The body weights of animals selected for the study were from 368 to 424 g in males and from 228 to 279 g in females.

Chemicals and Dosing

PFDoA was purchased from Exfluor Research Corporation (TX, USA). The PFDoA (Lot No. 4103) used in this study was 97 % pure, and was kept in an airtight container in a cold and dark place (2–9°C). The test article was suspended in a 0.5% aqueous solution of carboxymethylcellulose sodium (CMC-Na; Maruishi Pharmaceutical, Osaka, Japan), and administered to the animals by gastric intubation with a disposable gastric tube and disposable syringe. Before the start of the study, the stability of PFDoA in a 0.5% CMC-Na aqueous solution at concentrations of 0.01 and 100 mg/mL was confirmed after 4-h storage at room temperature following a 15-day refrigerated storage; therefore, dosing solutions were prepared at least once every 15 days throughout the study and were kept in a cool (2.0–7.2°C) and dark place under airtight conditions until dosing. The concentrations of PFDoA in the formulations were analyzed at the first and last preparation using high-performance liquid chromatography-tandem mass spectrometry, and were confirmed to be 96.4–99.0% of the target.

Experimental Design

Rats were administered PFDoA daily by repeated oral administration. The dose levels were determined based on the results of a 14-day dose-finding study, in which the liver weight was increased at all doses (1 mg/kg/day and above), and more clear toxic effects, including inhibition of body weight gain, changes in blood biochemical, and hematological parameters and brown discoloration of the liver, were observed in rats given 3 and 5 mg PFDoA/kg/day. Considering the longer administration period in the present combined study, the maximum dose of PFDoA was set at 2.5 mg/kg/day, and 0.1 and 0.5 mg/kg/day were derived by one-fifth divisions. The daily application volume (10 mL/kg body weight) was calculated according to the latest body weight. Control rats were given the same volume of vehicle alone.

Twelve males in each dose group (0, 0.1, 0.5, and 2.5 mg/kg/day) were dosed for a total of 42 days, beginning 14 days before mating. Seven males in the control and 2.5 mg/kg/day groups and all males in the 0.1 and 0.5 mg/kg/day groups were necropsied the day after day 42 of the dose (main group). The remaining five males in the control and 2.5 mg/kg/day groups were assigned to a recovery group, and after 42-day administration, they were kept without administration for 14 days (recovery period) and then necropsied. Twelve females/dose were dosed from 14 days prior to mating (main group). The pregnant females were dosed for gestation and nursing periods until 5 days after delivery and then necropsied on day 6 of nursing. Pregnant females which did not deliver by day 25 of gestation and females which showed abnormal delivery (stillbirth) were necropsied on day 26 of gestation and on day 0 of nursing, respectively. As a recovery group, other five females per dose were dosed with 0 or 2.5 mg PFDoA/kg/day for a total of 42 days without mating, and then kept without administration for 14 days (recovery period). All females in the recovery group were necropsied on the day after the 14-day recovery period, and therefore, females given PFDoA without mating were not examined fully at the end of administration period.

The first date of administration was defined as day 1 of the doing. In the main group females, the day of successful copulation was designated as day 0 of gestation and the end of deliver as day 0 of nursing or postnatal day (PND) 0. In recovery group males and females, the day following 42-day administration was defined as day 1 of recovery period.

Observation and Examination

Repeated Dose Toxicity Data

In all animals, general status, including life or death, appearance and behavior, of individual rats was observed twice daily during administration period (before and after the administration) and during the recovery period (morning and afternoon), and once in the morning of the day of necropsy. In addition, detailed clinical observations were conducted using a standardized scoring system for all of the animals before start of administration and once a week throughout the administration and recovery periods. Food consumption and body weight were measured at regular intervals throughout the administration and recovery periods.

Functional observations were performed on day 40 of the administration and on day 8 of recovery periods for males and for females of the recovery group, and on day 4 of nursing for females of main group. Subjects for the observations were 5 males/dose selected to approximate to the mean body weight of each dose group, 5 females/dose in recovery group

and 5 females/dose having delivered earlier in the main group. Evaluations were conducted using a predetermined standardized scoring system, as follows: (i) Sensorimotor reactivity to visual, tactile, auditory, pain, proprioceptive stimuli, and air righting reflex was assessed on an examination table, (ii) Forelimb and hindlimb grip strength was measured three times with a CPU gauge (Aikoh Engineering, Osaka, Japan), and (iii) Spontaneous motor activity was recorded for 1 h at intervals of 10 min using an automated activity monitor system [SUPERMEX and CompAct AMS (Muromachi Kikai, Tokyo, Japan)].

Urine was collected at the end of the administration and recovery periods in a nonfasted condition from five males per dose selected for functional observations and from five females per dose in the recovery group in the metabolism cage (KN-646, B-1 type, Natsume Seisakusho, Tokyo, Japan). Fresh urine (3 h) was examined for pH, protein, glucose, ketone body, urobilinogen, bilirubin, occult blood, and color, and urine volume and specific gravity were measured using collected urine (21 h).

Blood samples were collected for hematology and blood biochemistry from the abdominal aorta at necropsy under ether anesthesia after starvation for 16–22 h. In the main group, five males per dose not used in the functional observations and five females per dose used in the functional observations were selected for blood sampling. All animals in the recovery group were subjected to the blood sampling. One portion of the blood was treated with ethylenediaminetetraacetic acid dipotassium (EDTA-2K,TERUMO CORPORATION., Tokyo, Japan) and examined for the red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte, platelet count, white blood cell count (WBC), 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. Plasma obtained from blood treated with heparin sodium [HEPARIN SODIUM INJECTION-N "Ajinomoto", 1000 unit/mL (AJINOMOTO CO., INC., Tokyo, Japan)] was analyzed for aspartate aminotransferase (AST) and glucose. Serum prepared by centrifuging the blood collected in tubes filled with a serum separation agent (SEPACLEAN-A, EIKEN CHEMICAL CO., LTD., Tokyo, Japan) was analyzed for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GTP), total cholesterol (T-Cho), triglycerides (TG), total bilirubin (T-Bil), blood urea nitrogen (BUN), creatinine (Crea), sodium (Na), potassium (K), chlorine (Cl), calcium (Ca), inorganic phosphorus (IP), total protein (TP), protein fraction ratio, albumin/globulin (A/G) ratio, and albumin.

All surviving animals were euthanized by exsanguination after blood was collected under deep ether anesthesia and the external surfaces and organs and tissues of the whole body were examined macroscopically. The brain, pituitary gland, thyroid, heart, liver, kidney, spleen, adrenal gland, thymus, testes, epididymides, and ovaries were then weighed. The following organs and tissues were fixed and stored in 10% neutral-buffered formalin: the brain (cerebrum, cerebellum, and pons), spinal cord, pituitary gland, thymus, thyroid (including parathyroid), adrenal gland, spleen, heart, esophagus, stomach, liver, pancreas, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, trachea, lung, kidney, urinary bladder, prostate, seminal vesicle (including the coagulating gland), ovary, uterus (horn part and jugular), mammary gland (right abdomen), femur (right, including bone marrow), mesenteric lymph nodes, submandibular lymph nodes, sciatic nerve, and grossly abnormal tissues (including the boundary with normal tissues). 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 lungs were fixed with immersion following the injection of fixing solution. In principle, organs with right and left parts were both fixed and preserved.

Histopathological examinations were conducted on the preserved organs and tissues of five males and five females in the control and high dose groups, and also dead and euthanized animals during the study. All grossly abnormal tissues were also histopathologically examined regardless of the dose groups. Since treatment-related gross or histopathological abnormalities in the high dose group were observed in the forestomach, glandular stomach, pancreas, liver, testis, epididymis, prostate gland, seminal vesicle, coagulating gland, uterine horn part, spleen, thymus, born marrow, and adrenal gland, these organs from all animals in all groups were examined histopathologically. The organs were sectioned after paraffin embedding, stained with hematoxylin–eosin, and examined under a light microscope. To confirm the findings of the liver, specimens stained with Hall stain and Oil red O were additionally prepared and examined microscopically.

Reproductive/Developmental Toxicity Data

The estrous cycle of all females was evaluated by sampling the vaginal lavage daily from the first day of administration until evidence of copulation in the main group and until the necropsy day in the recovery group. Vaginal smear specimens made by the Giemsa stain were observed under an optical microscope. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles.

Each female in the main group 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 a 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. Gestation was confirmed by the

presence of delivery and by counting the number of implantation sites in the uterus at necropsy. 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 (9:00, 13:00, and 17:00) 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 the end of delivery. Gestational length was recorded, and the copulation index, fertility index, and gestation index were computed for each dose group.

All live and dead pups born were counted on PND 0, and the live birth index was calculated for each litter. Live pups were sexed and examined grossly on PNDs 0 and 4. Sex ratios were calculated for each litter. They were observed daily for general appearance and behavior, and the body weight of live pups was recorded on PNDs 0, 1, and 4. The viability index on PND 4 was calculated for each litter. All pups were euthanized on PND 4 by the inhalation of carbon dioxide and subjected to a gross external and internal (include the oral cavity) observation.

At necropsy of maternal animals, the numbers of corpora lutea and implantation in the uterus were recorded, and the implantation index and delivery index were calculated for each litter.

Statistical Analysis

The trend for detailed clinical and functional observations of each group, qualitative parameters of urinalysis, specific gravity of urine, and histopathological findings with multiple grades was evaluated by the Kruskal-Wallis test. If significant differences were identified ($p \leq 0.10$), 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. If significant differences were found ($p \leq 0.10$), the data were compared between the control and each dosage group using the chi-square two sample test or the Fisher's exact probability test.

The mean and standard deviation were calculated for the other parameters and evaluated by the Bartlett's test for homogeneity of variances. 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 ($p > 0.05$), a one-way analysis of variance was applied and data without homogeneity ($p \leq 0.05$) were subjected to the Kruskal-Wallis test. If a significant difference was identified ($p \leq 0.10$), the Dunnett's test or the Mann-Whitney test was used for pairwise comparisons between the control and individual treatment groups.

All statistical analyses comparing the control and individual treatment groups were conducted using the 5% level of probability as the criterion for significance.

RESULTS

Clinical and Functional Observations

In the 2.5 mg/kg/day group, soft feces was observed in one male on days 35–42 of the administration period and in another male only on day 4 of the recovery period. Although no deaths were observed in males, four females given 2.5 mg PFDoA/kg/day were found dead on days 18–22 of gestation. Hypothermia and vaginal hemorrhage were observed before death in one female. Three females were euthanized in the 2.5 mg/kg/day group due to a moribund condition on days 18–20 of gestation. In addition, two females were euthanized on day 26 of gestation because they did not deliver any pups, and two females were euthanized on nursing day 0 because of abnormal delivery (all pups stillborn). No clinical signs of toxicity were observed in females in the recovery group.

In detailed clinical observations, no significant difference was observed between the control and PFDoA-treated groups at any observation point. Although no significant differences were observed in functional observations for males on day 42 of the administration period between the control and PFDoA-treated groups, a significant decrease in forelimb grip strength was noted in males in the 2.5 mg/kg/day group at the end of the recovery period (Table I). A similar change was also observed in females of the recovery group. In females in the main group, the results of the 2.5 mg/kg/day group were exempt from statistical evaluation because only one female normally delivered pups and survived to the day of the functional observations (day 4 of nursing). Forelimb grip strength was slightly higher in this female than in control females. No significant change was observed in hindlimb grip strength in any of the treatment groups in either sex. In females given 2.5 mg PFDoA/kg/day in the recovery group, a significant decrease in motor activity was observed at 0–10 min, 10–20 min, and 20–30 min on week 6 of administration. Total motor activity for 60 min was also significantly decreased (Table I). Such an effect was not found on week 2 of the recovery period. No significant changes were observed in motor activity in any other groups.

Body Weight

Body weight was significantly lower in males at 2.5 mg/kg/day than in the controls from day 21 to the end of the 42-day administration period, and it remained significantly lower until the end of the 14-day recovery period (Fig. 1). Body weight in females in the recovery group showed similar time-dependent changes to those observed in the males, as shown in Figure 1. Body weight in females in the main group was significantly decreased at 2.5 mg/kg/day through the gestation period (Fig. 2). One female that normally delivered pups in the 2.5 mg/kg/day group had a lower body weight than the controls during the nursing period.

TABLE I. Grip strength and motor activity in male and female rats administered PFDoA

Dose (mg/kg/day)			0 (control)	0.1	0.5	2.5
MALES						
Number of animals examined			5	5	5	5
Administration week 6						
	Grip strength (g)	Forelimb	1459.28 ± 99.35	1532.14 ± 197.47	1632.80 ± 372.40	1313.72 ± 312.91
		Hindlimb	673.92 ± 240.73	636.16 ± 155.70	671.26 ± 84.61	526.92 ± 100.23
	Total motor activity	(60 min)	1459.3 ± 99.4	1169.4 ± 487.4	1340.0 ± 831.2	732.8 ± 442.6
Recovery week 2						
	Grip strength (g)	Forelimb	1576.54 ± 290.26			1217.00 ± 147.46*
		Hindlimb	669.16 ± 85.59			531.26 ± 119.71
	Total motor activity	(60 min)	1171.2 ± 344.9			9961.4 ± 362.2
FEMALES						
Number of animals examined			5	5	5	1
Day 4 of nursing (Main group)						
	Grip strength (g)	Forelimb	943.68 ± 140.49	922.20 ± 137.48	904.68 ± 124.73	1236.70[a]
		Hindlimb	502.66 ± 80.17	475.08 ± 88.55	478.46 ± 41.24	413.00[a]
	Total motor activity	(60 min)	1216.2 ± 234.2	1222.6 ± 494.5	1240.2 ± 853.1	1145.0[a]
Number of animals examined			5			5
Administration week 6 (Recovery group)						
	Grip strength (g)	Forelimb	1236.20 ± 260.53			998.20 ± 137.67
		Hindlimb	582.20 ± 51.34			472.54 ± 112.04
	Total motor activity	(60 min)	2785.6 ± 1000.2			1044.2 ± 563.3**
Recovery week 2 (Recovery group)						
	Grip strength (g)	Forelimb	1233.28 ± 221.67			834.58 ± 87.45**
		Hindlimb	630.68 ± 70.19			537.22 ± 105.37
	Total motor activity	(60 min)	1933.0 ± 1171.4			1117.8 ± 764.9

*Significantly different from the control, at $p \leqq 0.05$.
**Significantly different from the control, at $p \leqq 0.01$.
[a]Data from only one animal. In this group, other females did not deliver pups normally or survive to the day of the functional observations.

Food Consumption

A remarkable and significant decrease in food consumption was observed on days 28, 35, and 42 of the administration period at 2.5 mg/kg/day in males. However, no significant decreases were noted in food consumption in the 0.1 and 0.5 mg/kg/day groups. During the recovery period, no significant difference was observed in food consumption between the control and PFDoA-treated groups in males.

Females given 2.5 mg PFDoA/kg/day in the main group also consumed a significantly smaller amount of food from day 3 through day 20 of gestation. The amount of food consumed by one female that normally delivered pups in this group was lower than that of the control group during the nursing period. Food consumption in females in the 2.5 mg/kg/day recovery group showed similar time-dependent changes to those in the males.

Urinalysis

No significant difference was seen in any urinalysis parameters between the control and PFDoA-treated groups either at the end of the administration period or at the end of the recovery period.

Hematology

MCV and the reticulocyte ratio were significantly lower and MCHC was significantly higher in males given 2.5 mg

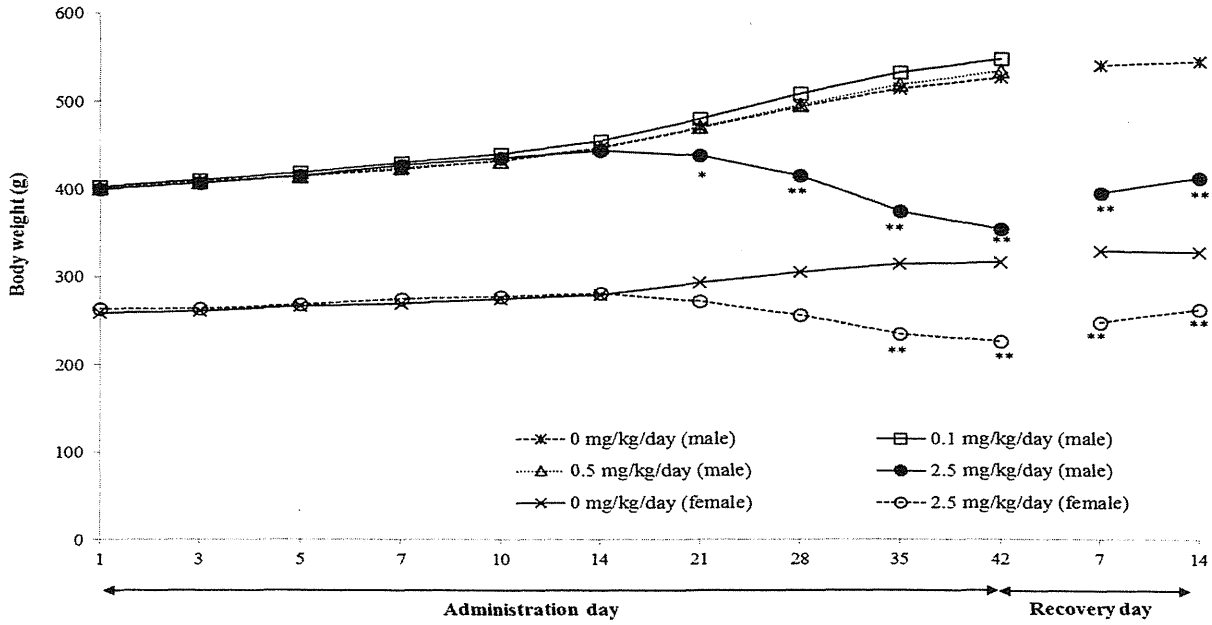

Fig. 1. Body weight changes in male rats in the main and recovery groups and female rats in the recovery group in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for perfluorododecanoic acid. *Significantly different from the 0 mg/kg/day group at $p \leq 0.05$, **Significantly different from the 0 mg/kg/day group at $p \leq 0.01$.

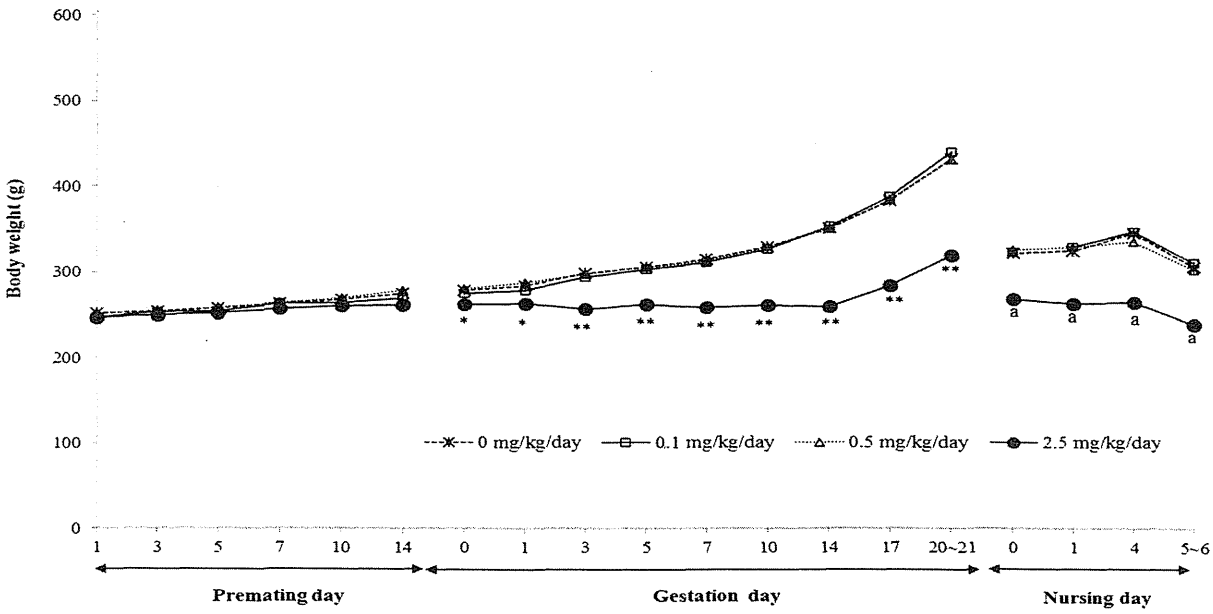

Fig. 2. Body weight changes in female rats in the main group in the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test for perfluorododecanoic acid. *Significantly different from the 0 mg/kg/day group at $p \leq 0.05$, **Significantly different from the 0 mg/kg/day group at $p \leq 0.01$. a: The data were exempt from statistical evaluation because only one female normally delivered pups.

PFDoA/kg/day than in the control group at the end of the administration period (Table II). In this dose group, significant decreases were noted in WBC, RBC, HGB, HCT, and lymphocyte, monocyte, and eosinophil counts on a differential counts of WBC, and a significant increase was observed in the reticulocyte counts at the end of the recovery period.

Many changes, including decreases in HCT, MCV, and the reticulocyte count and an increase in MCHC, were found in one female given 2.5 mg/kg/day, as shown in Table II. In the 0.1 and 0.5 mg/kg/day groups, no significant changes were found in any parameters. In the recovery group, HGB, HCT, MCV, and MCH were significantly lower, while the neutrophil differential count of WBC was significantly higher in females given 2.5 mg PFDoA/kg/day than in the control group.

Blood Biochemistry

Serum TP and albumin levels were significantly decreased in males at 2.5 mg/kg/day at the completion of the administration period (Table III). Significant increases were observed in the albumin and γ-globulin ratios in the protein fraction, as well as a significant decrease in the α_1-globulin ratio at 2.5 mg/kg/day and a significant decrease in the α_2-globulin ratio at 0.5 mg/kg/day and above. ALP activity was

TABLE II. Hematological findings in male and female rats administered PFDoA

	Main Group				Recovery Group	
Dose (mg/kg/day)	0 (control)	0.1	0.5	2.5	0 (control)	2.5
MALES						
Number of animals examined	5	5	5	5	5	5
Red blood cells (10^4/μL)	918 ± 37	914 ± 18	927 ± 31	943 ± 58	978 ± 56	859 ± 41**
Hemoglobin (g/dL)	16.3 ± 0.6	16.2 ± 0.4	17.0 ± 0.6	16.1 ± 0.9	16.4 ± 0.6	14.2 ± 0.6**
Hematocrit (%)	46.3 ± 1.4	46.2 ± 1.6	48.2 ± 2.2	44.0 ± 1.9	46.0 ± 1.3	40.3 ± 13.0**
MCV (fL)	50.5 ± 2.8	50.6 ± 2.2	52.0 ± 2.0	46.7 ± 1.6	47.1 ± 1.6	47.0 ± 1.4
MCH (pg)	17.8 ± 1.1	17.7 ± 0.7	18.3 ± 0.5	17.1 ± 0.4	16.8 ± 0.4	16.5 ± 0.4
MCHC (g/dL)	35.2 ± 0.3	35.0 ± 0.4	35.2 ± 0.5	36.7 ± 0.8**	35.7 ± 0.5	35.2 ± 0.5
Reticulocytes (%)	3.18 ± 0.47	3.26 ± 0.55	3.35 ± 0.63	1.17 ± 0.67**	3.15 ± 0.31	4.63 ± 0.56**
Platelets (10^4/μL)	124.8 ± 8.6	112.5 ± 11.8	118.0 ± 9.0	116.7 ± 33.2	127.6 ± 15.6	169.9 ± 41.2
White blood cells (10^2/μL)	113.4 ± 28.2	148.2 ± 33.9	108.4 ± 19.6	135.0 ± 36.9	139.8 ± 14.2	112.5 ± 6.9**
Neutrophils (10^2/μL)	16.8 ± 4.1	17.9 ± 6.2	13.8 ± 3.1	21.2 ± 8.6	19.3 ± 8.4	15.3 ± 5.0
Lymphocytes (10^2/μL)	90.6 ± 24.5	123.7 ± 30.6	89.8 ± 17.9	108.0 ± 32.4	112.5 ± 15.0	93.0 ± 6.0*
Monocytes (10^2/μL)	4.24 ± 1.22	4.28 ± 0.65	3.56 ± 1.18	4.76 ± 3.85	5.36 ± 1.90	2.54 ± 0.78*
Eosinophils (10^2/μL)	1.68 ± 0.78	2.26 ± 0.73	1.22 ± 0.55	1.00 ± 0.58	2.60 ± 0.62	1.56 ± 0.56*
Basophils (10^2/μL)	0.04 ± 0.09	0.04 ± 0.05	0.02 ± 0.04	0.06 ± 0.09	0.06 ± 0.05	0.00 ± 0.00
PT (sec)	21.3 ± 4.3	22.7 ± 3.9	20.7 ± 3.5	19.1 ± 0.9	20.1 ± 3.4	18.5 ± 1.0
APTT (sec)	26.0 ± 2.3	26.9 ± 2.2	25.9 ± 1.6	22.0 ± 3.5	25.5 ± 4.5	23.0 ± 1.2
FEMALES						
Number of animals examined	5	5	5	1	5	5
Red blood cells (10^4/μL)	812 ± 24	828 ± 44	837 ± 26	803[a]	868 ± 27	836 ± 43
Hemoglobin (g/dL)	15.3 ± 0.5	15.5 ± 0.5	15.6 ± 0.4	14.5[a]	15.8 ± 0.4	14.2 ± 0.7**
Hematocrit (%)	44.6 ± 1.9	45.6 ± 1.5	45.2 ± 1.2	40.5[a]	45.2 ± 1.1	41.1 ± 2.2**
MCV (fL)	54.9 ± 2.2	55.1 ± 1.6	54.1 ± 1.7	50.4[a]	52.1 ± 1.6	49.2 ± 2.1*
MCH (pg)	18.8 ± 0.5	18.8 ± 0.6	18.6 ± 0.3	18.1[a]	18.3 ± 0.5	17.0 ± 0.6**
MCHC (g/dL)	34.3 ± 0.5	34.0 ± 0.3	34.4 ± 0.6	35.8[a]	35.1 ± 0.5	34.6 ± 0.4
Reticulocytes (%)	9.79 ± 1.26	10.20 ± 1.14	8.67 ± 1.46	4.05[a]	3.00 ± 0.56	4.00 ± 0.90
Platelets (10^4/μL)	124.3 ± 11.6	144.5 ± 9.9*	136.7 ± 9.5	143.0[a]	112.8 ± 14.3	118.8 ± 35.4
White blood cells (10^2/μL)	116.5 ± 34.4	101.9 ± 37.0	102.0 ± 17.4	95.7[a]	82.7 ± 24.7	104.9 ± 31.8
Neutrophils (10^2/μL)	41.0 ± 28.6	24.6 ± 13.8	28.6 ± 7.8	14.2[a]	10.1 ± 4.0	17.5 ± 5.2*
Lymphocytes (10^2/μL)	67.8 ± 14.9	70.2 ± 21.5	64.6 ± 9.0	72.3[a]	68.5 ± 20.7	81.1 ± 32.2
Monocytes (10^2/μL)	5.60 ± 1.39	5.50 ± 2.09	6.86 ± 2.58	8.20[a]	2.82 ± 0.84	5.18 ± 2.28
Eosinophils (10^2/μL)	2.02 ± 0.66	1.54 ± 0.76	1.90 ± 0.32	0.90[a]	1.28 ± 0.38	1.16 ± 0.73
Basophils (10^2/μL)	0.04 ± 0.05	0.06 ± 0.05	0.04 ± 0.05	0.10[a]	0.00 ± 0.00	0.02 ± 0.04
PT (sec)	18.7 ± 1.0	18.0 ± 0.8	17.8 ± 1.1	17.2[a]	17.4 ± 0.9	18.9 ± 4.6
APTT (sec)	20.4 ± 1.0	20.2 ± 1.2	20.7 ± 1.9	21.8[a]	18.9 ± 1.6	25.3 ± 8.6

Values are given as the mean ± S.D.

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

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

[a]Data from only one animal. In this group, other females did not deliver pups normally or survive to the end of the study.

TABLE III. Blood biochemical findings in male and female rats administered PFDoA

	Main Group				Recovery Group	
Dose (mg/kg/day)	0 (control)	0.1	0.5	2.5	0 (control)	2.5
MALES						
Number of animals examined	5	5	5	5	5	5
Total protein (g/dL)	5.62 ± 0.13	5.54 ± 0.24	5.50 ± 0.16	4.30 ± 0.43**	5.74 ± 0.11	4.70 ± 0.14**
Albumin (g/dL)	2.82 ± 0.12	2.85 ± 0.18	2.85 ± 0.06	2.32 ± 0.30**	2.78 ± 0.14	2.62 ± 0.12
A/G (ratio)	1.01 ± 0.09	1.06 ± 0.06	1.08 ± 0.08	1.17 ± 0.08*	0.95 ± 0.10	1.26 ± 0.12**
Protein fraction (%)						
Albumin	50.2 ± 2.2	51.4 ± 1.4	51.9 ± 1.8	53.8 ± 1.8*	48.5 ± 2.5	55.8 ± 2.5**
Globulin α_1	19.9 ± 3.9	19.9 ± 1.7	21.2 ± 2.3	14.3 ± 2.5*	23.7 ± 2.2	14.4 ± 2.3**
Globulin α_2	8.04 ± 0.80	7.14 ± 0.43	6.92 ± 0.40*	5.82 ± 0.86**	6.70 ± 0.20	6.60 ± 0.22
Globulin β	16.6 ± 1.9	16.3 ± 1.1	15.0 ± 0.8	16.9 ± 0.7	16.2 ± 0.8	16.4 ± 1.5
Globulin γ	5.28 ± 0.88	5.28 ± 0.96	4.92 ± 0.57	9.18 ± 2.40**	4.86 ± 0.59	6.90 ± 0.91**
AST (IU/L)	74.6 ± 11.1	83.0 ± 22.1	103.6 ± 84.8	125.4 ± 56.1	65.6 ± 12.0	69.0 ± 5.7
ALT (IU/L)	31.2 ± 3.1	34.0 ± 5.7	48.2 ± 41.0	53.2 ± 28.0	26.0 ± 6.2	28.0 ± 6.4
ALP (IU/L)	357.2 ± 28.6	366.6 ± 88.4	551.6 ± 95.2**	630.0 ± 72.0**	251.0 ± 30.2	534.4 ± 78.0**
γ-GTP (IU/L)	0.52 ± 0.25	0.60 ± 0.20	0.54 ± 0.15	2.56 ± 2.20	0.58 ± 0.19	1.08 ± 1.70
Total bilirubin (mg/dL)	0.066 ± 0.018	0.044 ± 0.011	0.052 ± 0.022	0.390 ± 0.260**	0.054 ± 0.009	0.080 ± 0.027
Glucose (mg/dL)	166.2 ± 6.9	173.2 ± 19.0	156.8 ± 3.4	122.0 ± 4.6**	180.0 ± 17.0	154.6 ± 28.0
Total cholesterol (mg/dL)	67.0 ± 9.7	44.6 ± 7.6*	40.6 ± 7.8*	53.4 ± 20.0	56.8 ± 8.6	58.2 ± 8.6
Triglycerides (mg/dL)	37.4 ± 14.3	45.2 ± 12.8	33.6 ± 12.0	27.2 ± 5.40	56.6 ± 21.7	19.0 ± 5.50**
BUN (mg/dL)	15.7 ± 0.8	15.6 ± 0.9	16.2 ± 3.5	21.9 ± 1.2**	14.9 ± 1.1	19.7 ± 2.3**
Crea (mg/dL)	0.57 ± 0.03	0.56 ± 0.03	0.54 ± 0.04	0.48 ± 0.05**	0.54 ± 0.04	0.49 ± 0.05
Na (mEq/L)	143.8 ± 0.8	143.8 ± 0.8	143.8 ± 1.3	144.4 ± 2.4	140.6 ± 0.5	140.2 ± 0.8
K (mEq/L)	4.68 ± 0.32	4.86 ± 0.28	4.98 ± 0.22	4.83 ± 0.75	4.74 ± 0.62	5.32 ± 0.38
CI (mEq/L)	109.0 ± 0.7	108.2 ± 1.1	108.8 ± 1.8	110.2 ± 0.8	103.6 ± 1.1	105.4 ± 1.7
Ca (mg/dL)	9.52 ± 0.38	9.62 ± 0.36	9.40 ± 0.32	8.38 ± 0.26**	10.0± 0.05	9.04 ± 0.37**
IP (mg/dL)	6.60 ± 0.27	7.06 ± 0.74	6.64 ± 0.74	7.28 ± 0.36	6.92 ± 0.59	8.12 ± 0.73*
FEMALES						
Number of animals examined	5	5	5	1	5	5
Total protein (g/dL)	6.20 ± 0.23	6.50 ± 0.31	6.16 ± 0.19	5.30[a]	6.20 ± 0.23	4.56 ± 0.77**
Albumin (g/dL)	2.96 ± 0.23	3.34 ± 0.25*	3.26 ± 0.13	2.38[a]	3.53 ± 0.26	2.68 ± 0.43**
A/G (ratio)	0.92 ± 0.09	1.05 ± 0.09	1.13 ± 0.08**	0.81[a]	1.32 ± 0.11	1.45 ± 0.12
Protein fraction (%)						
Albumin	47.7 ± 2.6	51.3 ± 2.2*	52.9 ± 1.7**	44.8[a]	56.9 ± 2.1	59.1 ± 1.9
Globulin α_1	19.9 ± 2.1	18.8 ± 1.6	17.6 ± 1.9	16.3[a]	15.7± 1.5	10.6 ± 3.5*
Globulin α_2	7.98 ± 0.61	6.82 ± 0.69*	7.22 ± 0.73	7.80[a]	5.72 ± 0.64	6.54 ± 1.41
Globulin β	19.0 ± 1.5	17.7± 1.6	16.9 ± 0.7	17.2[a]	14.7± 1.0	14.8 ± 2.2
Globulin γ	5.46 ± 1.30	5.32 ± 0.88	5.36 ± 0.89	13.90[a]	7.06 ± 1.60	9.00 ± 3.80
AST (IU/L)	79.4 ± 12.8	66.8 ± 4.7	87.0 ± 48.1	147.0[a]	66.2 ± 8.7	143.6 ± 104.0**
ALT (IU/L)	26.2 ± 4.0	24.4 ± 2.3	28.2 ± 6.2	32.0[a]	27.2 ± 7.5	53.4 ± 61.0
ALP (IU/L)	199.6± 21.6	180.0 ± 62.0	174.6 ± 40.7	499.0[a]	144.4± 44.7	657.4 ± 439.0**
y-GTP (IU/L)	0.62 ± 0.28	0.62 ± 0.36	0.54 ± 0.11	1.50[a]	0.50 ± 0.20	15.4 ± 17.0**
Total bilirubin (mg/dL)	0.048 ± 0.008	0.046 ± 0.009	0.038 ± 0.008	0.070[a]	0.074± 0.011	1.240 ± 2.300
Glucose (mg/dL)	145.0± 23.0	150.4 ± 8.7	160.8 ± 13.0	121.0[a]	140.4± 19.0	115.4 ± 25.0
Total cholesterol (mg/dL)	67.2 ± 19.0	55.6 ± 16.0	45.8 ± 12.0	54.0[a]	69.2 ± 15.0	55.2 ± 22.0
Triglycerides (mg/dL)	26.8 ± 8.9	67.4 ± 86.9	33.0 ± 15.2	19.0[a]	17.4 ± 4.5	21.2 ± 10.0
BUN (mg/dL)	26.52 ± 4.52	22.74 ± 3.84	23.34 ± 2.77	29.70[a]	15.98± 1.04	17.06 ± 4.14
Crea (mg/dL)	0.62 ± 0.03	0.60 ± 0.01	0.59 ± 0.03	0.48[a]	0.60 ± 0.02	0.52 ± 0.03**
Na (mEq/L)	139.0± 2.0	139.2 ± 1.9	138.2 ± 2.0	139.0[a]	140.2± 1.3	139.6 ± 0.9
K (mEq/L)	4.97 ± 0.17	5.23 ± 0.20	5.11 ± 0.28	5.44[a]	4.50 ± 0.56	4.97 ± 0.30
CI (mEq/L)	103.6± 1.3	104.4 ± 1.5	103.4 ± 1.1	106.0[a]	104.8± 0.8	104.8 ± 0.8
Ca (mg/dL)	10.5 ± 0.5	10.6 ± 0.5	10.4 ± 0.3	9.70[a]	10.3 ± 0.2	9.2 ± 0.8**
IP (mg/dL)	8.96 ± 0.71	8.42 ± 0.40	8.76 ± 0.86	7.80[a]	6.62 ± 0.51	7.82 ± 1.40

Values are given as the mean ± S.D.

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

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

[a]Data from only one animal. In this group, other females did not deliver pups normally or survive to the end of the study.

significantly increased at 0.5 and 2.5 mg/kg/day in males. Significant increases in T-Bil and BUN and decreases in glucose, Crea, and Ca were noted in the 2.5 mg/kg/day group. Furthermore, T-Cho was significantly decreased at 0.1 and 0.5 mg/kg/day in males. Significant decreases in TP, the α_1-globulin fraction ratio, TG, and Ca, and significant increases in albumin and the γ-globulin fraction ratios, ALP, BUN, and IP were observed in males of the 2.5 mg/kg/day group at the end of recovery periods.

In females in the main group, many changes, including decreases in TP, albumin, and Crea, and increases in AST, ALP, γ-GTP, and T-Bil, were found in one female in the 2.5 mg/kg/day group (Table III). The albumin fraction ratio was significantly increased in females in the 0.1 and 0.5 mg/kg/day groups. Significant decreases in TP, albumin, α_1-globulin fraction ratio, Crea, and Ca, and significant increases in AST, ALP, and γ-GTP were observed in females in the recovery group at 2.5 mg/kg/day.

Necropsy Findings

In the main group, gross observations at necropsy revealed atrophy of the thymus in 3/7 males and 10/12 females, atrophy of the spleen in 3/7 males and 5/12 females, yellowish brown discoloration of the liver in 7/7 males and 6/12 females, black patches on the glandular stomach mucosa in 4/12 females, pancreas edema in 2/12 females, small-sized epididymis in 4/7 males, small-sized seminal vesicle in 4/7 males, pale yellow discoloration of the subcutis of general skin in 1/12 females, and atrophy of the lateral great muscle in 3/7 males at 2.5 mg/kg/day. In addition, opacity of the eye ball, granular surface on the forestomach mucosa, thickening of the forestomach mucosa, diverticulum of the ileum, yellow mass of the epididymis cauda, and yellow patches on the epididymis corpus were each observed in 1 male given 2.5 mg PFDoA/kg/day. Blood retention was identified in the uterus in five of seven females that died or were euthanized due to a moribund condition at the end of the gestation period in the 2.5 mg/kg/day group.

Yellowish brown or yellow discoloration in the liver (5/5 males and 4/5 females) and enlarged liver (4/5 males and 2/5 females), atrophy of the thymus (1/5 males and 1/5 females), spleen atrophy (1/5 females), edema of the submandibular gland and sublingual gland (1/5 females), atrophy of the lateral great muscle (1 female), pale yellow discoloration of the subcutis of the general skin (1 female), and small-sized seminal vesicle (1 male) were observed in the 2.5 mg/kg/day recovery group.

Organ Weight

The relative weight of the liver in males was significantly higher at 0.5 mg/kg/day and 2.5 mg/kg/day at the end of the administration period (Table IV). The absolute and relative weights of the thymus and absolute weights of the kidney, spleen, heart, pituitary gland, thyroid, adrenal gland, and epididymis in males given 2.5 mg PFDoA/kg/day were significantly decreased while the relative weights of the kidney and brain were significantly increased.

In females in the main group, many changes, including an increase in the relative liver weight and decreases in the absolute and relative weights of the spleen and thymus, were found in one surviving female given 2.5 mg/kg/day (Table IV). A significant increase in the relative liver weight and significant decreases in absolute weights of spleen, heart, pituitary gland, and thymus were observed at the end of the administration period in females given 0.5 mg PFDoA/kg/day.

Most changes observed in males given 2.5 mg PFDoA/kg/day at the end of the administration period remained after the 14-day recovery period (Table IV). In addition, the relative weights of testes and epididymides were significantly increased in the male recovery group. Significant increases in the relative weights of the brain, liver, and kidney and significant decreases in the absolute and relative weights of the ovary and absolute weights of the heart, pituitary gland, and adrenal gland were observed in females in the 2.5 mg/kg/day recovery group.

Histopathological Findings

Upon completion of the administration period, hepatic changes were observed in all males and females given 2.5 mg PFDoA/kg/day (Table V). They included diffuse hepatocyte hypertrophy, peribiliary inflammatory cellular infiltration, single cell necrosis of hepatocytes, focal necrosis, bilirubin deposition, and bile duct proliferation. The incidence of diffuse hepatocyte hypertrophy in both sexes and single cell necrosis of hepatocytes in females was significantly higher in the 2.5 mg/kg/day group. Focal necrosis was also detected in two females given 0.5 mg PFDoA/kg/day.

Histopathological changes were observed not only in the liver, but also in various organs in the 2.5 mg/kg/day group (Table V). A reduction in zymogen granules was seen in the pancreas in both sexes, and the frequency of the reduction in males was significantly increased. The incidence of edema of the interstitium in the pancreas was significantly higher in females in the 2.5 mg/kg/day group than in the controls. Atrophic changes were observed in the spleen, thymus, adrenal gland, muscle fibers, and male reproductive organs. The incidences of adrenal cortex atrophy in males and thymus cortex atrophy in females were significantly increased. Furthermore, a decrease in hematopoiesis in the bone marrow, ulcers in the glandular stomach, and erosion, hyperkeratosis, squamous cell hyperplasia, and inflammatory cellular infiltration, edema, and fibrosis of submucosa in the forestomach were noted.

As for male reproductive organs, atrophy of the glandular epithelium was observed in the prostate, seminal vesicle, and coagulating gland (Table V). In addition, cell debris at

TABLE IV. Organ weights of male and female rats administered PFDoA

		Main Group				Recovery Group	
Dose (mg/kg/day)		0 (control)	0.1	0.5	2.5	0 (control)	2.5
MALES							
Number of animals examined		5	5	5	5	5	5
Liver	(g)	12.0 ± 1.3	13.5 ±2.1	14.7 ±2.6	13.6 ±3.1	13.1 ± 1.2	15.2 ±2.8
	(%)[a]	2.51 ±0.14	2.67 ±0.21	3.00 ± 0.30*	4.30 ±0.27**	2.56 ±0.18	3.94 ±0.61**
Kidney[b]	(g)	3.01 ±0.27	3.28 ±0.22	3.26 ± 0.43	2.39 ±0.45*	3.15 ±0.14	2.76 ±0.21**
	(%)[a]	0.628 ±0.032	0.656 ±0.052	0.670 ± 0.058	0.760 ± 0.060**	0.614 ±0.029	0.718 ± 0.050**
Spleen	(g)	0.784 ±0.102	0.802 ±0.091	0.724 ± 0.123	0.488 ±0.155**	0.862 ±0.074	0.650 ± 0.025**
	(%)[a]	0.166 ±0.015	0.158 ±0.015	0.148 ± 0.013	0.152 ±0.029	0.170 ±0.019	0.170 ±0.010
Heart	(g)	1.480 ±0.180	1.520 ±0.150	1.390 ± 0.160	0.864 ±0.180**	1.480 ±0.094	1.110 ±0.110**
	(%)[a]	0.312 ±0.041	0.300 ±0.016	0.288 ± 0.029	0.274 ±0.013	0.292 ±0.023	0.286 ±0.011
Brain	(g)	2.21 ±0.13	2.14 ±0.10	2.16 ± 0.03	2.10 ±0.12	2.21 ±0.01	2.15 ±0.08
	(%)[a]	0.460 ±0.020	0.428 ±0.033	0.446 ± 0.034	0.688 ±0.160**	0.430 ±0.032	0.562 ±0.049**
Pituitary gland	(mg)	12.3 ±0.8	12.3 ±0.6	13.0 ±2.1	7.5 ± 1.6**	12.2 ±2.2	9.4 ± 1.5*
	(10^{-3}%)[a]	2.57 ±0.05	2.46 ±0.27	2.68 ± 0.51	2.37 ±0.30	2.40 ±0.43	2.44 ±0.33
Thymus	(mg)	325 ±97	442 ±75	328 ± 86	133 ±79**	382 ± 114	239 ±62*
	(10^{-3}%)[a]	67.6 ± 19.9	87.8 ± 12.7	67.0 ± 15.3	39.1 ± 18.9*	75.3 ±24.2	62.5 ± 19.0
Thyroid	(mg)	23.3 ±3.7	23.7 ± 1.5	22.3 ± 4.3	14.4 ±4.1**	22.4 ±3.7	16.2 ±3.8*
	(10^{-3}%)[a]	4.87 ±0.81	4.73 ±0.30	4.61 ± 1.00	4.53 ±0.77	4.38 ±0.61	4.22 ±0.96
Adrenal gland	(mg)	65.0 ±8.5	68.4 ±9.8	63.4 ± 17.0	38.4 ±5.9**	62.4 ±8.6	40.4 ±6.2**
	(10^{-3}%)[a]	13.5 ± 1.3	13.6 ± 1.9	13.2 ±3.9	12.5 ±2.9	12.3 ± 1.9	10.5 ± 1.5
Testis[b,c]	(g)	3.36 ±0.37	3.34 ±0.23	3.39 ± 0.30	2.81 ±0.65	3.43 ±0.28	3.39 ±0.42
	(%)[a]	0.671 ±0.054	0.647 ±0.049	0.677 ± 0.080	0.839 ±0.160	0.672 ±0.077	0.882 ±0.110**
Epididymis[b,c]	(g)	1.33 ±0.17	1.35 ±0.12	1.32 ± 0.10	0.90 ±0.31**	1.43 ±0.090	1.23 ±0.13*
	(%)[a]	0.267 ±0.033	0.263 ±0.024	0.263 ± 0.020	0.266 ±0.073	0.280 ±0.025	0.316 ±0.017*
FEMALES							
Number of animals examined		5	5	5	1	5	5
Liver	(g)	9.9 ±0.6	10.8 ±0.9	10.9 ± 0.9	10.9[d]	7.6 ±0.4	8.7 ± 1.8
	(%)[a]	3.23 ±0.19	3.43 ±0.11	3.70 ± 0.22**	4.52[d]	2.45 ±0.11	3.61 ±0.35**
Kidney[b]	(g)	2.19 ±0.17	2.06 ±0.22	1.97 ± 0.09	1.86[d]	2.02 ±0.08	1.99 ±0.29
	(%)[a]	0.720 ±0.093	0.656 ±0.038	0.668 ± 0.022	0.780[d]	0.656 ±0.022	0.836 ± 0.063**
Spleen	(g)	0.780 ±0.052	0.750 ±0.046	0.674 ± 0.081*	0.400[d]	0.560 ±0.100	0.522 ±0.110
	(%)[a]	0.256 ±0.040	0.240 ±0.019	0.232 ± 0.023	0.170[d]	0.182 ±0.035	0.218 ±0.035
Heart	(g)	1.040 ±0.090	1.080 ±0.054	0.928 ± 0.048*	0.770[d]	0.994 ±0.036	0.796 ±0.160*
	(%)[a]	0.342 ±0.022	0.346 ±0.018	0.316 ± 0.017	0.320[d]	0.324 ±0.011	0.334 ±0.034
Brain	(g)	2.02 ±0.11	2.00 ±0.06	2.00 ± 0.02	2.14[d]	2.10 ±0.09	1.99 ±0.09
	(%)[a]	0.662 ±0.057	0.640 ±0.041	0.680 ± 0.025	0.890[d]	0.682 ±0.028	0.852 ±0.150*
Pituitary gland	(mg)	17.3 ± 1.7	16.0 ±2.3	14.1 ± 1.9*	12.0[d]	17.7 ±2.2	10.4 ±4.5*
	(10^{-3}%)[a]	5.67 ±0.71	5.13 ±0.87	4.80 ± 0.60	5.00[d]	5.75 ±0.75	4.27 ± 1.3
Thymus	(mg)	310 ±27	297 ± 102	264 ± 16**	66.0[d]	298 ±70	241 ± 137
	(10^{-3}%)[a]	101.0 ± 11.2	93.7 ±27.8	89.5 ±3.8	27.5[d]	96.9 ±23.9	95.9 ±50.3
Thyroid	(mg)	17.8 ±4.7	18.8 ±3.7	18.3 ±4.2	11.0[d]	15.5 ± 1.5	13.1 ±5.6
	(10^{-3}%)[a]	5.74 ± 1.10	6.01 ± 1.20	6.21 ± 1.30	4.58[d]	5.02 ±0.47	5.32 ± 1.70
Adrenal gland	(mg)	84.2 ± 13.0	77.2 ± 8.6	79.4 ± 5.0	53.0[d]	70.8 ±9.2	47.4 ± 12.0**
	(10^{-3}%)[a]	27.5 ± 4.3	24.7 ± 3.3	27.0 ± 1.5	22.1[d]	23.0 ±3.1	19.9 ± 3.3
Ovary[b]	(mg)	115.4 ± 14.3	114.0 ± 21.1	109.6 ± 10.0	119.0[d]	100.4 ±17.4	56.0 ± 7.3**
	(10^{-3}%)[a]	37.6 ± 3.3	36.2 ± 4.7	37.2 ± 3.1	49.6[d]	32.6 ±6.0	23.7 ± 3.1*

Values are given as the mean ± S.D.

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

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

[a]Ratio of absolute organ weight to body weight on the necropsy day (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 2.5 mg/kg/day and 12 at 0.1 and 0.5 mg/kg/day in the main group, and 5 at 0 and 2.5 mg/kg/day in the recovery group).

[d]Data from only one animal. In this group, other females did not deliver pups normally or survive to the end of the study.

TABLE V. Histopathological findings in male and female rats administered PFDoA

		Main Group				Recovery Group	
Dose (mg/kg/day)	Grade	0 (control)	0.1	0.5	2.5	0 (control)	2.5
MALES							
Number of animals examined		7	12	12	7	5	5
Forestomach							
Erosion	+	0	0	0	1	0	0
Hyperkeratosis	+	0	0	0	2	0	0
Hyperplasia in squamous cells	+	0	0	0	2	0	0
Infiltration of inflammatory cells in the submucosa	+	0	0	0	1	0	0
Fibrosis of the submucosa	+	0	0	0	2	0	0
Pancreas							
Decrease in zymogen granules	+	0	0	0	5]**	0	1
	++	0	0	0	1]	0	0
Liver							
Deposition of bilirubin	+	0	0	0	1	0	2
Peribiliary infiltration of inflammatory cells	+	0	0	0	4	0	4*
Single cell necrosis of hepatocytes	+	0	0	0	1	0	0
	++	0	0	0	1	0	0
Focal necrosis	+	0	0	0	3	0	0
	++	0	0	0	1	?0	0
Diffuse hepatocyte hypertrophy	+	0	0	0	5*	0	3
Centrolobular hepatocyte hypertrophy	+	0	0	0	0	0	2
Periportal fatty changes	+	0	0	0	1	0	0
Fatty changes in midzonal	+	0	0	0	1	0	0
Fatty changes in diffuse	+	0	0	0	1	0	0
Testis							
Cell debris (Stage VII–VIII)	+	0	0	0	1	0	0
Decrease in elongated spermatids (Stage XII–XIV)	+	0	0	0	2	0	0
Epididymis							
Decrease in spermatozoa	+	0	0	0	2	0	1
	++	0	0	0	1	0	0
	+++	0	0	0	1	0	0
Cell debris in the lumen	+	0	0	0	2	0	0
	++	0	0	0	1	0	0
Spermatic granuloma	+	0	0	0	2	0	0
Prostate							
Glandular epithelium atrophy	+	0	0	0	3	0	1
Fibrosis in the interstitium	+	0	0	0	1	0	0
Seminal vesicles							
Glandular epithelium atrophy	+	0	0	0	3	0	1
	++	0	0	0	1	0	0
Coagulating gland							
Glandular epithelium atrophy	+	0	0	0	3	0	1
	++	0	0	0	1	0	0
Spleen							
White pulp atrophy	+	0	0	0	1	0	0
Red pulp atrophy	+	0	0	0	3	0	0
Thymus							
Atrophy of the cortex	+	0	0	0	0	0	1
	++	0	0	0	1	0	0
	+++	0	0	0	2	0	0
Bone marrow							
Decrease in hematopoiesis	+	0	0	0	2	0	0
	++	0	0	0	2	0	0
Adrenal glands							

TABLE V. *Continued*

		Main Group				Recovery Group	
Dose (mg/kg/day)	Grade	0 (control)	0.1	0.5	2.5	0 (control)	2.5
Atrophy of the cortex	+	0	0	0	5*	0	0
Skeletal muscle							
Muscle fiber atrophy	+	–	–	–	2(3)	–	–
	++	–	–	–	1(3)	–	–
FEMALES							
Number of animals examined		12	12	12	12[a]	5	5
Forestomach							
Edema of the submucosa	+	0	0	0	1	0	0
Glandular stomach							
Ulcer	+	0	0	0	4	0	0
Pancreas							
Edema of the interstitium	+	0	0	0	5 *	0	0
	++	0	0	0	1	0	0
Decrease in zymogen granuled	+	0	0	0	2	0	2
	++	0	1	0	1	0	0
Liver							
Deposition of bilirubin	+	0	0	0	1	0	5**
Single cell necrosis of hepatocytes	+	0	0	0	2 *	0	3
	++	0	0	0	5	0	0
Focal necrosis	+	0	0	2	4	0	0
Centrilobular hepatocyte necrosis	++	0	0	0	1	0	0
Bile duct proliferation	+	0	0	0	1	0	2
	++	0	0	0	1	0	2 **
Diffuse hepatocyte hypertrophy	+	0	0	0	6 **	0	2
	++	0	0	0	6	0	3
Inflammatory cell infiltration in peribiliary	+	0	0	0	0	0	3
Increase in mitosis in hepatocytes	+	0	0	0	1	0	0
Fatty changes in periportal	+	1	0	0	0	0	0
Fatty changes in diffuse	++	0	0	0	0	0	1
Uterus							
Hemorrhage at the implantation site	+	0	0	0	7 **	0	0
	++	0	0	0	1	0	0
Congestion of the endometrium	+	0	0	0	4	0	0
	++	0	0	0	1	0	0
Atrophy of endometrium and myometrium	+	0	0	0	0	0	2
Spleen							
White pulp atrophy	+	0	0	0	1(9)	0	1
	++	0	0	0	2 (9)	0	0
Red pulp atrophy	+	0	0	0	2(9)	0	0
	++	0	0	0	1(9)	0	0
Thymus							
Atrophy of the cortex	+	0	0	0	3 (10) **	0	1
	++	0	0	0	4 (10)	0	0
	+++	0	0	0	2 (10)	0	0
Bone marrow							
Decrease in hematopoiesis	+	0	0	0	2 (10)	0	1
Adrenal glands							
Atrophy of the cortex	+	0	0	0	0	0	5**
Skeletal muscle							
Muscle fiber atrophy	+	–	–	–	–	–	1(1)

Values are the number of animals with findings.
Values in parentheses are the number of animals examined.
–, Not examined; Grade +, slight change; ++, moderate change; +++, severe change.
[a]Including animals euthanized and found dead.
*Significantly different from the control, at $p \leq 0.05$.
**Significantly different from the control, at $p \leq 0.01$.

TABLE VI. Reproductive performance and developmental findings in rats administered PFDoA

Dose (mg/kg/day)			0 (control)	0.1	0.5	2.5
MAIN GROUP						
Premating period						
Estrous cycle normality			12/12	12/12	11/12	12/12
Length (days)[a]			4.13 ±0.30	4.29 ±0.54	4.18 ±0.43	4.23 ±0.41
Number of pairs			12	12	12	12
Copulation index (%)		Male	100	100	100	100
		Female	100	100	100	100
Fertility index (%)			100	91.7	100	100
Gestation index (%)			100	100	100	8.33**
Gestation length (days)[a]			22.1 ±0.3	22.2 ±0.4	22.1 ±0.3	23.0[b]
Number of pregnant animals			12	11	12	12
Number of corpora lutea[a]			17.1 ± 1.6	16.9 ± 1.3	15.8 ± 1.5	15.4 ±2.2
Number of implantation sites[a]			16.0 ± 1.3	16.6 ± 1.3	15.5 ± 1.4	14.5 ±2.1
Implantation index (%)[a]			93.9 ±4.8	98.4 ±2.7[d]	98.5 ±3.7[d]	94.4 ±8.9
Delivery index (%)[a]			94.3 ±6.3	91.7 ±6.5	89.7 ±9.7	31.4 ±54.0[c]
Number of litters			12	11	12	1
Number of pups delivered[a]		Total	15.1 ± 1.5	15.3 ± 1.7	13.9 ±2.0	16.0[b]
		Alive	15.1 ± 1.5	14.9 ± 1.8	13.8 ±2.1	14.0[b]
		Dead	0.0 ±0.0	0.4 ±0.7	0.1 ±0.3	2.0[b]
Sex ratio of live pups[a]			0.55 ±0.10	0.61 ±0.10	0.57 ±0.13	0.43[b]
Live birth index (%)[a]			100.0 ±0.0	97.6 ±4.6	99.3 ±2.4	87.5[b]
Number of live pups[a]	on nursing day 4		14.8 ± 1.3	14.6 ± 1.7	13.6 ±2.0	14.0[b]
Viability index (%)[a]			98.5 ±2.8	98.3 ±3.0	98.3 ±3.0	100[b]
Male pups						
Body weight (g)[a]	PND 0		6.48 ±0.30	6.52 ±0.64	6.70 ±0.52	4.70[b]
	PND 1		7.08 ±0.33	7.12 ±0.79	7.33 ±0.71	4.90[b]
	PND 4		10.50 ±0.63	10.70 ± 1.20	10.70 ± 1.40	6.20[b]
Female pups						
Body weight (g)[a]	PND 0		6.19 ±0.28	6.17 ±0.51	6.26 ±0.65	4.70[b]
	PND 1		6.81 ±0.32	6.75 ±0.62	7.01 ±0.68	5.00[b]
	PND 4		10.10 ±0.50	10.00 ± 1.10	10.20 ± 1.40	6.50[b]
RECOVERY GROUP						
Administration period						
Estrous cycle normality			5/5			0/5[e]
Length (days)[a]			4.24 ±0.43			-[d]
Recovery period						
Estrous cycle normality			5/5			1/5*
Length (days)[a]			4.10 ±0.22			4.00[e]

Estrous cycle normality, number of females with a normal estrous cycle / number of females examined; Copulation index, (number of animals with successful copulation / number of animals mated) × 100; Fertility index, (number of pregnant females/number of pairs with successful copulation) × 100; Gestation index, (number of females with live pups/number of pregnant females) × 100; Implantation index, (number of implantation sites/number of corpora lutea) × 100; Delivery index, (number of pups born/number of implantation sites) × 100; Sex ratio, (number of live male pups/number of live pups); Live birth index, (number of live pups on nursing day 0/number of pups born) × 100; Viability index, (number of live pups on nursing day 4/number of live pups on nursing day 0) × 100.

[a]Values are means and S.D.

[b]The number of dams or litters examined was one because only one dam normally delivered pups. The data were excluded from statistical evaluation.

[c]The number of litters examined was three because seven animals were found dead or moribund at the end of pregnancy and two females did not deliver pups normally. The data were excluded from statistical evaluation.

[d]Since continuous diestrous was observed in all five females, the length of the estrous cycle could not be calculated.

[e]The length of the estrous cycle was only calculated for one female because continuous diestrous was observed in the four other females. The data were excluded from statistical evaluation.

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

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

stages VII–VIII and a decrease in the number of elongate spermatids at stages XII–XIV in the testis, decrease in spermatozoa, cell debris in the lumen and spermatic granuloma in the epididymis, and fibrosis of the interstitium in the prostate were found. In female reproductive organs, hemorrhage on the implantation site and/or congestion on the endometrium were detected in the uterus of all 7 females found dead or moribund at the end of the gestation period. Hemorrhage at the implantation site was also found in one female that did not deliver live pups (all pups were stillborn).

Most hepatic changes remained after the 14-day recovery period (Table V). The incidences of peribiliary inflammatory cellular infiltration in males and bilirubin deposition and diffuse hepatocyte hypertrophy in females were significantly higher in the 2.5 mg/kg/day recovery group. Atrophy of the adrenal cortex was observed in all females in the 2.5 mg/kg/day recovery group with a significantly higher incidence. Endometrium and myometrium atrophy was noted in the uterus in 2 of 5 females given 2.5 mg PFDoA/kg/day after the 14-day recovery period. Although histopathological changes were also observed in the pancreas, thymus, spleen, bone marrow, skeletal fibers, and male reproductive organs after the 14-day recovery period, their incidences or degree was generally lower than those at the end of the administration period.

Reproductive and Developmental Findings

All females in the main group exhibited a normal estrous cycles during the premating period, except for one female in the 0.5 mg/kg/day group in which persistent diestrous was noted (Table VI). No significant deviations were observed in the incidence of a normal estrous cycle and length of the estrous cycle during the premating period. On the other hand, continuous diestrous was observed in the recovery group from day 27 of the administration period in all females given 2.5 mg/kg/day. A normal estrous cycle could not be recovered in four of the five females, even after termination of the administration period.

All males and females in the main groups were successfully copulated (Table VI). Although one female was not impregnated in the 0.1 mg/kg/day group, all other females became pregnant. No significant changes were found in the fertility index, the number of corpora lutea, or the number of implantation sites between the control and PFDoA-treated groups. In the 2.5 mg/kg/day group, 7 of 12 females given 2.5 mg PFDoA/kg/day were found dead or fell into a moribund state at the end of pregnancy, as mentioned above. Two of five surviving pregnant females did not deliver any pups, and 2 other females did not deliver live pups (all pups were stillborn). Consequently, only one female delivered live pups in the 2.5 mg/kg/day group; therefore, the gestation and delivery indices in this group were markedly lower than those of the control group. The gestation length of this one female in the 2.5 mg/kg/day group did not differ from that in the other groups.

The number of normally delivered pups in the 2.5 mg/kg/day group was 16 in one litter; however, two of them were found dead on nursing day 0 (Table VI). Although the other 14 pups survived to the end of the study, their body weights on PNDs 0, 1, and 4 were markedly lower than those of the control group. Necropsy of dead pups revealed renal pelvis dilatation and ascites in one pup in the 0.5 mg/kg/day group, while no other gross external or internal alterations were found in pups that survived until PND 4 or pups found dead during the postnatal period. No significant changes were observed in any reproductive/developmental parameters in the 0.1 and 0.5 mg/kg/day groups.

DISCUSSION

In this study, 7/12 females receiving 2.5 mg PFDoA/kg/day were found dead or moribund at the end of pregnancy. In contrast, no clear dose-related clinical signs of toxicity were observed in females of the recovery group or in males, which suggested that the cause of death involved factors that associated with pregnancy or delivery. Vaginal hemorrhage and/or blood retention in the uterus were observed in the dead and moribund females. Histopathological examinations of the uterus revealed hemorrhage in the implantation sites and congestion of the endometrium. These findings demonstrated that these females could not maintain a pregnancy, and excessive bleeding after placental separation may worsen their general condition.

Food consumption and body weight gain were markedly decreased in both sexes in the 2.5 mg/kg/day group. The effects on body weight are typically observed in rodents given PFCAs at relatively high doses, but they were not accompanied with reduced food intake necessarily (ATSDR, 2009; Hirata-Koizumi et al., 2012). Interestingly, Yang et al. (2002) reported that a 7-day dietary treatment with PFOA lowered the body weight of mice and this effect disappeared when peroxisome proliferator activated receptor (PPAR) α, a nuclear receptor important in regulating fatty acid metabolism in tissues such as liver, kidney, heart, and intestinal mucosa (Corton et al., 2000), was knocked out. PFDoA was recently shown to activate mouse PPARα in transiently transfected COS-1 cells (Wolf et al., 2012). Although no data are currently available on the interaction between PFDoA and rat PPARα, the significant induction of the mRNA levels of important PPARα target genes, acyl CoA oxidase and CYP4A1, was demonstrated in male rats orally dosed with PFDoA at 1 mg/kg and higher for 14 days (Zhang et al., 2008) and at 0.2 mg/kg/day and higher for 110 days (Ding et al., 2009). Taken together, these findings suggest that PFDoA may inhibit body weight gain via the activation of PPARα. In our studies for PFDoA and PFOdA, hepatic necrosis was observed at a dose affecting the body

weight (Hirata-Koizumi et al., 2012); therefore, there is also the possibility that hepatic necrosis is one factor for inhibition of body weight gain.

As with PFOA and the other PFCAs, the primary target of PFDoA was the liver. Relative liver weights increased in both sexes in the 0.5 and 2.5 mg/kg/day groups. Various histopathological changes, including hepatocyte hypertrophy and necrosis, were observed in the liver in both sexes given 2.5 mg PFDoA/kg/day, and focal necrosis was also found in the liver of 2/12 females receiving 0.5 mg PFDoA/kg/day. These changes have been attributed, at least in part, to PPARα activation by PFDoA because PPARα is considered to mediate the biological effects of peroxisome proliferators, such as increases in liver weight due to hepatocyte hypertrophy and hyperplasia, transcriptional increases in enzymes involved in the metabolism of fatty acids, and hepatocarcinogenesis (Green, 1995; Holden et al., 1999; Corton et al., 2000). On the other hand, the following findings suggest that a different mechanism from PPARα activation is involved in the hepatotoxicity of PFDoA. The peribiliary infiltration of inflammatory cells, bilirubin deposition, and proliferation of the bile duct were observed in the 2.5 mg/kg/day group, and blood biochemical examinations revealed an increased level of T-Bil and γ-GTP activity at 2.5 mg/kg/day and increased ALP activity at 0.5 and 2.5 mg/kg/day. These changes indicate inflammatory cholestasis. Yellow brown discoloration of the liver and subcutis, and yellow mass and patch on the epididymis observed in some animals given 2.5 mg PFDoA/kg/day may have resulted from the accumulation of yellow bilirubin pigment. The dose-independent changes in serum T-Cho observed in males suggest that the hypocholesterolemic action of PFDoA via PPARα activation may have been countervailed by impaired cholesterol excretion associated with cholestasis in the high dose group.

Most of the other changes observed in the 2.5 mg/kg/day group may be secondary effects that occur with the pronounced reduction in body weight gain and food consumption. A reduction in motor activity and grip strength may reflect muscle weakness accompanying decreases in body weight rather than neurotoxicity. Atrophy of the lateral great muscle in the 2.5 mg/kg/day group supports this hypothesis. Histopathological changes observed in the stomach, thymus, pancreas, and bone marrow are known to be associated with nutrient deficiencies and/or stress. The prolonged administration of PFDoA, which had a marked influence on food consumption and body weight, must have been stressful for animals. On the other hand, atrophy of the adrenal gland cannot only be explained by changes in body weight and food consumption because previous food restriction studies demonstrated that the adrenal gland was hypertrophied (Moriyama et al., 2008; Shallie et al., 2012). Such atrophic changes in the adrenal gland were shown to be induced by adrenal steroidogenesis inhibitors such as 1-(o-chlorophenyl)−1-(p-chlorophenyl)−2,2-dichloroethane (o,p'-DDD), and α-[1,4-dioxido-3-methylquinoxalin-2-yl]-*N*-methylnitrone (DMNM) (Hamid et al., 1974; Rosol et al., 2001). Because PFDoA was demonstrated to inhibit steroidogenesis in the testis and ovary (Shi et al., 2007; Shi et al., 2009a,b; 2010a,b), it may also alter adrenal steroidogenesis to cause atrophy of the adrenal cortex.

PFDoA affected the male and female reproductive systems. In males, cell debris and a reduction in the number of spermatid or spermatozoa were observed in the testis and epididymis, and atrophic changes were identified in the prostate, seminal vesicle, and coagulating gland in the 2.5 mg/kg/day group. Although these changes may have been due to the inhibition of body weight gain, a previous study demonstrated that the oral administration of PFDoA to rats for 110 days at a dose as low as 0.2 mg/kg/day decreased serum testosterone levels without affecting body weight (Shi et al., 2009a). An *in vitro* study reported the dose-dependent inhibition of steroidogenesis in mouse Leydig tumor cells and primary rat Leydig cells (Shi et al., 2010a), which indicated that PFDoA directly affected testicular testosterone synthesis, and not via the hypothalamic-pituitary-testicular axis. Since decreased testosterone biosynthesis is known to result in the degeneration and reduction in the number of germ cells as well as decreased size of accessory sex glands (O'Connor et al., 2002; OECD, 2009), the histopathological changes observed in the male reproductive organs in this study were attributed, at least in part, to the disruption of steroidogenesis. Shi et al. (2007, 2009a) reported that levels of the steroidogenic acute regulatory protein (StAR), which is responsible for cholesterol transport to the inner mitochondrial membrane, and StAR mRNA were markedly decreased in the testes of rats exposed to PFDoA, and treatment with the hydrosoluble form of cholesterol, which readily enters the inner mitochondrial membrane without the help of StAR, to mouse Leydig tumor cells prevented the inhibitory effect of PFDoA on steroidogenesis (Shi et al., 2010a). These results suggest that StAR is one of the target proteins for PFDoA activity in Leydig cells. A recently conducted proteomic analysis on the testis of rats exposed to PFDoA indicated that alterations in multiple pathways, including mitochondrial disruption and oxidative stress, may be associated with the testicular toxicity of PFDoA in rats (Shi et al., 2010b). Decreased testosterone levels in the testes and/or blood was also caused by PFOA, perfluorononanonic acid (PFNA, C9) and perfluorodecanoic acid (PFDeA, C10) (Bookstaff et al., 1990; Biegel et al., 1995; Jensen et al., 2008; Feng et al., 2009; Feng et al., 2010), which may involve the same mechanism as PFDoA. Recent study on PFOA-induced disruption of testosterone biosynthesis suggests the involvement of PPARα (Li et al., 2011).

A previous study demonstrated that PFDoA decreased serum estradiol levels in female rats following a 28-day oral administration period at a dose that affected body weight (Shi et al., 2009b). Alterations in the ovarian expression of genes responsible for cholesterol transport and steroidogenesis (StAR protein, cholesterol side-chain cleavage enzyme, and 17-beta-hydroxysteroid dehydrogenase) were also found