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Toxicity comparison of surface treatment agent APFO and its alternative substance APFHx

メタデータ	言語: English 出版者: 公開日: 2018-01-11 キーワード (Ja): キーワード (En): 作成者: 岩井, 宏之 メールアドレス: 所属:
URL	https://doi.org/10.24729/00000668

大阪府立大学博士（獣医学）学位論文

Toxicity comparison of surface treatment agent APFO and its alternative substance APFHx

(界面活性剤 APFO とその代替物質 APFHx の毒性比較)

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2017 年

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Preface

Perfluorooctanoic acid (PFOA: $C_7F_{15}COO^-$), its salts and PFOA-related compounds are used in a wide variety of applications and consumer products across many manufacturing industries. PFOA and its salts, especially ammonium salt (APFO), have been used most widely used as a polymerization aid for fluoroelastomers and fluoropolymers, with polytetrafluoroethylene (PTFE) being an important fluoropolymer used in producing, e.g. non-stick kitchen ware (UNEP 2017). PFOA-related compounds incorporating PFOA moieties in the side chains of hydrocarbon-based polymers become hydrophobic and lipophobic nature. Due to their physicochemical nature, PFOA, its salts and PFOA related compounds have been used in a wide variety of applications and consumer products such as water- and stain-resistant coatings for textiles, oil-resistant coatings for food packaging and cookware (Karsa 1995, Kissa 2001). On the other hand, some of PFOA and PFOA related compounds have been released into the environment. PFOA-related compounds released into the air, water and soil will decompose into PFOA in the environment and in the organism (Wang et al 2005). PFOA itself is not degradable in the environment and in biota (Liou et al 2010, Martin et al 2003b, Olsen et al 2007). Therefore, PFOA which is the final decomposition product can transport long-range in the environment. There is widespread PFOA in environmental compartments, organisms (Dietz et al 2008, Gonzalez-Gaya et al 2014). According to some studies of the U.S. general population from 1999 to 2015, PFOA was found about 1-5 ng / mL in the serum (CDC 2009, Olsen et al 2017). PFOA has been detected in human umbilical cord blood and breast milk (Apelberg et al 2007, Mondal et al 2014). Children up to about 12 years of age have higher serum PFOA concentrations than their mothers (Mondal et al., 2012). These facts demonstrate that exposures relevant to potential developmental effects occur during prenatal and postnatal development.

Due to concerns about PFOA's human and environmental impacts, structurally similar substances were considered candidates for substitutes. The substitutes with similar structures were designed to reduce the number of carbons as bioaccumulation increases as the number of carbons increases. Products with Perfluorohexanoic acid (PFHxA: $C_5F_{11}COO^-$) -related compounds may meet technical performance and cost. PFOA-related compounds have been treated on the surface of textile, carpet and paper resulting in breathability and water repellency. These days we have confirmed that the PFHxA-related products have the same performance as PFOA related ones.

In this thesis, the author investigated the toxicity of PFOA and the accumulation and toxicity of PFHxA which can be replaced industrially. Since PFOA and PFHxA have high acidity, they are made into ammonium salts for industrial use to make it neutral and easy to handle. Therefore, in this thesis, it was carried out with the ammonium salts APFO and APFHx. In the first chapter, the authors repeatedly orally administrated APFO to rats and investigated the influence on liver weight, peroxisomal enzyme activity. For the dose setting, 50 mg/kg was made the highest concentration with reference to the guidance value of the United Nations Global Harmonization System (GHS) (UNECE 2005). Our repeated toxicity test of male cynomolgus monkey also showed increased liver weight at 3 mg/kg (Butenhoff et al 2002). So the common ratio 10 was set to 5 and 0.5 mg/kg so as to include these doses. In addition, since immune suppression by PFOA was reported in mice, changes in the number of immune cells were examined in rats. In the second chapter, the author investigated the bioaccumulation of APFHx using rat and mouse of both sexes because of the species difference and sex difference in APFO bioaccumulation. The toxicity of APFO was considered to be due to accumulation in the body, and the accumulation property of the substitute substance APFHx was examined. We decided to examine the proportion of excretion patterns after administration of single and multiple

(14 days) oral doses of APFHx to male and female mice and rats. Dose setting is needed no toxic effect and to be low enough high to identify metabolites in excrement, usually the NOAEL from the critical toxicology study (US EPA 1998). Since there was a report that the NOAEL of PFHxA was 50 mg/kg/day in the 90-day repeated test, this amount was set as the dose. In the last chapter, the author examined the developmental and reproduction toxicity of APFHx to compare with APFO in mice. From the 90-day repeated toxicity study of PFHxA in rats, the NOAEL was 50 mg/kg/day (Chengelis et al 2009b). And we conducted a 24-month chronic toxicity study of PFHxA in rats, no tumor was observed even at 200 mg/kg/day administration (Klaunig et al 2015). The authors concluded that the toxicity of APFHx in repeated administration is low. Since PFOA has been exposed to fetuses and infants in human, the author decided to conduct developmental and reproduction toxicity study. PFOA has high bioaccumulation in male rats, but it disappears from the body quickly in female rats. Therefore, the developmental and reproduction toxicity of rats is estimated to be low. It is necessary to judge by developmental and reproduction toxicity in mice with no gender difference in bioaccumulation. Although APFHx had no gender or species difference in bioaccumulation, this substance was examined in mice for comparison with APFO.

Acronyms and formulas of perfluoroalkyl acids

Chemical	Formula	Acronym
Perfluoroalkyl acids	$F(CF_2)_nCOO^-$ & $F(CF_2)_nSO_3^-$	PFAAs
Perfluoroalkyl carboxylic acids		PFCAs
Perfluorobutyric acid	$F(CF_2)_3COO^-$	PFBA
Perfluorohexanoic acid	$F(CF_2)_5COO^-$	PFHxA
Perfluoroheptanoic acid	$F(CF_2)_6COO^-$	PFHpA
Perfluorooctanoic acid	$F(CF_2)_7COO^-$	PFOA
Perfluorodecanoic acid	$F(CF_2)_9COO^-$	PFDA
Perfluorocarboxylates		PFCs
Ammonium perfluorohexanoate	$F(CF_2)_5COO\ NH_4$	APFHx
Ammonium perfluorooctanoate	$F(CF_2)_7COO\ NH_4$	APFO
Sodium perfluorooctanoate	$F(CF_2)_5COO\ Na$	NaPFHx
Perfluoroalkyl sulfonic acids		PFSAs
Perfluorohexane sulfonic acid	$F(CF_2)_6SO_3^-$	PFHxS
Perfluorooctane sulfonic acid	$F(CF_2)_8SO_3^-$	PFOS

Chapter 1

A fourteen-day repeated dose oral toxicity study of ammonium perfluorooctanoate (APFO) in male rats

Introduction

Perfluorooctanoic acid (PFOA: $C_7F_{15}COO^-$) is acidic but is neutral in the form of the ammonium salt (ammonium perfluoroocanoate, APFO; $C_7F_{15}COONH_4$, CAS Registry no. 3825-26-1). Industrially, it has been used particularly and frequently as an additive to promote polymerization of fluorine-based resin or rubber. The results of past studies in rats, mice, and monkeys given this substance with diet show that the target organ of APFO is the liver (Biegel et al 2001, Butenhoff et al 2002, Kennedy 1987). PFOA which is the dissociation product of APFO is a peroxisome proliferator (PPs) (Kennedy et al 2004, Takagi et al 1992). A major activity of PPs is to increase liver peroxisomes, activate their ß-oxidation, and cause liver thickening (Cattley et al 1998, Corton et al 2000). Attention has been paid to possible adverse effects of PPs on immune function as well as on lipid metabolism (Yang et al 2000). Treatment of male C57B1/6 mice with PFOA resulted in a decrease of thymocytes and splenocytes (Yang et al 2001). Oral administration of WY-14,643 (a PPAR ligand) to C57BL/6 mice markedly reduced the number of splenocytes (Cunard et al 2002). PPARα ligand seems to serve as a profound immunosuppressive agent in mice. Perfluorodecanoic acid (PFDA), a homologue of PFOA, has also been considered as a PP. Injection of Fischer 344 rats with PFDA resulted in some influence on the immune system, although the influence was less marked than that in mice (Nelson et al 1992). It has been reported that the half-life in vivo is longer with PFDA than with PFOA, and that the effects in vivo are greater with PFDA (Kudo et al 2000). The current study was undertaken to examine whether or not the reported influence on mouse immune system would appear in rats treated with APFO and whether immunosuppression would be the action of APFO.

Materials and methods

Test material

The test material, ammonium perfluorohexanoate (APFO), was 10% aqueous solution received from Daikin Industries Ltd. (Osaka, Japan).

Animals and treatment

All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals in the animal facilities at LSI Medience Corporation Central Laboratory (American Association for the Accreditation of Laboratory Animal Care [AAALAC] accredited). Male Crj:CD(SD)IGS strain rats was chosen because this strain is widely used in toxicity studies, and there is an abundance of background data available. Rats were quarantined for 5 days and were acclimated for 8 days (from their arrival until use in the experiments). Rats were given clinical observations once a day, and the body weight was measured on the day of their arrival and on the final day of quarantine. It was confirmed that all rats were in good health. Age at the start of administration was 6 weeks. Body weight at the start of administration was in the range of 170 to 202 g. These rats were maintained on a 12-h light/dark cycle at 22°C to 24°C, a relative humidity of 47.3% to 63.0%, a ventilation of 6 to 20 air changes per hour with fresh, filtered air.

The test substance was administered orally, using a disposable syringe with a gastric tube attached, once a day for 14 days. For the dose setting, 50 mg/kg was made the highest concentration with reference to the guidance value of the United Nations Global Harmonization System (GHS) (UNECE 2005). Our repeated toxicity test of male cynomolgus monkey also showed increased liver weight at 3 mg/kg (Butenhoff et al 2002). So the common ratio 10 was set to 5 and 0.5 mg/kg so as to include these doses. A control group (0 mg/kg), administered the vehicle alone (purified water), was also used for the study. The dose volume was set at 10 mL/kg and was calculated for each rat based on the most recently measured body weight.

Hematology and blood chemistry

Hematological and blood chemistry examinations were performed on the day scheduled for necropsy (day 15). All rats were fasted from the evening the day before examination. Blood samples were obtained from the inferior vena cava of all rats under intraperitoneal pentobarbital sodium anesthesia. Erythrocyte (red blood cell) count, hemoglobin concentration, hematocrit, reticulocyte ratio, leukocyte (white blood cell) count, and differential leukocyte count were measured.

The serum samples were examined for the level of glucose, total cholesterol, HDL cholesterol (HDL), LDL cholesterol (LDL), phospholipids, and triglycerides in the blood.

Pathological examinations

Organ Weight

Liver, kidney, and spleen from all rats were weighed. An electronic balance (AW-120, Shimadzu Corp.) was used for weighing. Body weight was measured on the day of necropsy and used to calculate the relative organ weights.

Necropsy

After the blood sampling, all rats were euthanized by exsanguination by severing the abdominal aorta under anesthesia and then were subjected to necropsy. Liver, kidney, spleen, and gross lesion from all rats were removed and preserved in a 10 vol% neutral phosphate-buffered formalin. Liver of scheduled sacrifice animals was fixed after a portion was collected for the measurement of peroxisomal enzyme activity.

Measurement of lymphocyte subsets

At the scheduled sacrifice on day 15, 0.3 mL of blood samples were collected at the same time as those for hematology. Blood samples were treated with anticoagulant heparin sodium. To confirm the protocol for the flow cytometry, the blood from the remaining 3 animals at grouping were used. Red blood cells were lysed using ACK Lysing Solution (0.16 mol/L NH_4Cl, 10mmol/L $KHCO_3$, 0.1 mmol/L EDTA-2Na, pH 7.2–7.4). Two-color staining with FITC-labeled anti-rat CD3 antibody (G4.18; BD Pharmingen, lot no. 0000061473) and PE-labeled anti-rat NKR-P1A antibody (10/78; BD Pharmingen, lot no. 0000049085), two-color staining with FITC-labeled anti-rat CD4 antibody (OX-38; BD Pharmingen, lot no. 0000047781) and PE-labeled anti-rat CD8a antibody (OX-8; BD Pharmingen, lot no. 0000035815) and single-color staining with PE-labeled anti-rat CD45RA antibody (OX-33; BD Pharmingen, lot no. 0000038597) was performed, and the ratio of the surface marker expression (on T cells, B cells, NK cells, helper T cells, or killer T cells) was evaluated using a flow cytometer (EPICS XL-MCL; Beckman Coulter). As control antibodies, FITC-labeled anti-mouse IgG2a, κ antibody (BD Pharmingen, lot no. 0000043133), FITC-labeled anti-mouse IgG3, κ antibody (BD Pharmingen, lot no. M076476), and PE-labeled anti-mouse IgG1, κ antibody (BD Pharmingen, lot no. 0000049663) were used. The results were expressed as the ratio and the absolute numbers (cellularity multiplied by the ratio) for each positive cell within the lymphocyte subsets.

Measurement of peroxisomal enzyme activity in the liver

Portions of the liver collected from all surviving animals at the scheduled sacrifice (day 15) were perfused with buffer solution (154 mmol/L KCl, 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA-2K) to remove blood. After weighing, the portions were rapidly frozen in liquid nitrogen and were kept frozen at −80°C until preparation for measurement. After defrosting, the portions were homogenized in the same buffer solution using Polytron homogenizer (Kinematica)

and Teflon homogenizer (GTR-1000, Tokyo Rikakikai Co., Ltd.) and were centrifuged at 700 g, at approximately 4°C for 10 min to obtain the supernatant (sample). Cyanide-insensitive palmitoyl CoA ß-oxidation (FAOS) activity, carnitine acetyltransferase (CAT) activity, and carnitine palmitoyltransferase (CPT) activity were quantified using an auto-analyzer (TBA-2000FR, Toshiba Co., Ltd.), and activity per wet tissue weight and per protein were calculated.

As for FAOS, the amount of deoxidized NAD by ß-oxidation of palmitoyl CoA was quantified (Lazarow & De Duve 1976) after inhibiting the oxidizing system in the mitochondria by KCN.

As for CAT, acetyl CoA was used as a substrate, CoA-SH formed by transition of acetyl was reacted with 5,5′-dithio-bis-2-nitrobenzoate, and the amount of 5-thio-2-nitrobenzoate formed was quantified (Markwell et al 1973).

For CPT, using palmitoyl CoA as a substrate, CoA-SH formed by transition of palmitoyl was reacted with 5,5′-dithio-bis-2-nitrobenzoate, and the amount of 5-thio-2-nitrobenzoate formed was quantified (Markwell et al 1973). The protein content of sample was measured by the Lowry methods using an auto-analyzer (TBA-2000FR, Toshiba Co., Ltd.).

Statistical analysis

Numerical data were analyzed by multiple comparison test for statistical significance. In the analysis, the homogeneity of the variance among the groups was first tested by Bartlett's test. When the variance was demonstrated to be homogeneous, one-way analysis of variance was applied. When the variance was heterogeneous, Kruskal–Wallis test was applied. When a significant difference was detected among the groups, Dunnett's test or Dunnett- type multiple comparison test was applied. Bartlett's test, one-way analysis of variance, and Kruskal–

Wallis test were conducted at the significance level of 5%; Dunnett's test and Dunnett-type multiple comparison test were conducted at the significance level of 5% and 1%. MiTOX® (Mitsui Zosen Systems Research Inc.) was used for statistical analysis. Statistical analysis was performed on the items listed below. The analysis was not performed on results of the clinical observation, or necropsy.

Multiple comparison tests were performed on body weight, hematology data, blood chemistry data, organ weight (absolute and relative weight), lymphocyte subsets, and peroxisomal enzyme activity in the liver

Results

After APFO was repeatedly administered orally to male Crj:CD(SD)IGS rats for 14 days, no animal died and no abnormality was observed in any dose group. A tendency for weight loss was observed in the 50 mg/kg group on days 4, 8, and 14 but not at 0.5 or 5 mg/kg (data not shown).

In all groups, a significant increase or a tendency for increase was observed in the absolute and relative weights of the liver, while no change was observed in the weight of the spleen (Table 1). At necropsy, enlargement of the liver was observed in the APFO 5 and 50 mg/kg dose groups, while no effect was observed in the spleen and the thymus.

The mean FAOS level (per wet tissue weight and per protein), a marker for peroxisomes, in the 5 and 50 mg/kg groups was more than 4 times the level in the control group. The mean CAT level (per wet tissue weight and per protein), a marker for peroxisome and mitochondria, in the 5 and 50 mg/kg groups was more than 10 times the level in the control group. The mean CPT level (per wet tissue weight and per protein), a marker for mitochondria, in the 50 mg/kg was about twice the control level. Significantly higher FAOS (per wet tissue weight and

per protein) and CAT (per wet tissue weight and per protein) were observed in the 50 mg/kg group (Table 2). The magnitude of stimulation induced by APFO on CPT was low. APFO stimulated ß-oxidation in the liver. It was more pronounced in peroxisome activity than in mitochondria.

Significant elevation in glucose level was observed in the 5 and 50 mg/kg group, and a significant reduction in glucose level was observed in the 0.5 mg/kg group. The reduction in glucose level observed in the 0.5 mg/kg group was not considered to be related to treatment with APFO because of the magnitude, and no such change was observed in either the 5 or 50 mg/kg groups (Figure 1). ß-oxidizing activity in the liver rose significantly, but no change was observed in the blood level of total cholesterol, HDL-cholesterol, LDL-cholesterol, phospholipids, or triglycerides. Blood glucose level showed a significant elevation.

A significant reduction or a tendency for reduction in red blood cell count, hemoglobin concentration, and hematocrit was seen in most APFO dose groups. Anemia was also revealed by the hematological examination. However, anemia observed was not rated as severe. In addition, hematopoiesis was sustained, because the reticulocyte ratio did not decrease. No anemic symptom such as discoloration of eyes or pale skin was observed in the clinical observation. White blood cell count tended to rise after APFO treatment, whereas the percentages of lymphocytes and monocytes remained unchanged (Table 3). There was no change in the ratio or absolute number (cellularity multiplied by the ratio) for each positive cell within the lymphocyte subsets. APFO did not affect the numbers of T cells, B cells, NK cells, helper T cells, or killer T cells (Tables 4a, b).

Discussion

Treatment of rats with APFO resulted in liver weight gain and stimulation of

ß-oxidation, in agreement with the previous reports (Biegel et al 2001). Also, in a previous study of mice, treatment with PFOA resulted in liver weight gain and elevation of fatty acid oxidizing activity (Yang et al 2001). In another study, treatment of mice with PFOA resulted in decreases in thymus and spleen weights (Yang et al 2002), but no such change was observed in the current study of rats. Regarding the decrease in thymus and spleen weights of mice after treatment with PFOA, Yang et al. additionally reported that direct exposure of splenocytes and thymocytes to PFOA in vitro exerted no effect (Yang et al 2000). They thus conclude that suppression of immunocompetent cells of mice after PFOA treatment is a secondary effect of the chemical. In fact, PPARα was found in mouse lymphocytes, and ligands to PPARα induced transrepression of NF-κB, while the expression of several PPARα-regulated genes (involved in lipid oxidation, etc., in the liver) was not confirmed in this study (Jones et al 2002).

In rats, treatment with APFO elevated ß-oxidation but did not affect any parameter of blood lipids. This suggests that lipid supply may be greater in rats than in mice. The phenomenon of peroxisome proliferation in the liver varies greatly among different species. This phenomenon is not as intense in guinea pigs, cats, dogs, and primates (including humans) as in rodents (Cattley et al 1998, Kurata et al 1998, Pugh et al 2000). Suppression of immunocompetent cells, which is thought to be secondary to PPARα ligand-induced increase of fat metabolism in the liver, seems to be specific to rodents.

Among rodents, rats showed little change in blood lipids. This suggests that the secondary effect of the chemical on immunocompetent cells was absent in rats. It appears possible that the effect of PPARα ligands (e.g., APFO) in suppressing activity of immunocompetent cells is specific to mice.

Summary

APFO was repeatedly administered orally to male Crj:CD(SD)IGS rats for 14 days. Doses of APFO were 0, 0.5, 5, and 50 mg/kg. Significant increases and increasing tendencies in absolute/relative weight of the liver and no change in weight of the spleen were observed in all groups. Although inductions of mitochondrion- and peroxisome-specific enzymes were increased, no decrease was seen in any hematological parameter of lipid metabolism. Red blood cell count, hemoglobin concentration, and hematocrit or these tendencies showed a significant decrease or a tendency to decrease, but no influence on lymphocyte subsets was noted. Secondary inhibition of immunocompetent cells, previously reported for mice, was not seen in this study of rats.

Tables

Table 1. The effects of APFO on body, liver and spleen weights in rats.

APFO Dose (mg/kg/day)	Final body weight (g) Day 15	Liver weight (g) Day15	Relative liver weight (g/100 g body weight) Day15	Spleen weight (g) Day15	Relative spleen weight (g/100 g body weight) Day15
0	270±17	8.43±0.27	3.13±0.10	0.74±0.14	0.28±0.05
0.5	263±16	7.72±0.18	2.94±0.11	0.62±0.07	0.24±0.03
5	257±9	12.39±0.18	4.82±0.13	0.72±0.07	0.28±0.03
50	233±9	14.72±1.75	6.32±0.62*	0.56±0.10*	0.24±0.03

All values are means ± S.D. for four animals. *p <0.05; **p<0.01 compared with the control group.

Table 2. Peroxisomal enzyme activity.

APFO Dose (mg/kg/day)	FAOS (U/g ·wet tissue)	CAT (U/g ·wet tissue)	CPT (U/g ·wet tissue)	FAOS (U/g ·protein)	CAT (U/g ·protein)	CPT (U/g ·protein)	Protein (g/g ·wet tissue)
0	0.490±0.079	0.42±0.05	2.68±0.59	3.13±0.25	2.7±0.5	17.19±3.45	0.156±0.020
0.5	0.438±0.031	0.49±0.11	2.50±0.26	3.02±0.21	3.4±0.7	17.32±2.35	0.145±0.013
5	2.056±0.059	4.65±0.66	4.16±2.03	14.02±0.69	31.7±4.6	28.41±14.16	0.147±0.003
50	3.288±0.275*	8.33±1.85**	5.23±2.11	21.89±2.09*	55.3±11.8**	34.74±13.89	0.151±0.007

All values are means ± S.D. for four animals. *p <0.05; **p<0.01 compared with the control group.

Table 3. The effects of APFO on hematology in rats.

APFO Dose (mg/kg/day)	Red blood cell count (x 10^6/ml)	Hemoglobin conc. (g/dL)	Hematocrit (%)	Reticulocyte ratio (%)	White blood cell count (x 10^3/ml)	Lymphcyte (%)	Monocyte (%)
0	7.44±0.16	14.8±0.3	45.1±1.2	5.6±1.2	7.72±0.55	86.1±2.0	2.1±0.4
0.5	7.02±0.32	13.9±0.7	42.7±1.3	5.6±0.9	8.87±1.77	81.2±1.6	1.8±0.2
5	6.83±0.12	14.0±0.4	43.1±1.0	5.9±0.6	12.71±2.66*	82.6±4.7	2.5±1.0
50	6.65±0.21*	13.2±0.5**	40.7±1.9*	6.0±1.3	10.15±2.76	89.1±2.9	1.5±0.3

All values are means ± S.D. for four animals. *p <0.05; **p<0.01 compared with the control group.

Table 4a. Lymphocytes subset in blood.

APFO Dose (mg/kg/day)	$CD3^+$ (%)	$CD45RA^+$ (%)	$NKR\text{-}P1A^+$ (%)	$CD4^+$ (%)	$CD8a^+$ (%)
0	50.0±3.9	36.1±4.1	3.36±0.44	35.5±3.4	13.1±2.3
0.5	52.1±5.8	31.4±5.8	4.07±1.61	38.4±6.2	11.2±2.6
5	48.4±4.1	28.3±3.7	4.24±1.01	34.3±3.3	12.8±3.3
50	53.7±1.3	32.6±1.7	3.17±0.31	37.0±2.4	13.2±2.7

All values are means ± S.D. for four animals. *p <0.05; **p<0.01 compared with the control group.

Table 4b. Number of lymphocytes staining with a panel of monoclonal antibodies.

APFO Dose (mg/kg/day)	$CD3^+$ (x 10^6 cells/ml)	$CD45RA^+$ (x 10^6 cells/ml)	$NKR\text{-}P1A^+$ (x 10^6 cells/ml)	$CD4^+$ (x 10^6 cells/ml)	$CD8a^+$ (x 10^6 cells/ml)
0	0.65±0.18	0.48±0.18	0.05±0.01	0.46±0.13	0.17±0.05
0.5	0.91±0.25	0.56±0.22	0.07±0.03	0.68±0.22	0.19±0.02
5	0.90±0.25	0.53±0.17	0.08±0.01	0.65±0.22	0.23±0.07
50	1.14±0.21	0.69±0.15	0.07±0.01	0.79±0.18	0.28±0.07

All values are means ± S.D. for four animals. *p <0.05; **p<0.01 compared with the control group.

Figure

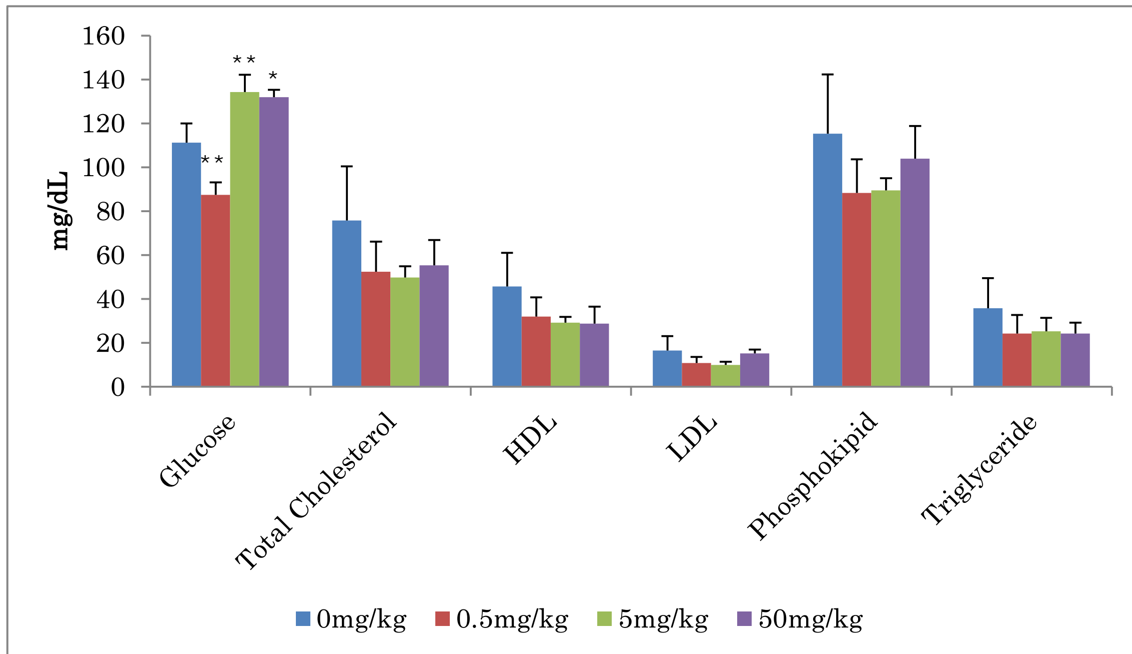

Figure 1

Effects of APFO on blood chemistry. Four rats per group were treated for 14 days. Glucose, total cholesterol, HDL cholesterol (HDL), LDL cholesterol (LDL), phospholipids and triglycerides were measured using the methods shown in the Methods and Material section. Their values are expressed in mean ± S.D. * $p < 0.05$, ** $p < 0.01$ compared to the corresponding control value. Significantly higher glucose was observed in the 5 and 50 mg/kg groups.

Chapter 2
Toxicokinetics of ammonium perfluorohexanoate (APFHx) in rats and mice

Introduction

Ammonium perfluorooctanoate (APFO) has long been used as a polymerization aid to manufacture fluorocarbon resin and fluorocarbon rubber. During its use, it is rapidly transformed to perfluorooctanoic acid (PFOA). PFOA can also be produced as a by-product of the production of certain PFOA-related compounds (particularly those now being phased out of commercial production in the United States and Europe, with a carbon chain of eight carbon atoms), which have been used as water and oil repellents.

PFOA has been reported to persist in the environment (Kennedy et al 2004, Lau et al 2007, Prevedouros et al 2006). In response to this property of the compound, industrial companies have been working to replace PFOA and PFOA-related compounds with alternatives. In particular, industrial companies have investigated the manufacturing and use properties of perfluorocarboxylate (PFC) with a carbon chain length of six carbon atoms and related compounds. Because of the acceptable performance characteristics of these substances, industrial companies are also studying the health and environmental profile of these promising alternatives.

Kudo et al. (Kudo et al 2006) compared PFCs of different carbon chain lengths, reporting that those with seven carbon atoms or fewer were pharmacologically cleared from mice more quickly than carboxylates of C8 and greater lengths. Chengelis et al. (Chengelis et al 2009b) and Loveless et al. (Loveless et al 2009) examined the toxicity of perfluorohexanoic acid (PFHxA, C6) and concluded that the toxicity was low, compared to C8.

Developmental toxicity of PFOA in the rat showed that small postnatal weight gain deficits, slight delays of sexual maturation, and postweaning mortality (likely related to immaturity) were noted only in the F1 generation animals of the highest dose group (30 mg/kg). While results indicated that PFOA was less developmentally toxic in rat, the developmental toxicity of PFOA in the CD-1 mouse were reported significant alterations of postnatal growth and

development were seen at even lower doses (1 and 3 mg/kg). This was probably due to the bioaccumulation of PFOA in the female rat with a half-life estimate of 3-4 h compared to that of 6-8 days in the males. In contrast, no significant differences were seen between serum PFOA concentrations of male and female mice. A different profile of developmental toxicity for PFOA in the mouse from in the rat are considered most likely related to the pharmacokinetic differences between these two rodent species.

The aim of this study was to examine the bioaccumulation potential of APFHx in the both sexes and species.

Materials and methods

Test guidelines

Environmental Protection Agency (EPA), Office of Prevention, Pesticides, and Toxic Substances (OPPTS) Health Effects Test Guidelines, 850.7485 Metabolism and Pharmacokinetics were the test guidelines used for this study.

Study design

This study was designed as a single-dose study, followed by a multiple-dose study. The multiple-dose study was conducted in two phases: phase 1, in which the elimination of the test compound was observed, and phase 2, in which the organ distribution was observed. This study design complies with the guidelines of the United States EPA.

Dose-setting should be determined by a low dose at which no toxic effects are observed, but which is high enough to allow identification of metabolites in excreta. Chengelis et al. reported that the no-observed-adverse-effect level (NOAEL) of PFHxA was 50 mg/kg/day in the subchronic study (Chengelis et al 2009b). The author adopted 50 mg/kg/day for the dose

on account of this.

Test material

Test materials were labeled and unlabeled APFHx. Unlabeled APFHx was a 50% aqueous solution received from Daikin Industries, Ltd. (Osaka, Japan). Carbon 14 (^{14}C)-labeled APFHx was supplied by GE Healthcare, Ltd., (Waukesha, WI) and stored at −20°C in the dark. The radiolabeled material was supplied as a nominal 100% APFHx powder (purity, 99.6%; high-performance liquid chromatography analysis) with a stated specific activity of 6.59 MBq/mg. The terminal carbon, which binds two oxygen atoms at the end of the carbon chain, was labeled.

Animals

All cage sizes and housing conditions were in compliance with the Guide for the Care and Use of Laboratory Animals. Male and female Sprague-Dawley (Crl:CD[SD]) rats and CD-1 mice were supplied by Charles River Ltd. (Margate, UK) Ltd. All animals were 7‐10 weeks of age at the time of dosing.

Single oral administration

Compound and dose administration

The formulations were administered by gastric gavage at a target volume of 10 mL/kg body weight to achieve a target concentration of 50 mg/kg body weight (target radioactive dose: 3-5 MBq/kg body weight). Two male and 2 female rats, and 2 male and 2 female mice, received a single oral administration of [^{14}C]-APFHx.

Urine and feces samples from each animal were collected into containers cooled

with solid carbon dioxide for the following periods: 0-6 hours after dosing (urine only), 6-24 hours after dosing, and then at 24-hour intervals until 72 hours after dosing. Cages were washed with water at the time of each feces collection, and the water was saved as sample. Expired air was collected over 0-24 and 24-48 hours after dosing. The expired air was collected into an absorbing solution of ethanolamine:ethanediol (3:7; v/v).

At the end of the 72-hour collection period, each animal was humanely killed by CO_2 asphyxiation. The gastrointestinal tract and residual carcass from each rat were weighed and retained as samples.

Levels of total radioactivity were determined in each sample collected.

Multiple oral administrations

Compound and dose administration

The formulations were administered by gastric gavage at a target volume of 10 mL/kg body weight to achieve a target concentration of 50 mg/kg body weight (target radioactive dose: 3-5 MBq/kg body weight).

APFHx was orally administered at a target daily dose of 50 mg/kg body weight for 13 consecutive days, followed by a single oral dose of [^{14}C]-APFHx (day 14). The dose was administered to 8 male and 8 female rats (4 animals each for the phase I and II studies) and 16 male and 17 female mice (4 animals each for the phase I study, and 12 animals each for the phase II study; 1 female mouse died as a result of gavage error).

Phase I

After administration of the last dose (day 14), urine samples were collected into containers cooled with solid carbon dioxide from each animal for the following periods: 0-6 hours

after dosing, 6-24 hours after dosing, and then at 24-hour intervals until 168 hours after dosing. Feces samples were collected into containers cooled with solid carbon dioxide at 24-hour intervals until 168 hours after dosing. Cages were washed with water at the time of each feces collection, and the water was saved as sample. At the end of the 168-hour collection period, each animal was humanely killed by CO_2 asphyxiation and body weight was measured. A terminal whole blood sample was collected into heparinized tubes (approximately 5-10 mL for rats and 0.5-1 mL for mice from the vena cava and heart, respectively).

Phase II

After administration of the last dose (day 14), serial blood samples were taken from all rats (approximately 0.4 mL). Samples were collected from the tail vein at 12 hours after dosing. A terminal whole blood sample was taken (approximately 5-10 mL) from the vena cava at 24 hours after dosing. All blood samples were collected into heparinized tubes. Plasma was separated from the whole blood samples. After taking the blood sample, each animal was humanely killed by CO_2 asphyxiation and body weight was measured.

Blood samples were taken from the hearts of mice (approximately 0.5-1 mL). Samples were taken from 4 male and 4 female mice at predosing and 12 and 24 hours after dosing. All blood samples were collected into heparinized tubes. Plasma was separated from all blood samples by centrifugation. Before sampling, each animal was humanely killed by CO_2 asphyxiation and body weight was measured.

The gastrointestinal tract, white fat, kidney, liver, spleen, and residual carcass were separately weighed and retained separately as samples.

Levels of total radioactivity were determined in each sample collected.

Preparation of samples for total radioactivity analysis

Liquid samples

Duplicate aliquots of liquid samples were made up to 1 mL with water (if necessary) and mixed with scintillation fluid. Duplicate aliquots of each blood sample were combusted using a PerkinElmer 307 Sample Oxidizer (PerkinElmer Life Science and Analytical Instruments Inc, Sears Green, UK).

Solid samples

Feces samples were weighed, an appropriate amount of water was added, and total weight was recorded before homogenization. Duplicate aliquots of fecal material from each sample (approximately 0.2‑0.3 g) were combusted using a PerkinElmer 307 Sample Oxidizer. Carcass samples were minced and then analyzed as previously described for feces. All gastrointestinal-tract and tissue samples were finely chopped using scissors and then analyzed as previously described for feces.

All aliquots were combusted using a PerkinElmer 307 Sample Oxidizer. The [^{14}C]-carbon dioxide generated was absorbed and mixed with scintillant before analysis by liquid scintillation counting. The efficiency of oxidation of the test samples was determined relative to [^{14}C]-standard oxidation efficiencies at regular intervals during each series of oxidations. Combustion of standards showed recovery efficiencies of >97%.

Quantification of radioactivity

All samples prepared in scintillation fluid were subjected to liquid scintillation counting for 5 minutes, together with representative blank samples, using a Packard TR 2100 Liquid Scintillation Analyzer with automatic quench correction by an external method. Where

possible, samples were analyzed in duplicate and allowed to heat- and light-stabilize before analysis. Before calculation of each result, a background count was determined and subtracted from each sample count. For scintillation counting, a limit of reliable determination of 30 disintegrations per minute (dpm) above background has been instituted in these laboratories. At the specific activity used, the limit of reliable measurement was approximately 0.06 µg equiv/g for a tissue and blood weight of approximately 0.1 g. The calculated limit of reliable measurement was 0.1 µg equiv/g for a mean plasma weight of approximately 0.05 g. Where results have been derived from data below the limit of reliable determination, this fact was noted.

Results

Single oral administration

Excretion of total radioactivity after a single oral administration of [^{14}C]-APFHx to male and female rats and mice are shown in Figure 2. Irrespective of sex or species, excretion of radioactive material was rapid, with mean recoveries of >90% of the dose at 24 hours after dosing. The major route of elimination was via the urine (means of recovery: 73.0-90.2% of the administered dose), followed by the feces (means of recovery: 7.0-15.5%). Elimination via expired air was negligible.

At 72 hours after dosing, mean recoveries of total radioactivity were 97.4 and 100.8% in male and female rats, respectively, with approximately 0.2% remaining in the gastrointestinal tract and carcass.

At 72 hours after dosing, mean recoveries of total radioactivity were 95.4 and 97.3% in male and female mice, respectively, with approximately 0.6-0.9% remaining in the gastrointestinal tract and carcass.

Multiple oral administrations (14 daily doses)

Irrespective of sex or species, in multiple oral administrations (13 daily doses) of APFHx followed by a single oral administration of [^{14}C]-APFHx, excretion of total radioactivity was rapid, with mean recoveries of >90% of the dose administered (mean values >95% of the ultimately recovered material) at 24 hours after dosing. The major route of elimination was via the urine (means of recovery: 77.8-83.4%), followed by the feces (means of recovery: 9.6-12.9%), indicating that the majority of the administered dose had been absorbed (Figure 3).

At 168 hours after dosing in rats and mice, radioactivity in most tissues was generally very low or below the detection limit. Tissue concentrations were below those of blood concentrations, with the exception of the liver, in which the concentration was approximately 4-8 times higher than that of the circulating blood level. Elevated levels of measurable radioactivity in the liver were consistent with its role in metabolism and excretion (Table 5).

At 12 hours after dosing, mean plasma concentrations were 0.8 and 0.4 mg/mL in male and female rats, respectively. At 24 hours after dosing, mean plasma values decreased to 0.5 and 0.3 mg/mL in male and female rats, respectively. At 12 hours after dosing, mean plasma concentrations were 1.3 and 1.0 mg/mL in male and female mice, respectively. At 24 hours after dosing, mean plasma values decreased to 1.0 and 0.5 mg/mL in male and female mice, respectively (Table 6).

Discussion

Our investigation on rats and mice showed that PFHxA administered as a single dose of APFHx was rapidly eliminated. Its body distribution in rats and mice was similar. Further, its elimination showed the same pattern in the repeated-dose study. This result is similar to findings reported by Chengelis et al., who examined the toxicokinetics of PFHxA in rats and monkeys and

found that it was rapidly eliminated in both species (Chengelis et al 2009b).

Our findings suggest that either the association of PFHxA with toxicokinetic mechanisms seen for PFOA and higher chain length PFCs is either very weak or that the mechanism is different altogether. Further investigations are warranted to determine which of these hypotheses is true.

In contrast to PFHxA, elimination rates of PFOA in rats were highly sex dependent: Female rats showed rapid elimination, whereas male rats did not (Kennedy et al 2004, Kudo et al 2001, Lau et al 2007, Ohmori et al 2003, Vanden Heuvel et al 1991).

Katakura et al. (Katakura et al 2007) reported that organic anion transport peptide is involved in excretion of PFOA from the kidney in rats. An organic anion-transporting polypeptide (Oatp) is a membrane transport protein or transporter that mediates the transport of mainly organic anions across the cell membrane and the family of organic anion transporters (Oat) are expressed in the renal epithelial cells to regulate the excretion and reabsorption of endogenous and exogenous organic anions. The Oatp1a1, which is one of the Oatp family, and Oat 3, which is one kind of Oat, might play a role as transporters of PFOA in the rat kidney, suggesting that these transporters reabsorb PFOA. They discussed the theory of Oat1a1 transport, noting that the gender-dependent distribution in rats may be explained by the fact that the distribution of Oatp1 in rat kidney is sex dependent, although that of Oat3 is not (Kato et al 2002). PFHxA does not show the same gender dependency in rats.

A study in rats comparing the elimination of PFCs with carbon chain lengths ranging from C7 to C10 showed that those with a longer carbon chain are eliminated more slowly (Ohmori et al 2003). Yang et al. (Yang et al 2009) reported that among PFCs with chain lengths between 6 and 10 carbon atoms, those with a longer carbon chain have higher affinities to Oatp1a1. A study of rat kidneys showed that Oatp1a1 levels were higher in male than in female

rats. It would fit into this framework that PFHxA, which has a C6 chain and, therefore, is shorter than those PFCs studied by Yang et al., may have an even lower affinity to Oatp1a1.

Weaver et al. (Weaver et al 2010) considered that Oatp1al plays a key role in the reabsorption of PFCs, particularly for C8, C9, and C10 PFCs. At the same time, it did not influence the transportation of C6. It is unclear whether this was attributable to the low affinity, as mentioned above, or simply to the low retention time, reducing the opportunity for transport and reabsorption.

In mice, elimination rates of PFOA are less likely to be dependent on sex (Lau et al 2006), but the distribution of Oatp1 in the kidney and liver is sex dependent (Cheng & Klaassen 2008). Therefore, the distribution of Oatp1 can probably not account for the pharmacokinetics of PFOA. It is likely that other transporters are involved. For perfluoroheptanoic acid (PFHpA, C7), Weaver et al. (Weaver et al 2010) indicated that there is no uptake by human embryonic kidney cells, which lack the Oat1 transporter. This may be because C7 is hydrophilic and, therefore, less likely to permeate cell membranes without a transporter.

With regard to PFCs in general, 1) the effect of change of the electric potential of short-chain substances is lessened (Kleszczynski & Skladanowski 2009) and 2) suppression of gap junctional intercellular communication also becomes weaker as the carbon chain becomes shorter (Upham et al 1998).

In PFHxA, which has a relatively short chain, the cell membrane is either not traversed because it is hydrophilic and lacks a transporter, or the ion is not taken up because it has no intracellular usefulness.

Further, the measured bioaccumulation of PFHxA is low. The value of the apparent inhibition constant [Ki(app)] is 1,857.8 in Chinese hamster ovary (CHO) cells (Yang et al 2009). Since Kudo et al. reported that β-oxidation activity in the liver of rats and mice is not in proportion

to the carbon chain length of PFCs, but to its concentration in the liver (Kudo & Kawashima 2003, Kudo et al 2006), and because the bioaccumulation of PFHxA is low, its toxic effects, such as β-oxidation activity, can be considered low.

Although it seems likely that Oat1 plays no part in the toxicokinetic mechanisms of PFHxA, it is probably important to clarify whether the mechanisms of PFHxA are replicas of other, secondary mechanisms of PFOA and higher chain PFCs or whether the mechanisms are entirely different.

Summary

Excretion patterns and rates of APFHx after administration of a single and multiple (14 days) oral dose(s) at 50 mg/kg to male and female mice and rats were examined. The test substance was [^{14}C]-labeled APFHx. After a single oral administration, total excretion was rapid, with mean recoveries of over 90% of the dose at 24 hours after administration, irrespective of gender or species. The major route of elimination was via the urine (means of percentage recovery between 73.0 and 90.2% of the dose), followed by the feces (means of percentage recovery between 7.0 and 15.5% of the dose). Elimination via expired air was negligible. For the multiple dose tests, multiple (13 daily doses) oral administration of APFHx was followed by a single oral administration of [^{14}C]-APFHx. Excretion was rapid, with mean recoveries of over 90% of the administered dose (mean values >95% of the ultimately recovered material) at 24 hours after dosing, irrespective of gender or species. The major route of elimination was via the urine (means of percentage recovery between 77.8 and 83.4% of the dose), followed by the feces (means of percentage recovery between 9.6 and 12.9% of the dose).

Tables

Table 5. Concentration of total radioactivity in tissues at 168 hours after dosing.

Sample	Male rats	Female rats	Male mice	Female mice
White fat	0.03±0.01	0.03±0.00	0.03±0.03	0.04±0.02
Kidneys	0.11±0.03	0.13±0.02	0.05±0.02	0.05±0.02
Liver	1.16±0.30	0.85±0.06	0.70±0.11	0.61±0.07
Spleen	0.03±0.01	0.04±0.00	0.02±0.05	0.02±0.02
Gastrointestinal tract	0.03±0.01	0.03±0.00	0.04±0.02	0.03±0.01
Carcass	0.10±0.03	0.10±0.05	0.06±0.02	0.04±0.02
Whole blood	0.15±0.03	0.16±0.02	0.17±0.03	0.17±0.02

All values are means ± SD for four animals.

Table 6. Plasma concentrations of total radioactivity following multiple oral administration.

Time point	Male rats	Female rats	Male mice	Female mice
12 hour	0.8 ± 0.1	0.4 ± 0.1	1.3 ± 0.7	1.0 ± 0.5
24 hour	0.5 ± 0.0	0.3 ± 0.1	1.0 ± 0.3	0.5 ± 0.1

All values are presented as mean ±standard deviation of 4 animals.

Figures

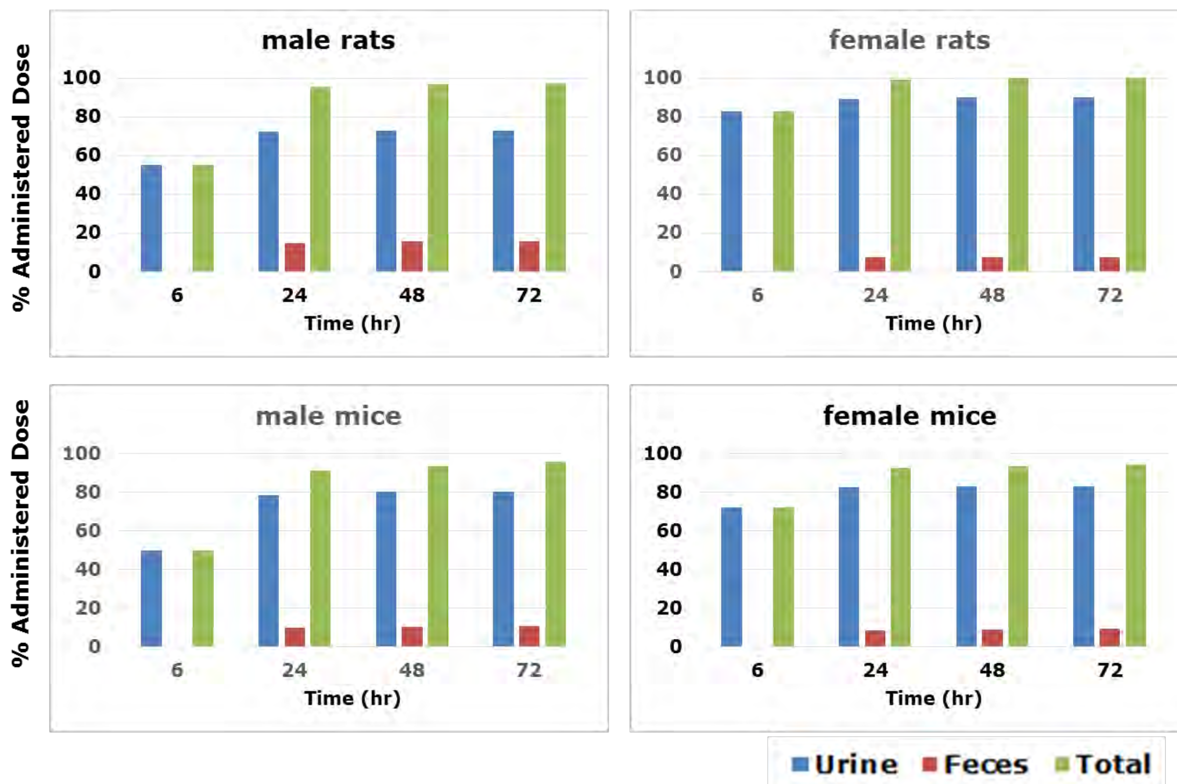

Figure 2

The excretion of total radioactivity following a single oral administration of [^{14}C]-APFHx to male and female rats are shown with mean cumulative results presented graphically in the upper row, and male and female mice are shown in the lower row. All values are means for two animals. Following a single oral administration, the major route of elimination was via the urine with means of 73.0 and 90.2% of the dose in males and female rats respectively. Faecal elimination accounted for 15.5% in males and 7.3% in females. Excretion of total radioactivity was with means of 95.6 and 99.2% recovered by 24 hours post dose.

Following a single oral administration, the major route of elimination was via the urine with means of 80.3 and 84.0% of the dose in males and female mice respectively. Fecal elimination accounted for 10.5% in males and 7.0% in females. Excretion of total radioactivity was with means of 90.9 and 94.1% recovered by 24 hours post dose.

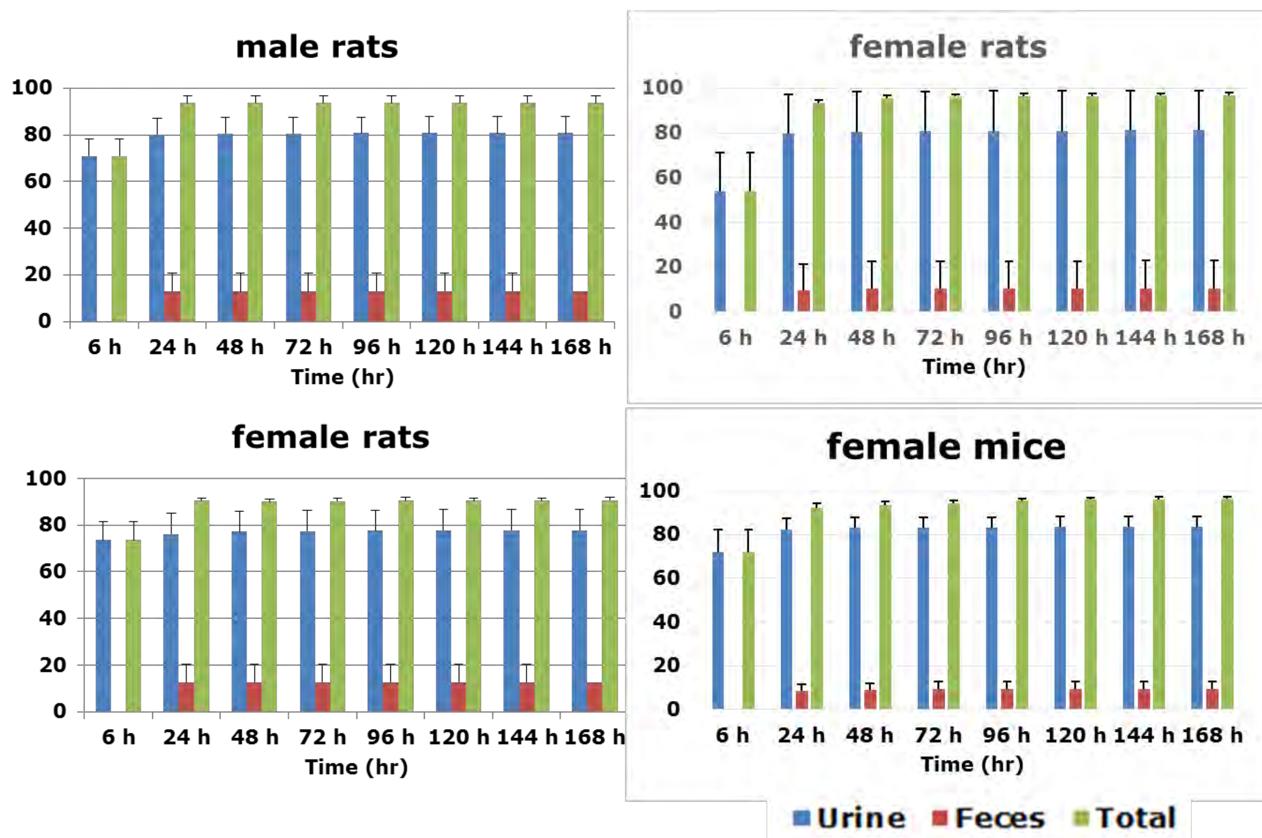

Figure 3

The excretion of total radioactivity following a multiple (13 daily doses) oral administration of APFHx followed by a single oral administration of [^{14}C]-APFHx to male and female rats are shown with mean cumulative results presented graphically in the upper row, and male and female mice are shown in the lower row.. All values are means ± SD for four animals.

Following the radiolabelled dose administration, the major route of elimination of radioactivity was via the urine with means of 80.7 and 77.8% of the dose in males and female rats respectively. Fecal elimination accounted for 12.9% in males and 12.6% in females.

Excretion of total radioactivity was rapid with means of 93.7 and 90.4% recovered by 24 hours post dose.

Chapter 3
Developmental and perinatal/postnatal reproduction toxicity study of ammonium perfluorohexanoate (APFHx) in mice

Introduction

PFHxA is a highly stable, ultimate degradant of the class of 'C6' surfactant chemicals which is currently being developed to be used as a durable water and oil repellent for textiles, carpets, nonwoven, and paper applications. In the environment, any activity of PFHxA will undoubtedly be due to the dissociated PFHxA ion. Since the PFHxA salt ,like the APFHx, readily dissociates in water to yield the PFHxA ion, it is a good vehicle to test the toxicity of 'C6' chemicals without introducing a confounding factor of acid toxicity at higher concentrations that are not environmentally relevant. Further, in some applications, the APFHx which is neutral may find application as an industrial surfactant. The developmental toxicity of perfluorobutyric acid (PFBA) and perfluorooctane sulfonic acid (PFOS) has been extensively studied (Das et al 2008, Lau et al 2003). The mouse has been used to evaluate the developmental toxicity of PFOA. Unlike the rat, sex differences in exposure levels in the mouse do not appear to exist (Lau et al 2006). For this reason, the mouse appears to be an appropriate and sensitive model for evaluation of PFHxA. Developmental toxicity observed in rodents showed a lack of teratological findings (structural anomalies) from PFHxA. Effects on pup survival and postnatal growth have been consistently observed in both rats and mice in a dose-dependent pattern (Lau et al 2007). The general toxicity and toxicokinetics of PFHxA were investigated in a 90-day repeat-dose toxicity and metabolism studies in rats (Chengelis et al 2009a, Chengelis et al 2009b). The toxicity and toxicokinetics of other PFHxA, including sodium perfluorohexanoate (NaPFHx) and APFHx, have also been studied (Iwai 2011, Loveless et al 2009). The purpose of this research, conducted in 2 phases, was to test for toxic effects resulting from APFHx treatment of Crl: CD1(ICR) pregnant female mice and evaluate the development of the embryo and fetus consequent to exposure of the dam from implantation to closure of the hard palate and during lactation. Since PFHxA has high acidity, APFHx was used to avoid this. This study was designed to evaluate the International Conference

on Harmonisation Harmonised Tripartite Guideline stages C through F of the reproductive process and detect effects on gestation, parturition, lactation, and maternal behavior in female mice and on the development of the offspring of the treated female mice. Because manifestations of effects induced during this period may be delayed in the offspring, observations were continued through sexual maturity of the F1 generation mice.

The data presented here are from research conducted in 2 phases. The first phase used doses of the test substance that did not allow determination of a clear no observable adverse effect level (NOAEL). The second phase was conducted at lower doses to determine a clear NOAEL.

Materials and methods

Study design

These studies were conducted in compliance with the Good Laboratory Practice (GLP) regulations of the US Environmental Protection Agency, the Japanese Ministry of Agriculture, Forestry and Fisheries, and the Organization for Economic Cooperation and Development (OECD). The pharmacokinetic analysis and analytical portion of the study were conducted in accordance with the appropriate Food and Drug Administration and OECD Principles of GLP (ENV/MC/CHEM(98)17).

Animals and husbandry

The methodology was comparable for each phase with the exception of the dosage levels. Eighty Crl:CD1(ICR) (Charles River Laboratories, Inc., St. Constant Canada or Kingston New York) mice were used in each phase. Mice were approximately 61 to 63 days of age and weighed 24 to 33 grams at arrival at the Testing Facility. They were bred with male

mice of the same strain and source that were maintained as a breeding colony at the Testing Facility. Female F0 generation mice were given temporary numbers at receipt and given permanent identification numbers when assigned to the study on the basis of body weights obtained on the first day of gestation (Day 0). Female mice were permanently identified using a tail tattoo. Pups were not individually identified during the lactation period; all parameters were evaluated in terms of the litter. At weaning, F1 generation mice were identified by tail tattoo.

All husbandry, cage sizes and housing conditions were in compliance with the Guide for the Care and Use of Laboratory Animals. The study rooms were maintained under conditions of positive airflow relative to a hallway and independently supplied with a minimum of ten changes per hour of 100% fresh air that had been passed through 99.97% HEPA filters. Room temperature and humidity were monitored constantly throughout the studies. Room temperature was targeted at 64°F to 79°F (18°C to 26°C); relative humidity was targeted at 30% to 70%.

F0 generation mice were individually housed in stainless steel, wire-bottomed cages, except during the cohabitation and postpartum periods. During cohabitation, each pair of male and female mice was housed in the male mouse's cage. Each dam and delivered litter were housed in a common nesting box during the postpartum period. After weaning (PPD 20), F1 generation mice were housed in nesting boxes. Mice were pair housed until at least PPD27, after which they were individually housed. An automatically controlled 12-hours light:12-hours dark fluorescent light cycle was maintained. Each dark period began at 19:00 hours (± 30 minutes).

Cages were changed approximately every other week. Bedding was changed as often as necessary to keep the mice dry and clean. Mice were given ad libitum access to Certified Rodent Diet® #5002 (PMI® Nutrition International, Inc., St. Louis, MO, USA) in individual feeders.

Local water that had been processed by passage through a reverse osmosis

membrane (R.O. water) was available to the mice ad libitum from individual water bottles attached to the cages. Chlorine was added to the processed water as a bacteriostat. Bed-o'cobs bedding (The Andersons Industrial Products Group, Maumee, OH, USA) was used as the nesting material. Each lot was certified for contaminates by the Testing Facility.

Test substance

APFHx, a granular white solid, is also known as ammonium salt of perfluorohexanoic acid. The test substance was supplied as a 50% aqueous solution in R.O. deionized water. The aqueous solution was a colorless liquid. Purity was 93.4%. Solutions of the test substance were prepared once weekly at the Testing Facility and stirred continuously for at least 24 hours prior to dosage administration and stored at room temperature. The vehicle (R.O. water) was available from a continuous source at the Testing Facility and maintained at room temperature.

The study samples analyzed were within the acceptance criteria of ±10% of their mean nominal concentrations. For homogeneity, the relative standard deviation (RSD) for the formulation for the grand mean of the average value for the top, middle and bottom formulations for each group was ± 5%. Homogeneity results showed that the formulation technique used produced homogenous preparations. Stability was demonstrated in a separate study for 10 days at room temperature from 7 mg/mL to 70 mg/mL.

Mating and treatment

After acclimation of approximately one week, 100 virgin female mice were cohabitated with 100 breeder male mice, one male mouse per female mouse. The cohabitation period consisted of a maximum of 5 days. Female mice with a copulatory plug observed in situ

were considered to be at day 0 of gestation (DG 0) and assigned to individual housing. F0 generation female mice were administered the test substance and/or vehicle once daily via oral gavage from DG 6 through DG 18. Dosages were adjusted daily for body weight changes and given at approximately the same time each day. Dams in the process of delivering pups were not adminstered the test substance or vehicle in order to preclude possible disruption to maternal behavior and/or cannibalization of the pups. F1 generation pups were not directly administered the test substance and/or vehicle, but may have been possibly exposed to the test substance and/or vehicle during maternal gestation (in utero exposure) or via maternal milk during the lactation period. The treatment regimen for each of the two phases is summarized in Table 7.

F0 generation mice were observed for viability at least twice each day of the study and for clinical observations and general appearance once weekly during acclimation and on DG 0. The mice were also examined for clinical observations, abortions, premature deliveries and deaths prior to dosage administration and between one and two hours after dosage administration and once daily during the postdosage period. Body weights were recorded once weekly during the acclimation period, on DG 0, and daily during the dosage and postdosage periods.

F0 generation mice were evaluated for adverse clinical signs observed during parturition, duration of gestation, litter sizes (all pups delivered) and pup viability at birth, fertility index (percentage of matings that result in pregnancies), gestation index (percentage of pregnancies that result in birth of live litters), number of offspring per litter (live and dead pups), number of implantation sites, general condition of dam and litter during the postpartum period, viability indices (percentage of pups born that survive 4 and 7 days) and lactation index (percentage of pups born that survive 20 days). Maternal behavior was evaluated on lactation days (DLs) 0, 4, 7, 14 and 20.

Day 0 of lactation (postpartum) was defined as the day of birth and was also the first

day on which all pups in a litter were individually weighed (pup body weights were recorded after all pups in a litter were delivered and groomed by the dam). Each litter was evaluated for viability and general appearance at least twice daily. The pups in each litter were counted once daily. Clinical observations were recorded once daily during the preweaning period. Pup body weights were recorded on PPD 0 (birth), 4, 7, 14 and 20. During the preweaning period, pups were evaluated for eye opening beginning PPD 10.

F1 generation mice postweaning were observed for viability daily. These mice were also examined for clinical observations and general appearance once daily. Body weights were recorded weekly. Female mice were evaluated for the age of vaginal patency, beginning on PPD 20. Male mice were evaluated for the age of preputial separation, beginning on PPD 26.

Gross necropsy

All mice were necropsied and gross lesions were retained in neutral buffered 10% formalin for possible future evaluation. Unless specifically cited below, all other tissues were discarded. F0 generation mice were sacrificed by carbon dioxide asphyxiation. Pups were sacrificed by an intraperitoneal injection of sodium pentobarbital (pups ≤14 days of age) or by carbon dioxide asphyxiation (pups ≥15 days of age).

At weaning of the F1 generation litters, five livers per group from the F0 generation mice were excised, weighed and frozen on dry ice. Livers were maintained frozen (≤ -70 ^{o}C) for analysis. Mice that did not deliver a litter were sacrificed on DG 23 and examined for gross lesions. The number and distribution of implantation sites were recorded after staining with 10% ammonium sulfide to confirm the absence of implantation sites.

Dams with no surviving pups were sacrificed after the last pup was found dead or missing, presumed cannibalized. A gross necropsy of the thoracic, abdominal and pelvic viscera

was performed and implantation sites were recorded after staining with 10% ammonium sulfide.

Mice that died before scheduled termination were examined for the cause of death as soon as possible after the observation was made. The mice were examined for gross lesions. The lungs, trachea and esophagus were perfused and saved in neutral buffered 10% formalin for possible future evaluation. The heart, kidneys, stomach and spleen were retained in neutral buffered 10% formalin for possible histological evaluation. Gravid uterine weights were recorded. Pregnancy status and uterine contents of female mice were recorded. Conceptuses in utero were examined to the extent possible, using the same methods described for term fetuses/pups. The livers were excised, weighed and frozen on dry ice. Livers were maintained frozen ($\leq$ -70 ^{o}C) until shipped for analysis to Charles River Preclinical Services Montreal (PCS-MTL).

Pups that died before initial examination of the litter for pup viability were evaluated for vital status at birth. The lungs were removed and immersed in water. Pups with lungs that sank were considered stillborn; pups with lungs that floated were considered liveborn and to have died shortly after birth.

Pups found dead were examined for gross lesions and for the cause of death as soon as possible. As any histopathology would conducted on the whole pup dead on PPD 1 to 3, the bone tissue needs to be de-calcified. As Bouin solution is used to both fix tissue and de-calcify the bone, all pups were preserved in the solution for possible future evaluation. The PPD 4 and older pups will be dissected (organs removed) for processing. In the reason, all pups found dead on PPD 4 to 20 were preserved in neutral buffered 10% formalin.

On PPD 20, all pups not selected for continued evaluation were sacrificed by carbon dioxide asphyxiation and examined for gross lesions. Necropsy of the pups included a single cut at the suture of the frontal and parietal bones of the skull, and the cross-sectioned brain was

examined for hydrocephaly.

One male mouse that died before scheduled termination was examined for the cause of death as soon as possible after the observation was made. The mouse was examined for gross lesions. The heart, kidneys, lungs, stomach and spleen were retained in neutral buffered 10% formalin for possible histological evaluation. The liver was excised, weighed and frozen on dry ice.

Five F1 generation mice per sex per group (total 40 mice) in each phase were sacrificed on PPD 41 for sample collection for body burden determination. Blood samples (0.5 mL to 1.0 mL) and livers were collected from these mice. Blood samples were collected via the vena cava after sacrifice. The blood samples were transferred into uncoated (red top) tubes and spun in a refrigerated (4 ^{o}C) centrifuge for 10 minutes at 3500 RPM. The resulting serum was transferred into appropriately labeled polypropylene tubes. All samples were frozen on dry ice as soon as possible and maintained frozen ($\leq$ -70 ^{o}C) for analysis. The remaining mice were sacrificed by carbon dioxide asphyxiation on PPD 41. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed.

Statistical Analyses

Statistical analysis, including regression analysis, and descriptive statistics such as arithmetic means and standard deviations, accuracy and precision were performed using Watson laboratory Information Management system (LIMS) and Microsoft Excel. Litter values were used where appropriate. Clinical observations and other proportional data were analyzed using the variance test for homogeneity of the binomial distribution.

Continuous data, such as body weights, organ weights, percentage of litter reaching a developmental landmark and percent mortality per litter were analyzed as described under the

parametric heading of the schematic. Bartlett's test of homogeneity of variances was used to estimate the probability that the dosage groups had different variances. A non-significant result ($p>0.001$) indicated that an assumption of homogeneity of variance was not inappropriate, and the data were compared using the analysis of variance. If that test was significant ($p\leq0.05$), the groups given the test substance were compared with the control group using Dunnett test. If Bartlett test was significant ($p>0.001$), the analysis of variance test was inappropriate, and the data were analyzed as described under the nonparametric heading of the schematic. When 75% or fewer of the scores were tied, the Kruskal-Wallis Test was used to analyze the data, and in the event of a significant result ($p\leq0.05$), Dunn's method of multiple comparisons was used to compare the groups given the test substance with the control group. When more than 75% of the scores were tied, Fisher exact test was used to compare the proportion of ties in the dosage group.

Variables with graded count scores, such as litter size were analyzed using the nonparametric procedures described above. The pup weights for all weigh days were covaried against litter size on at birth.

Results

Mortality, clinical and necropsy observations

Phase 1

A total of 3, 6, 1 and 3 F0 generation female mice were found dead in the 0, 100, 350 and 500 mg/kg/day dosage groups, respectively (Table 8). Single deaths that occurred in the mid dosage and high dosage groups during the gestation period appear to have been related to administration of the test substance based on the timing of the deaths (DGs 8 and 13). No other mortality related to APFHx occurred. The deaths in the control and low dosage group all occurred

between days 13 and 16 of lactation (DLs 13 to 16). These deaths, which was within the historical range for this Testing Facility, and possibly two of the three deaths that occurred in the highest dosage group appeared to be due to the stress of nursing which is known to occur in mice (Dagnaes-Hansen et al 2010, Feinstein et al 2008, Krugner-Higby et al 2006, Kunstyr 1986). Additionally, a total of 1, 0, 2 and 6 mice in the 0, 100, 350 and 500 mg/kg/day dosage groups, respectively, were sacrificed due to no surviving pups.

Phase 2

No mortality related to APFHx occurred. All mice survived until scheduled killing, with the exception of 1 mouse in the 7 mg/kg/d dosage group that was killed on DG 17 when it delivered its litter; and 1 mouse in the 35 mg/kg/d dosage group that was killed on PPD 2 due to no surviving pups.

All clinical observations during the gestation and lactation periods were considered unrelated to the test substance because: (1) the incidences were not dosage dependent and (2) the observations occurred in only 1 mouse in a group. These clinical observations included a red perivaginal substance and urine-stained abdominal fur.

There were no test substance-related necropsy observations. All necropsy observations were considered unrelated to the test substance because (1) the incidences were not dosage dependent or (2) the observations occurred in only 1 mouse. These necropsy observations included numerous clear cysts in the liver (one 7 mg/kg/d dosage group mouse), clear fluid-filled cyst in the capsule of the kidney (one 35 mg/kg/d dosage group mouse), thick walls of the uterus (one 7 mg/kg/d dosage group mouse), and clear fluid-filled cysts in the uterus (one 35 mg/kg/d dosage group mouse).

Body weights and body weight gains

Phase 1

Body weights and body weight gains during the gestation period and body weights during the lactation period were unaffected by dosages of the test substance as high as 500 mg/kg/day. All values were comparable among the 4 dosage groups and did not differ significantly (Figure 4).

Body weight gains during lactation were significantly reduced ($p \leq 0.05$ to $p \leq 0.01$) for DLs 0 to 4 in the 350 and 500 mg/kg/day dosage groups compared to the control group value. Although no additional significant differences occurred among the groups for body weight gain during lactation, the average gain during the entire lactation period was reduced in the 500 mg/kg/day dosage group compared to the control group value. Body weight gains from DLs 0 to 20 were 97.7%, 110.3% and 64.4% of the control group value.

Phase 2

Body weights and body weight gains during the gestation and lactation periods were unaffected by dosages of the test substance as high as 175 mg/kg/day. All values were comparable among the four dosage groups and did not differ significantly (Figure 5).

Pregnancy, fertility and litter observations

Phase 1

Pregnancy occurred in 19, 19, 20 and 18 of the 20 mated female mice in the 0 (Vehicle), 100, 350 and 500 mg/kg/day dosage groups, respectively (Table 9). All pregnant dams delivered litters, with the exception of one mouse in each of Groups III and IV that died during

gestation. All mated mice were pregnant and all that survived delivered a litter.

The number of pups dying on PPDs 1 to 4 in the 350 and 500 mg/kg/day dosage groups was significantly increased ($p \leq 0.01$) compared to the control group (Table 10). The number of pups dying on PPD 0 was increased in the 300 mg/kg/day dosage group and significantly increased ($p \leq 0.01$) in the 500 mg/kg/day dosage group compared to the control group.

The number of mice with stillborn pups was increased in the highest dosage group, and the number of mice with all pups dying on PPDs 0 to 3 was significantly increased ($p \leq 0.01$) in the 500 mg/kg/day dosage group compared to the control group values. The following additional effects occurred in the 500 mg/kg/day dosage group: the average litter size was reduced at birth and throughout the lactation period with a significant reduction ($p \leq 0.05$) on PPD 4; and the PPD 4 and 7 viability indices were significantly reduced ($p \leq 0.01$) compared to the control group value (Table 10). The day 7 viability index was significantly reduced ($p \leq 0.05$) in the 350 mg/kg/day dosage group. The average number of surviving pups per litter was significantly reduced ($p \leq 0.01$) in the 500 mg/kg/day dosage group for PPDs 4, 7, 14 and 20 compared to the control group values (Table 11).

Pup body weights in all treated groups were generally lower in the treated groups compared to the control group values. Pup body weights were significantly reduced ($p \leq 0.05$ to $p \leq 0.01$) on PPD 0 in the 100 mg/kg/day and higher dosage groups compared to the control group value (Table 11). The variance in litter size among the groups also had an effect on the pup body weights27. When pup body weights were covaried against the litter size on PPD 0, the statistically significant differences among the groups for pup body weights were no longer present. It is known that the background data of the laboratory that as the litter size increases the weight of the pup decreases. The litter size was largest in the 100 mg/kg/day dosage group and therefore

the reduced pup weight that occurred in this group was considered related to the litter size. The litter sizes in the 350 and 500 mg/kg/day dosage groups were affected by increased pup deaths that occurred in these dosage groups. Therefore, an effect on pup body weight was related to the test substance in these two dosage groups.

Statistically significantly reduced pup body weights persisted in the 350 mg/kg/day dosage group through PPD 7 and in the 500 mg/kg/day dosage group through PPD 4. On PPD 20 average pup weights per litter were 89.1%, 80.0% and 88.1% of the control group value. The lack of dosage-dependency can be attributed to the differences in litter size among the groups.

All other natural delivery and litter observations were unaffected by dosages of the test substance as high as 500 mg/kg/day. Values for the numbers of dams delivering litters, the duration of gestation, averages for implantation sites per delivered litter, the gestation index (number of dams with one or more liveborn pups/number of pregnant mice), total litter sizes, lactation index and percent male pups per number of pups sexed per litter, were comparable among the four dosage groups and did not significantly differ.

Phase 2

Pregnancy occurred in all of the 20 mated female mice in the 0 (Vehicle), 7, 35 and 175 mg/kg/day dosage groups, respectively. However, 20, 17, 20 and 20 mice respectively delivered litters (Table 12).

The number of stillborn pups and pups dying on day 1 postpartum were significantly increased ($p \leq 0.05$), and the average pup weight per litter was significantly reduced on day 1 postpartum in the 175 mg/kg/day dosage group compared to the 0 (Vehicle) mg/kg/day dosage group values (Tables 13 and 14).

All other natural delivery and litter observations were unaffected by dosages of the

test substance as high as 175 mg/kg/day (Table 14). Values for the numbers of dams delivering litters, the duration of gestation, averages for implantation sites per delivered litter, the gestation index (number of dams with one or more liveborn pups/number of pregnant mice), the numbers of dams with stillborn pups and of dams with all pups dying, litter sizes, viability index, surviving pups per litter, percent male pups per number of pups sexed per litter, live litter size at weighing and pup weight per litter were comparable among the four dosage groups and did not significantly differ (Table 14). A significant reduction ($p \leq 0.01$) in the lactation index in the 7 mg/kg/day dosage group was not considered related to the test substance because it was not dosage-dependent.

Terminal body weights

Phase 1

Terminal body weights were comparable among the four dosage groups (Table 15). The absolute weights of the liver and the ratio of the liver weight to the terminal body weight did not differ significantly among the groups.

Phase 2

Terminal body weights were comparable among the four dosage groups (Table 16). The absolute weights of the liver and the ratio of the liver weight to the terminal body weight did not differ significantly among the groups.

Necropsy observations

Phase 1

Tan areas in the liver occurred in one mouse in the 350 mg/kg/day dosage group and five ($p \leq 0.01$) of 20 mice in the 500 mg/kg/day dosage group.

There were no other test substance related necropsy observations. One mouse each in the 0 and 100 mg/kg/day dosage group had a bent sternum proximal to the xiphoid process and one mouse in the 500 mg/kg/day dosage group had intestines that were distended with gas.

Phase 2

There were no test substance related necropsy observations. Necropsy observations included numerous clear cysts in the liver (one 7 mg/kg/day dosage group mouse), clear fluid filled cyst in the capsule of the kidney (one 35 mg/kg/day dosage group mouse), thick walls of the uterus (one 7 mg/kg/day dosage group mouse) and clear fluid filled cysts in the uterus (one 35 mg/kg/day dosage group mouse).

Levels of PFHxA in liver homogenates

Phase 1

Results of analyses of liver homogenates from 10 F0 generation control group mice were all below the lower limit of quantitation with the exception of one mouse for which a detectable level was found. Based on the quantity found, (just above the lower limit of quantitaion) and the lack of any adverse clinical observations in this mouse, this result appeared to indicate cross contamination of the sample during collection and/or processing. This mouse was found dead on LD 17 and the liver was taken at approximately the same time as the livers from other early deaths in treated groups were taken.

All blood samples were below the limit of quantitation. In the 100 mg/kg/day dosage

group, all liver homogenates analyzed were below the lower limit of quantitation. In the 350 mg/kg/day dosage group, three of eight samples had analytical results that were quantifiable. The highest level (87.5 µg/mL) occurred in a mouse that was found dead on DG 13. The other mice had much lower levels of PFHxA, but it is interesting that both of these mice were sacrificed early after their litters had died off. These were the only mice in this group that lost their litters.

In the 500 mg/kg/day dosage group five of 16 samples had analytical results that were quantifiable. The highest level (98.4 µg/mL) occurred in a mouse that found dead on day 6 of gestation. The other mice had much lower levels of PFHxA but each of these mice had litters that died early. Two samples that were below the lower limit of quantitation were from mice that lost their litters.

Phase 2

All liver samples from F1 generation pups euthanized on postpartum day 42 were below the limit of quantitation.

F1 generation preweaning clinical observations

Phase 1

No clinical observations in the F1 generation pups were attributed to dosages of the test substance as high as 500 mg/kg/d because (1) the incidences were not dosage dependent; (2) the observation occurred in only 1 to 3 litters; and/or (3) the observation occurred only in the vehicle control group. These clinical observations included scab, dehydration, tip of tail red or missing, not nursing, not nesting, and ungroomed coat.

The average day that 50% of the pups had open eyes was significantly longer

($p \leq 0.01$) in the 350 mg/kg/day dosage group compared to the control group value (Table 17). The lack of a significant difference in the 500 mg/kg/day dosage group may be related to the higher number of early pups deaths and reduced litter size in this group. The percentage of pups per litter with open eyes was significantly reduced ($p \leq 0.05$ to $p \leq 0.01$) in the 350 and 500 mg/kg/day dosage groups on PPD 14 compared to the control group value.

An increased number of pups in the 500 mg/kg/day dosage group that were found dead or stillborn in the 500 mg/kg/day dosage group had no milk in the stomach. All pups that survived to PPD 20, and were not continued on study appeared normal at necropsy.

Phase 2

Two litters in the 175 mg/kg/day dosage group had a pup each with corneal opacity and one pup each with microphthalmia. One litter in this dosage group also had a pup with a lenticular opacity. No other clinical observations in the F1 generation pups were attributed to dosages of the test substance as high as 175 mg/kg/day.

No other clinical observations in the F1 generation pups were attributed to dosages of the test substance as high as 175 mg/kg/d because (1) the incidences were not dosage dependent; (2) the observation occurred in only 1 to 3 litters; and/or (3) the observation occurred only in the vehicle control group.

These clinical observations included dehydration cold to touch, tip of tail red, pale body, not nesting, a head laceration, scab on the lower midline or head and a mass on the back. The day of eye opening did not differ among the groups (Table 18).

No necropsy observations in the F1 generation pups were attributed to dosages of the test substance as high as 175 mg/kg/day. There was only a single finding (gas filled intestine) in a 7 mg/kg/day dosage group mouse for mice found dead and all other mice appeared normal for

those mice sacrificed on day 20 postpartum.

F1 generation postweaning

Phase 1

One male mouse in the 350 mg/kg/day maternal dosage group was found dead on PPD 23. There were no clinical signs noted during the postweaning period. This mouse had the lowest body weight in its group. At necropsy, all tissues appeared normal for a moderate degree of autolysis. This mouse apparently did not thrive postweaning. All other F1 generation male and female mice survived to scheduled sacrifice.

All clinical observations in the F1 generation male and female mice were considered unrelated to maternal administration of the test substance because (1) the incidences were not dosage dependent; (2) the observation occurred in only 1 mouse; and/or (3) the observation is common in this species and strain. These clinical observations were limited to common findings in the tail including constricted, bent, missing or purple.

All F1 generation male mice appeared normal at necropsy. One F1 generation female mouse in the 100 mg/kg/day dosage group had a small left kidney. No other necropsy observations occurred in these mice.

Phase 2

All F1 generation male and female mice survived to scheduled killing. All clinical observations in the F1 generation male and female mice were considered unrelated to maternal administration of the test substance because (1) the incidences were not dosage dependent; (2) the observation occurred in only 1 mouse; and/or (3) the observation is common in this species and strain. These clinical observations included lacrimation, ptosis, scab on the tail, and a lenticular

opacity.

Necropsy observations in the F1 generation female and male mice occurred in 1 mouse each in the 35 and 175mg/kg/d dosage groups, respectively. One male mouse had a clear fluid-filled cyst in the liver and 1 female mouse had a dark flat red mass in the mesentery.

F1 generation body weights and body weight gains

Phase 1

The only significant difference that occurred among the groups were significantly reduced ($p \leq 0.05$) body weights on PPD 21 in the 100 and 350 mg/kg/day dosage groups compared to the control group value. In conclusion, body weights and body weight gains of the F1 generation male and female mice were unaffected by maternal dosages of the test substance as high as 500 mg/kg/day (Figures 6 and 7).

Phase 2

Body weights and body weight gains of the F1 generation male and female mice were unaffected by maternal dosages of the test substance as high as 175 mg/kg/day (Figures 8 and 9). No significant differences occurred among the groups during the postweaning period (PPD 21 to 41).

F1 generation sexual maturity

Phase 1

Sexual maturation was unaffected by maternal dosages of the test substance as high as 500 mg/kg/day (Table 19). The average day on which preputial separation or vaginal patency occurred was comparable among the 4 dosage groups.

Phase 2

Sexual maturation was unaffected by maternal dosages of the test substance as high as 175 mg/kg/day (Table 20). The average day on which preputial separation or vaginal patency occurred was comparable among the four dosage groups.

F1 generation terminal body weights

Phase 1

Terminal body weights in the F1 generation male mice were comparable among the four groups (Table 21). The ratio of the liver weight to the terminal body weight was significantly reduced ($p \leq 0.05$) in the 500 mg/kg/day dosage group compared to the control group value. This was considered related to APFHx.

Terminal body weights in the F1 generation female mice were significantly reduced ($p \leq 0.05$) in the 350 mg/kg/day dosage group compared to the control group value. Maternal dosages of the test substance as high as 500 mg/kg/day did not affect the liver weights or the ratio of liver weights to the terminal body weight (Table 22).

Phase 2

Terminal body weights were comparable among the four groups (Table 23). Maternal dosages of the test substance as high as 175 mg/kg/day did not affect the liver weights or the ratio of liver weights to the terminal body weight (Table 24) for either sex. There were no significant differences among the groups.

Levels of PFHxA in blood and liver homogenates in F1 generation

No detectable level of PFHxA was found in the blood or liver homogenate from any F1 generation male or female pup.

Discussion

Over the two phases of study conduct, F0 generation female mice were administered PFHxA and/or vehicle once daily from DG 6 through DG 18 at dosages of 0, 7, 35, 100, 175, 350 or 500 mg/kg/day. Dosages as high as 100 mg/kg/day produced no maternal or developmental toxicity. Maternally toxic doses of 175 mg/kg/day and higher produced increased number of stillborn pups and pups dying day 1 along with a reduction in pup weights on PPD 1, 2 litters with pups with corneal opacity and delays in physical development.

The developmental toxicity of perfluoroalkyl acids (PFAAs) and their derivatives was reviewed by Lau in 2004 (Lau et al 2004)and again in 2007 (Abbott et al 2007). Lau noted that developmental toxicity from PFAAs exposure in rodents during gestation included embryo fetal death, teratological findings (malformations and variations), and reductions in fetal weight and these toxicities appeared only in the presence of maternal toxicity. Neonatal mortality as observed with PFAAs was also observed with perfluorooctanesulfonic acid (PFOS), again in a dose-dependent pattern. Cross-fostering of PFOS mouse pups did not improve survival(Lau et al 2004). The mouse offspring exposed to PFOS at doses as low as 15 mg/kg/day did not survive 24 hours.

The mechanism of action of PFAAs toxicity is still being explored with reports of activation of PPAR-α demonstrated with PFOA (Abbott et al 2007, Iwai & Yamashita 2006) and

PFOS(Shipley et al 2004, Takacs & Abbott 2007) and interference with the normal physiology function of pulmonary surfactants being two potential mechanisms (Xie et al 2007).

Results of this study support what has been generally observed for other PFAAs in that developmental toxicity has generally only been seen in the presence of maternal toxicity. Similar to other PFAAs the developmental toxicity observed has been related to pup survival and growth and not malformations, indicating that late gestational exposure to the test substance may be producing the toxicity observed. The dose of PFHxA that resulted in an effect on mouse pups was approximately 5 times the dose of PFOS that produced similar effects.

The PFHxA doses (175 and 350 mg/kg) in this study were chosen to approximate the area under the curves (AUCs) estimated to have resulted from doses of PFOA (5 and 10 mg/kg), at which severe developmental toxicity was previously indicated in CD-1 mice (Lau et al 2006). In studies of CD-1 mice, the developmental toxicity induced by exposure to PFOA throughout gestation included dose-related full litter resorptions, reduced postnatal survival, delayed eye opening, growth deficits, and sex-specific alterations in pubertal maturation (Lau et al 2006). Exposure to the C-4 chemical perfluorobutyric acid (PFBA) during pregnancy in the mouse did not recapitulate the profound adverse developmental effects previously reported with the C-8 chemicals PFOA and PFOS (neonatal morbidity and mortality, and postnatal growth deficits), although some subtler effects were still detectable. The milder responses of PFBA are likely accounted for by the rapid elimination of the chemical as well as its lower potency compared with the PFAAs with longer carbon chains. Similar to PFBA exposure, the absolute weights of the liver and the ratio of the liver weight to the terminal body weight did not differ significantly among all groups, and sexual maturation was also unaffected by maternal dosages PFHxA.

There are two 90-day repeated oral studies in rats of PFHxA and related salts. These studies included some reproductive end points. The NOAEL following 90 days of dosing with

PFHxA was 50 mg/kg/d in male rats, based on liver weight increases and hepatocellular hypertrophy (Chengelis et al 2009a). The NOAEL following 90 days of dosing with NaPFHx was 20 mg/kg/d, based on nasal lesions in male rats (Loveless et al 2009). Both studies demonstrated higher liver weights, minimal hepatocellular hypertrophy, and slightly higher hepatic peroxisomal ß-oxidation activity. The maternal and reproductive/developmental NOAELs for these studies was 100 mg/kg/d, based on reduced F1 pup weights. This NOAEL was consistent with this study in mice.

Butenhoff and his colleagues conducted a comprehensive 2 generation reproductive toxicity study on PFOA with Sprague-Dawley rats and reported little toxicity, small postnatal weight gain deficits, slight delays of sexual maturation, and postweaning mortality (likely related to immaturity) were noted only in the F1 generation animals of the highest dose group (30 mg/kg) (Butenhoff et al 2004). The PFOA was less developmentally toxic than PFOS probably due, in part, to the efficient renal elimination of PFOA in the female rat, with a half-life estimate of 3 to 4 hours, compared to that of 6 to 8 days in the males (Kim et al 2016, Kudo et al 2002, Ohmori et al 2003). In contrast, no significant differences were seen between serum PFOA concentrations of male and female mice.

The toxicity of another C6 chemical, perfluorohexanesulfonic acid (PFHxS), has also been studied. Mean serum elimination half-life values in PFHxS were about 30 days between male and female mice (Sundstrom et al 2012). The reproductive and developmental toxicity study of PFHxS demonstrated, at 3 and 10 mg/kg/d, increased liver to body weight and liver to brain weight ratios, centrilobular hepatocellular hypertrophy, hyperplasia of thyroid follicular cells, and decreased hematocrit but no effects on reproduction or developmental toxicity (Butenhoff et al 2009). However, it should be noted that because of the selectivity of the end points in this reproductive/developmental screening study, no definitive evidence of reproductive or

developmental effects could be excluded.

Cassone and colleagues reported that in chick eggs effects of PFHxS and PFHxA exposure (maximum dose = 38 000 and 9700 ng/g egg, respectively) on embryonic death, developmental end points, tissue accumulation, messenger RNA expression in liver and cerebral cortex, and plasma thyroid hormone occurred (Cassone et al 2012b). Pipping success was reduced (37%) and tarsus length and embryo mass were reduced at the highest dose of PFHxS (38 000 ng/g). However, no effects were observed for PFHxA (Cassone et al 2012b). Free thyroxine (free T4) levels in the plasma of developing chicken embryos exposed to PFHxS and PFHxA. PFHxS significantly decreased plasma-free T4 levels in a dose-dependent manner, whereas PFHxA had no effect on circulating thyroid hormone levels.

The depleted free T4 status observed in PFHxS-exposed embryos is in agreement with several of the altered end points presented in their study (e.g., reduced pipping success, embryo growth, and increased deiodinase expression). Thyroid hormone-dependent neurodevelopmental pathways were affected in developing chicken embryos exposed to PFHxS. One thyroid hormone-independent mode of PFHxS action identified was gap junction intercellular communication (GJIC) via nephroblastoma-overexpressed gene (*nov*) downregulation (Cassone et al 2012a). The PFHxS was found to inhibit GJIC in a dose-dependent fashion, and this inhibition occurred rapidly and was reversible(Hu et al 2002). Upham and colleagues reported the effect of Perfluoroalkyl carboxylic acids, such as PFOA, on GJIC in WB-rat liver epithelial cells. The chain length of the Perfluoroalkyl carboxylic acids of 7 to 10 inhibited GJIC in a dose–response fashion, whereas carbon lengths of 2 to 5, 16, and 18 did not appreciably inhibit GJIC. Unfortunately, PFHxA was not specifically tested (Upham et al 1998).

Martin and colleagues compared bioconcentration factors (BCFs) for perfluoroalkyl

carboxylates and sulfonates with the number of CF_2 moieties. The PFHxS ($F(CF_2)_6SO_3^-$), which has 6 CF_2 moieties, had a bioconcentration factor (BCF) of about 10, whereas PFHpA ($F(CF_2)_6COO^-$), which also has 6 perfluoroalkyl carbons, could not be detected in most tissues, despite a higher exposure concentration (Martin et al 2003a).

Some reports estimated that the BCF for PFHxA ($F(CF_2)_5COO^-$), which has 5 CF_2 moieties, was about 0.1 (Rayne et al 2009, Webster & Ellis 2011). There is a 100 times difference in BCF between PFHxS and PFHxA. It appears that hydrophobicity of PFHxA is low and it hardly interacts with/permeates through the cell membrane. Therefore, this also suggests that PFHxA does not inhibit GJIC.

In this study, the body burden of PFHxA in F1 mice after weaning at postnatal day 41 was measured with detectable amount of the chemical found in the liver. Analysis of data from another study showed that the pharmacokinetics of PFHxA was characterized in female mice when administered once by oral gavage at dose levels of 35, 175, and 350 mg/kg (Table 25). The PFHxA was rapidly absorbed (C_{max} reached within 30 minutes) and in general was not quantifiable at 24 hours after dose administration. The terminal elimination half-life of PFHxA ranged from 0.889 to 1.24 hours and was dose independent. The increase in C_{max} (from 96.6 to 454 mg/mL) was lower than proportional, whereas the increase in $AUC_{(0\text{-inf})}$ (from 178 to 1893 h mg/mL) was proportional to the increase in the dose from 35 to 350 mg/kg. In a mouse multipledose study,13 daily doses of orally administered APFHx was followed by a single oral administration of [^{14}C]-APFHx. Excretion was rapid, with mean recoveries of over 90% of the administered dose (mean values >95% of the ultimately recovered material) at 24 hours after dosing, irrespective of gender; thus, by PD 22, most of the APFHx would eliminated, even if one assumes a preferential accumulation of PFHxA in the liver (like PFOA). This most likely has to do with the rapid elimination of PFHxA as with PFBA.

Kudo and colleagues reported that the potency of the induction of hepatomegaly, peroxisomal β-oxidation, and microsomal 1-acylglycerophoshocholine(1-acyl-GPC) acyltransferase were compared among PFAAs with 6 to 9 carbon chain length in the liver of male and female mice (Kudo et al 2006). The longer the perfluoroalkyl chain, the more PFAAs accumulates in the liver of both male and female mice. The accumulated PFAAs induced hepatomegaly, peroxisomal β-oxidation, and microsomal 1-acyl-GPC acyltransferase, with little PFHxA accumulation in the liver. Log octanol–water partition coefficient (Po/w) of PFBA, PFHxA, and PFOA using liquid chromatography–tandem mass spectrometry is 1.43, 2.51, and 3.60 (in our data). The half-life of PFBA would be faster than PFHxA. Mean terminal serum PFBA elimination half-lives were 1 to 10 hours in the rat, about 10 hours in the mouse, 40 hours in monkey, and approximately 3 days in humans. The elimination half-life of PFHxA in rats ranged between 0.4 and 9.8 hours, in mouse about 1 hour, in monkeys 14-47 hours, and in humans it is 14-49 days. It seems there was no difference in the elimination half-life of PFBA and PFHxA. A comparison of elimination rates of PFHxA among various species (including mouse and humans) has been made by Russell and colleagues and Chang and colleagues (Chang et al 2008, Russell et al 2013). Unlike PFHxS, PFOS, and PFOA and like PFBA, PFHxA is rapidly eliminated, which may contribute to higher NOAELS for developmental and reproductive parameters in mammalian species.

On the basis of the data from this study, the maternal NOAEL for APFHx is 100 mg/kg/d. The NOAEL in the F1 generation is also 100 mg/kg/d. None of the effects observed in the pups preweaning at any dose level persisted into the postweaning period.

Summary

The reproductive toxicity potential of APFHx in pregnant Crl:CD1(ICR) mice was investigated.

Twenty females / group were administered the test substance or vehicle once daily from gestation day (DG) 6 through 18. Doses in phase 1: 0, 100, 350 and 500 mg/kg/day; phase 2: 0, 7, 35 and 175 mg/kg/day. Parameters evaluated: mortality, viability, body weights, clinical signs, abortions, premature deliveries, pregnancy and fertility, litter observations, maternal behavior and sexual maturity in the F1 generation. The level of APFHx was measured in the liver of F0 and F1 mice. At doses of 350 and 500 mg/kg/day maternal mortalities, excess salivation and changes in body weight gains occurred. Pup body weights were reduced on PPD 0 in the all dosage groups, but persisted only in the 350 and 500 mg/kg/day groups. Additional effects at 300 and 500 mg/kg/day included stillbirths, reductions in viability indices and delays in physical development. Levels of APFHx in the livers of the 100 mg/kg/day dams were all below the lower limit of quantization (0.02 µg/mL) and in the 350 mg/kg/day group, three of eight samples had analytical results that were quantifiable. In phase 2: no APFHx was found in the liver. Adverse effects occurred only in the 175 mg/kg/day group and consisted of increased stillborn pups, pups dying on postpartum day (PPD) 1 and reduced pup weights on PPD 1. Based on these data, the maternal and reproductive no-observable-adverse-effect-level (NOAEL) for APFHx is 100 mg/kg/day.

Tables

Table 7. Treatment regimen for phase 1 and phase 2.

Dosage group	Number of mice	Dosage, mg/kg/d	Concentration, mg/mL	Dosage volume, mL/kg
Phase 1				
I	20	0 (vehicle)	0	5
II	20	100	20	5
III	20	350	70	5
IV	20	500	100	5
Phase 2				
I	20	0 (vehicle)	0	5
II	20	7	1.4	5
III	20	35	7	5
IV	20	175	35	5

Table 8. Summary of mortality data for phase 1.

Dosage group	I	II	III	IV
Dosage, mg/kg/d	0 (vehicle)	100	350	500
Animals tested, N	20	20	20	20
Pregnant, N	19	19	20	18
Litters delivered, N	19	19	19	17
Found dead, N	3	6	1	3

Table 9. F0 generation female mice: Summary of natural delivery observations.

Dosage group	I	II	III	IV
Dosage, mg/kg/d[a]	0 (vehicle)	100	350	500
Mice assigned to natural delivery, N	20	20	20	20
Pregnant, N (%)	19 (95.0)	19 (95.0)	20 (100.0)	18 (90.0)
Included in analyses, N	19	19	19[b]	17[b]
Delivered a litter, N (%)	19 (100.0)	19 (100.0)	19 (100.0)	17 (100.0)
Duration of gestation,[c] mean ± SD	19.9 ± 0.6	19.9 ± 0.2	19.9 ± 0.6	20.2 ± 1.1
Implantation sites per delivered litter				
N	245	276	266	239
Mean ± SD	12.9 ± 4.3	14.5 ± 1.8	14.0 ± 3.6	14.0 ± 2.4
Dams with no stillborn pups, N (%)	2 (10.5)	0 (0.0)	5 (26.3)	7 (41.2)[d]
Dams with no liveborn pups, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)
Gestation index[e]				
N (%)	100.0	100.0	100.0	94.1
N/N	19/19	19/19	19/19	16/17
Dams with all pups dying days 0-3 postpartum, N (%)	1 (5.3)	0 (0.0)	2 (10.5)	5 (31.3)[d]
Dams with all pups dying days 4-20 postpartum, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Abbreviation: SD, standard deviation.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Excludes mice that were found dead before delivery.
[c]Calculated (in days) as the time elapsed between confirmed mating (arbitrarily defined as day 0 of gestation) and the day the first pup was delivered.
[d]Significantly different from the control group value ($P \leq 0.01$).
[e]Number of mice with live offspring/number of pregnant mice.

Table 10. Litter observations (naturally delivered pups): F1 generation litters.

Dosage group	I	II	III	IV
Dosage, mg/kg/d[a]	0 (vehicle)	100	350	500
Delivered litters with 1 or more liveborn pups, N	19	19	19	16
Pups delivered (total)				
N	221	250	245	177
Mean ± SD	11.6 ± 4.2	13.2 ± 1.6	12.9 ± 3.8	11.1 ± 2.4
Liveborn				
Mean ± SD	11.4 ± 4.5	13.2 ± 1.6	12.2 ± 3.4	9.4 ± 3.9
N (%)	217 (98.2)	250 (100.0)	232 (94.7)	150 (84.7)[b]
Stillborn				
Mean ± SD	0.2 ± 0.7	0.0 ± 0.0	0.3 ± 0.4	1.0 ± 2.2
N (%)	4 (1.8)	0 (0.0)	5 (2.0)	16 (9.0)
Unknown vital status,[c] N	0	0	8	11
Pups found dead or presumed cannibalized				
Day 0, N/N (%)	0/217 (0.0)	0/250 (0.0)	3/232 (1.3)	21/150 (14.0)[b]
Days 1-4, N/N (%)	2/217 (0.9)	3/250 (1.2)	25/229 (10.9)[b]	20/129 (15.5)[b]
Days 5-7, N/N (%)	1/215 (0.5)	1/247 (0.4)	3/204 (1.5)	0/109 (0.0)
Days 8-14, N/N (%)	0/214 (0.0)	1/244 (0.4)[d]	3/201 (1.5)	0/109 (0.0)
Days 15-20, N/N (%)	0/214 (0.0)	2/215 (0.9)[d]	0/198 (0.0)	0/109 (0.0)
Day 4 viability index[e]				
%	99.1	98.8	87.9	72.7[b]
N/N	215/217	247/250	204/232	109/150
Day 7 viability index[f]				
%	98.6	98.4	86.6	72.7[b]
N/N	214/217	246/250	201/232	109/150
Lactation index[g]				
%	99.5	98.2[c]	97.0	100.0
N/N	214/215	213/217[c]	198/204	109/109

Abbreviations: day(s), day(s) postpartum; SD, standard deviation.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Significantly different from the control group value ($P \leq 0.01$).
[c]Maternal cannibalization or autolysis precluded identification of vital status at birth.
[d]Excludes mortality of pups that remained on study after dam was found dead.
[e]Number of live pups on day 4 postpartum/number of liveborn pups on day 0 postpartum.
[f]Number of live pups on day 7 postpartum/number of liveborn pups on day 0 postpartum.
[g]Number of live pups on day 20 (weaning) postpartum/number of live pups on day 4 postpartum.

Table 11. Litter viability (naturally delivered pups): F1 generation litters.

Dosage group Dosage, mg/kg/d[a]	I 0 (vehicle)	II 100	III 350	IV 500
Delivered litters with 1 or more liveborn pups, N	19	19	19	16
Surviving pups/litter[b]				
Day 0,[c] mean ± SD	11.4 ± 4.5	13.2 ± 1.6	12.2 ± 3.4	9.4 ± 3.9
Day 20, mean ± SD	11.3 ± 4.6 [15][d]	12.3 ± 1.2	10.4 ± 4.9	6.8 ± 5.0[e]
Percent male pups per Number of pups sexed				
Day 0, mean ± SD	45.8 ± 18.6	48.8 ± 10.5	55.4 ± 16.2	44.0 ± 17.3
Day 20, mean ± SD	48.3 ± 15.3 [18][f]	52.3 ± 9.7 [15][d]	56.0 ± 16.5 [17][f]	47.6 ± 11.3 [11][f]
Live litter size at weighing				
Day 0, mean ± SD	11.4 ± 4.5	13.2 ± 1.6	12.0 ± 3.5	9.9 ± 2.9 [13][g]
Day 4, mean ± SD	11.9 ± 3.8 [18][g]	13.0 ± 1.7	12.0 ± 3.6 [17][g]	9.9 ± 2.0[h] [11][g]
Day 7, mean ± SD	11.9 ± 3.8 [18][g]	12.9 ± 1.6	11.8 ± 3.6 [17][g]	9.9 ± 2.0 [11][g]
Day 14, mean ± SD	11.9 ± 3.8 [18][g]	12.4 ± 1.4 [15][i]	11.6 ± 3.4 [17][g]	9.9 ± 2.0 [11][g]
Day 20, mean ± SD	11.9 ± 3.8 [18][g]	12.3 ± 1.2[15][i]	11.6 ± 3.4 [17][g]	9.9 ± 2.0 [11][g]
Pup weight/litter, g				
Day 0, mean ± SD	1.6 ± 0.2	1.5 ± 0.1[h,j]	1.4 ± 0.2[e]	1.4 ± 0.2[e] [13][g]
Day 4, mean ± SD	3.0 ± 0.4 [18][g]	2.8 ± 0.2	2.2 ± 0.6[e] [17][g]	2.4 ± 0.5[e] [11][g]
Day 7, mean ± SD	4.4 ± 0.8 [18][g]	4.1 ± 0.4	3.6 ± 1.0[e] [17][g]	3.9 ± 0.8 [11][g]
Day 14, mean ± SD	7.4 ± 1.9 [18][g]	6.8 ± 0.8 [15][i]	6.4 ± 1.4 [17][g]	6.8 ± 1.1 [11][g]
Day 20, mean ± SD	11.0 ± 3.0 [18][g]	9.8 ± 1.5 [15][i]	8.8 ± 2.7 [17][g]	9.7 ± 2.0 [11][g]

Abbreviations: day, day of postpartum; SD, standard deviation; [ ], number of values averaged.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Average number of live pups per litter, including litters with no surviving pups.
[c]Includes liveborn pups and pups that died before weighing on day 0 postpartum.
[d]Excludes litters with mortality of pups that remained on study after dam was found dead.
[e]Significantly different from the control group value ($P \leq 0.01$).
[f]Excludes values for litters that had no surviving pups.
[g]Excludes values for litters that had no surviving pups.
[h]Significantly different from the control group value ($P \leq 0.05$).
[i]Excludes litters with mortality of pups that remained on study after dam was found dead.
[j]With pup body weights per litter covaried with litter size per litter, the analyses were not significant.

Table 12. Natural delivery observations: F0 generation female mice.

Dosage group Dosage, mg/kg/d[a]	I 0 (vehicle)	II 7	III 35	IV 175
Mice assigned to natural delivery, N	20	20	20	20
Pregnant, N	20	20	20	20
Delivered a litter, N (%)	20 (100.0)	17 (100.0)	20 (100.0)	20 (100.0)
Included in analyses, N	20	17	19[b]	20
Duration of gestation,[c] mean ± SD	19.6 ± 0.5	19.8 ± 0.8	19.8 ± 0.4	19.7 ± 0.5
Implantation sites per delivered litter				
N	261	220	239	252
Mean ± SD	13.0 ± 2.3	12.9 ± 2.5	12.6 ± 1.8	12.6 ± 1.7
Mice with stillborn pups, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.0)
Mice with no liveborn pups, N	0	0	0	0
Gestation index[d]				
%	100.0	100.0	100.0	100.0
N/N	20/20	17/17	19/19	20/20
Mice with all pups dying days 0-3 postpartum, N (%)	0 (0.0)	1 (5.9)	0 (0.0)	0 (0.0)
Mice with all pups dying days 4-20 postpartum, N (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Abbreviation: SD, standard deviation.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Excludes values for a mouse that was killed on day 17 of gestation due to premature delivery.
[c]Calculated (in days) as the time elapsed between confirmed mating (arbitrarily defined as day 0 of gestation) and the day the first pup was delivered.
[d]Number of mice with live offspring/number of pregnant mice.

Table 13. Litter viability (naturally delivered pups): F1 generation litter.

Dosage group Dosage, mg/kg/d[a]	I 0 (vehicle)	II 7	III 35	IV 175
Delivered a litter with 1 or more liveborn pups, N	20	17	19	20
Pups delivered total				
N	249	213	232	241
Mean ± SD	12.4 ± 2.5	12.5 ± 3.0	12.2 ± 1.7	12.0 ± 2.1
Liveborn				
Mean ± SD	12.4 ± 2.5	12.4 ± 3.4	12.2 ± 1.7	11.9 ± 2.5
N (%)	249 (100.0)	211 (99.1	232 (100.0)	238 (98.8)
Stillborn				
Mean ± SD	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.2 ± 0.7
N (%)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.2)[b]
Unknown vital status, N	0	2	0	0
Pups found dead or presumed cannibalized				
Day 0, N/N (%)	0/249 (0.0)	0/211 (0.0)	0/232 (0.0)	4/238 (1.7)[b]
Days 1-4, N/N (%)	3/249 (1.2)	6/211 (2.8)	2/232 (0.9)	3/234 (1.3)
Days 5-7, N/N (%)	1/246 (0.4)	0/205 (0.0)	0/230 (0.0)	3/231 (1.3)
Days 8-14, N/N (%)	0/245 (0.0)	0/205 (0.0)	0/230 (0.0)	0/228 (0.0)
Days 15-20, N/N (%)	0/245 (0.0)	0/205 (0.0)	0/230 (0.0)	1/228 (0.4)
Day 4 viability index[c]				
%	98.8	97.2	99.1	97.0
N/N	246/249	205/211	230/232	231/238
Day 7 viability index[d]				
%	98.4	97.2	99.2	95.8
N/N	245/249	205/211	230/232	228/238
Lactation index[e]				
%	99.6	97.2	100.0	98.3
N/N	245/246	205/211[b]	230/230	227/231

Abbreviations: day(s), day(s) postpartum; SD, standard deviation.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Significantly different from the vehicle control group value ($P \leq 0.01$).
[c]Number of live pups on day 4 postpartum/number of liveborn pups on day 0 postpartum.
[d]Number of live pups on day 7 postpartum/number of liveborn pups on day 0 postpartum.
[e]Number of live pups on day 20 (weaning) postpartum/number of live pups on day 4 postpartum.

Table 14. Litter observations (naturally delivered pups): Summary; F1 generation litters.

Dosage group Dosage, mg/kg/d[a]	I 0 (vehicle)	II 7	III 35	IV 175
Delivered a litter with 1 or more liveborn pups, N	20	17	19	20
Day 0, mean ± SD	12.4 ± 2.5	12.4 ± 3.4	12.2 ± 1.7	11.9 ± 2.5
Day 20, mean ± SD	12.2 ± 2.5	12.0 ± 3.5	12.1 ± 1.7	11.4 ± 3.0
Percent male pups per number of pups sexed				
Day 0, mean ± SD	52.3 ± 13.2	54.0 ± 18.5	52.4 ± 15.0	53.1 ± 12.9
Day 20, mean ± SD	52.8 ± 13.2	51.4 ± 14.7 [16][b]	52.3 ± 15.0	53.2 ± 13.7
Live litter size at weighing				
Day 0, mean ± SD	12.4 ± 2.5	12.4 ± 3.4	12.2 ± 1.7	11.7 ± 2.8
Day 4, mean ± SD	12.3 ± 2.4 [16][c]	12.8 ± 1.7	12.1 ± 1.7	11.6 ± 3.0
Day 7, mean ± SD	12.3 ± 2.4 [16][c]	12.8 ± 1.7	12.1 ± .7	11.4 ± 3.0
Day 14, mean ± SD	12.3 ± 2.4 [16][c]	12.8 ± 1.7	12.1 ± .7	11.4 ± 3.0
Day 20, mean ± SD	12.3 ± 2.4 [16][c]	12.8 ± 1.7	12.1 ± .7	11.4 ± 3.0
Pup weight/litter, g				
Day 0, mean ± SD	1.6 ± 0.1 [16][c]	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.2[d]
Day 4, mean ± SD	2.8 ± 0.3 [16][c]	2.8 ± 0.3	3.0 ± 0.3	2.7 ± 0.5
Day 7, mean ± SD	4.2 ± 0.6 [16][c]	4.2 ± 0.4	4.4 ± 0.4	4.2 ± 0.6
Day 14, mean ± SD	6.8 ± 1.2 [16][c]	6.7 ± 0.6	7.0 ± 0.7	6.8 ± 0.9
Day 20, mean ± SD	10.2 ± 1.8 [16][c]	10.0 ± 1.2	10.8 ± 1.3	10.4 ± 1.4

Abbreviations: day, day postpartum; SD, standard deviation; [ ], number of values averaged.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Excludes values for a litter which had no surviving pups on day 2 postpartum.
[c]Average number of live pups per litter, including litters with no surviving pups.
[d]Significantly different from the vehicle control group value ($P \leq 0.05$).

Table 15. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F0 generation female mice.[a]

Dosage group Dosage, mg/kg/d[b]	I 0 (vehicle)	II 100	III 350	IV 500
Mice tested, N	10	12	8	16
Pregnant, N	9	11	8	14
Included in analyses, N	5[c]	5[c]	5[c]	5[c]
Terminal body weight, mean ± SD	43.8 ± 2.8	43.1 ± 3.1	45.7 ± 4.4	40.1 ± 3.6
Liver, mean ± SD	3.124 ± 0.252	3.200 ± 0.340	3.272 ± 0.321	2.866 ± 0.157
Liver, mean ± SD, %	7.136 ± 0.515	7.438 ± 0.685	7.178 ± 0.572	7.188 ± 0.772

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (liver weight/terminal body weight) × 100.
[b]Dosage occurred on days 6 through 18 of gestation.
[c]Excludes values for mice that were killed due to no surviving pups or found dead.

Table 16. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F0 generation female mice.[a]

Dosage group Dosage, mg/kg/d[b]	I 0 (vehicle)	II 7	III 35	IV 175
Mice tested, N	5	9	6	5
Pregnant, N	5	6	6	5
Included in analyses, N	5	5[c]	5[d]	5
Terminal body weight, mean ± SD	42.4 ± 4.4	43.1 ± 5.1	44.0 ± 4.4	45.8 ± 2.8
Liver, mean ± SD, g	3.030 ± 0.208	3.194 ± 0.356	3.506 ± 0.517	3.172 ± 0.345
Liver, mean ± SD, %	7.172 ± 0.361	7.418 ± 0.497	7.970 ± 0.964	6.906 ± 0.362

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (organ weight/terminal body weight) × 100.
[b]Dosage occurred on days 6 through 18 of gestation.
[c]Excludes values for mouse 430, which was killed on day 2 of lactation due to no surviving pups.
[d]Excludes values for mouse 448, which was killed on day 17 of gestation due to premature delivery.

Table 17. Eye opening by litter: F1 generation litters.

Maternal dosage group	I	II	III	IV
Maternal dosage, mg/kg/d[a]	0 (vehicle)	100	350	500
Litters delivered, N	19	19	19	17
Litters tested, N	18[b]	19	17[b]	11[b]
Percentage of pups meeting criterion				
Day 10, mean ± SD	0.4 ± 1.7	0.4 ± 1.5	0.5 ± 2.2	0.0 ± 0.0
Day 11, mean ± SD	0.4 ± 1.7	0.4 ± 1.5	0.5 ± 2.2	0.0 ± 0.0
Day 12, mean ± SD	6.8 ± 23.4	0.8 ± 2.4	1.1 ± 3.0	1.3 ± 4.3
Day 13, mean ± SD	31.7 ± 37.9	14.0 ± 19.2	13.2 ± 25.8	14.2 ± 29.4
Day 14, mean ± SD	82.5 ± 24.4	68.6 ± 34.9	42.0 ± 39.5[c]	50.2 ± 38.0[d]
Day 15, mean ± SD	98.4 ± 3.7	88.2 ± 25.6	76.1 ± 37.8	73.4 ± 42.4
Day 16, mean ± SD	100.0 ± 0.0	99.2 ± 3.3	91.1 ± 22.7	99.2 ± 2.5
Day 17, mean ± SD	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Criterion day,[e] mean ± SD	13.8 ± 0.7	14.2 ± 0.8	14.9 ± 1.1[c]	14.5 ± 1.0

Abbreviations: day, day postpartum; SD, standard deviation.
[a]Dosage occurred on days 6 through 18 of gestation.
[b]Excludes values for litters that had no surviving pups at time of testing.
[c]Significantly different from the control group value ($P \leq 0.01$).
[d]Significantly different from the control group value ($P \leq 0.05$).
[e]The average day postpartum that at least 50% of the pups had the developmental measure present.

Table 18. Eye opening by litter: F1 generation litters.

Maternal dosage group	I	II	III	IV
Maternal dosage, mg/kg/d	0 (vehicle)	7	35	175
Litters tested, N	20	16	19	20
Day 10, mean ± SD	0.4 ± 1.7	2.1 ± 4.8	1.0 ± 2.9	1.3 ± 3.2
Day 11, mean ± SD	0.4 ± 1.7	2.2 ± 4.1	1.7 ± 3.5	1.2 ± 3.1
Day 12, mean ± SD	3.5 ± 6.5	7.0 ± 10.7	5.5 ± 6.6	2.8 ± 4.0
Day 13, mean ± SD	37.6 ± 34.1	35.3 ± 23.5	50.4 ± 35.5	29.7 ± 25.6
Day 14, mean ± SD	85.5 ± 22.7	87.6 ± 24.4	89.3 ± 22.7	78.9 ± 27.4
Day 15, mean ± SD	99.6 ± 1.6	99.2 ± 3.3	99.6 ± 1.9	94.2 ± 22.3
Day 16, mean ± SD	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Criterion day,[a] mean ± SD	13.8 ± 0.7	13.8 ± 0.6	13.4 ± 0.6	14.0 ± 0.8

Abbreviations: day, day postpartum; SD, standard deviation.
[a]The average day postpartum that at least 50% of the pups had the developmental measure present.

Table 19. Sexual maturation: F1 generation mice.

Maternal dosage group	I	II	III	IV
Maternal dosage, mg/kg/d	0 (vehicle)	100	350	500
Male mice, N	20	20	19[a]	20
Preputial separation,[b] mean ± SD	29.4 ± 1.9 [18][c]	29.8 ± 2.2	29.3 ± 2.3	29.4 ± 1.7
Body weight at separation,[d] mean ± SD, g	24.49 ± 3.03 [18][c]	23.22 ± 2.11	22.40 ± 2.27	22.80 ± 2.68
Female mice, N	20	20	20	20
Vaginal patency,[e] mean ± SD	26.8 ± 2.0 [19][c]	27.5 ± 1.1 [19][c]	27.6 ± 2.0 [18][c,f]	27.5 ± 2.1
Body weight at vaginal patency,[g] mean ± SD, g	18.08 ± 1.44 [19][c]	17.38 ± 2.82 [19][c]	16.31 ± 1.70 [18][c,f]	17.57 ± 2.20

Abbreviations: SD, standard deviation; [ ], number of values averaged.
[a]Excludes values for a mouse that was found dead on day 23 postpartum.
[b]Average day postpartum that the prepuce was observed to be separated.
[c]Excludes mice for which the exact day of maturity could not be determined.
[d]Average body weight on day prepuce was first observed to be separated.
[e]Average day postpartum that the vagina was observed to be patent.
[f]Excludes a mouse that had not reached sexual maturity by day 41 postpartum, the day of scheduled killing.
[g]Average body weight on day vagina was first observed to be patent.

Table 20. Sexual maturation: F1 generation mice.

Maternal dosage group	I	II	III	IV
Maternal dosage, mg/kg/d	0 (vehicle)	7	35	175
Male mice, N	20	20	20	20
Preputial separation,[a] mean ± SD	29.2 ± 1.0	29.0 ± 1.1	28.0 ± 1.0[b]	28.4 ± 1.0
Body weight at separation,[c] mean ± SD, g	23.6 ± 2.2	23.9 ± 2.1	22.8 ± 2.3	22.6 ± 2.4
Female mice N	20	20	20	20
Vaginal patency,[d] mean ± SD	26.4 ± 2.8	25.8 ± 2.9	25.8 ± 1.6	25.2 ± 1.9
Body weight at vaginal patency,[e] mean ± SD, g	16.8 ± 2.6	16.2 ± 2.9	17.2 ± 1.7	15.7 ± 2.4

Abbreviation: SD, standard deviation.
[a]Average day postpartum that the prepuce was observed to be separated.
[b]Significantly different from the vehicle control group value ($P \leq 0.01$).
[c]Average body weight on day prepuce was first observed to be separated.
[d]Average day postpartum that the vagina was observed to be patent.
[e]Average body weight on day vagina was first observed to be patent.

Table 21. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F1 generation male mice.[a]

Maternal dosage group	I	II	III	IV
Dosage, mg/kg/d	0 (vehicle)	100	350	500
Mice tested, N	5	5	6	5
Included in analyses, N	5	5	5[b]	5
Terminal body weight, mean ± SD	30.9 ± 2.3	31.3 ± 3.4	30.9 ± 1.1	30.1 ± 2.0
Liver, mean ± SD	2.110 ± 0.140	2.121 ± 0.254	2.220 ± 0.126	1.930 ± 0.155
Liver, mean ± SD, %	6.830 ± 0.341	6.782 ± 0.277	7.192 ± 0.251	6.412 ± 0.267[c]

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (liver weight/terminal body weight) × 100.
[b]Excludes values for a mouse which was found dead on day 23 postpartum.
[c]Significantly different from the control group value ($P \leq 0.05$).

Table 22. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F1 generation female mice.[a]

Maternal dosage group	I	II	III	IV
Dosage, mg/kg/d	0 (vehicle)	100	350	500
Mice tested, N	5	5	5	5
Terminal body weight, mean ± SD	25.4 ± 1.4	26.1 ± 1.9	22.8 ± 1.2[b]	25.4 ± 1.4
Liver, mean ± SD	1.536 ± 0.104	1.549 ± 0.168	1.457 ± 0.174	1.569 ± 0.080
Liver, mean ± SD, %	6.056 ± 0.232	5.928 ± 0.239	6.392 ± 0.522	6.180 ± 0.395

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (liver weight/terminal body weight) × 100.
[b]Significantly different from the control group value ($P \leq 0.05$).

Table 23. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F1 generation male mice.[a]

Maternal dosage group Maternal dosage, mg/kg/d	I 0 (vehicle)	II 7	III 35	IV 175
Mice tested, N	5	5	5	5
Terminal body weight, mean ± SD	31.8 ± 1.7	32.0 ± 0.7	31.4 ± 2.1	31.4 ± 1.4
Liver, mean ± SD, g	2.231 ± 0.354	2.365 ± 0.116	2.134 ± 0.080	2.246 ± 0.151
Liver, mean ± SD, %	6.976 ± 0.762	7.396 ± 0.452	6.810 ± 0.419	7.156 ± 0.236

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (organ weight/terminal body weight) × 100.

Table 24. Terminal body weights, liver weights, and ratios (%) of liver weight to terminal body weight: F1 generation female mice.[a]

Maternal dosage group Maternal dosage, mg/kg/d	I 0 (vehicle)	II 7	III 35	IV 175
Mice tested, N	5	5	5	5
Terminal body weight, mean ± SD	23.9 ± 2.1	23.1 ± 1.6	23.5 ± 1.4	23.9 ± 2.1
Liver, mean ± SD, g	1.444 ± 0.184	1.479 ± 0.198	1.380 ± 0.149	1.503 ± 0.167
Liver, mean ± SD, %	6.018 ± 0.408	6.398 ± 0.504	5.866 ± 0.359	6.270 ± 0.256

Abbreviation: SD, standard deviation.
[a]All weights were recorded in g. Ratios (%) = (organ weight/terminal body weight) × 100.

Table 25. Pharmacokinetic exposure parameters of PFHxA in female Crl: CD1(ICR) mice serum following oral gavage of APFHx.

Dose level, mg/kg	T_{max}, h	C_{max}, µg/mL	$AUC_{(0-t)}$, µg·h/mL	$AUC_{(0-inf)}$, µg·h/mL	$T_{1/2}$, h
35	0.5	96.6	176	178	0.889
175	0.5	330	1121	1144	1.24
350	0.5	4454	1873	1893	0.924

Abbreviations: AUC, area under the curve;

Figures

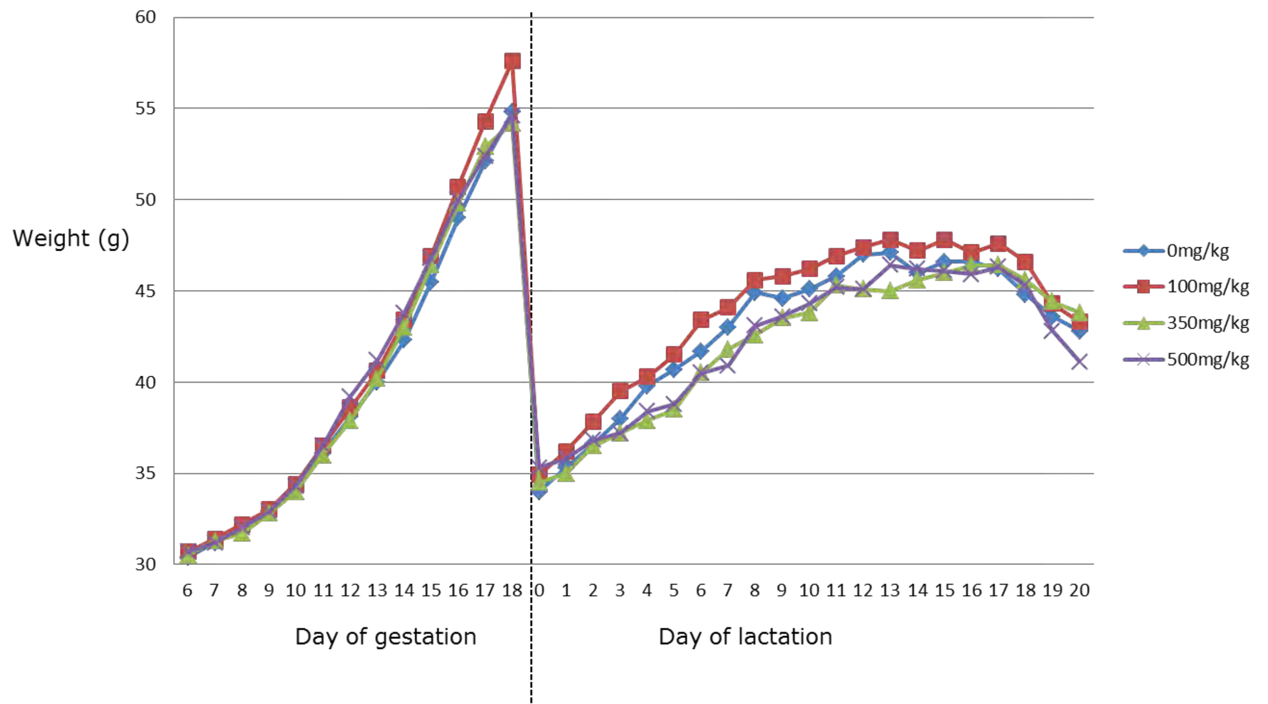

Figure 4.

Maternal body weights (phase I) : F0 generation female mice.

Mean maternal body weight for mice during the gestation period and during the lactation period dosing with APFHx. Body weight of maternal mice treated with 0, 100, 350, and 500mg/kg/day APFHx. All values were comparable among the 4 dosage groups and did not differ significantly.

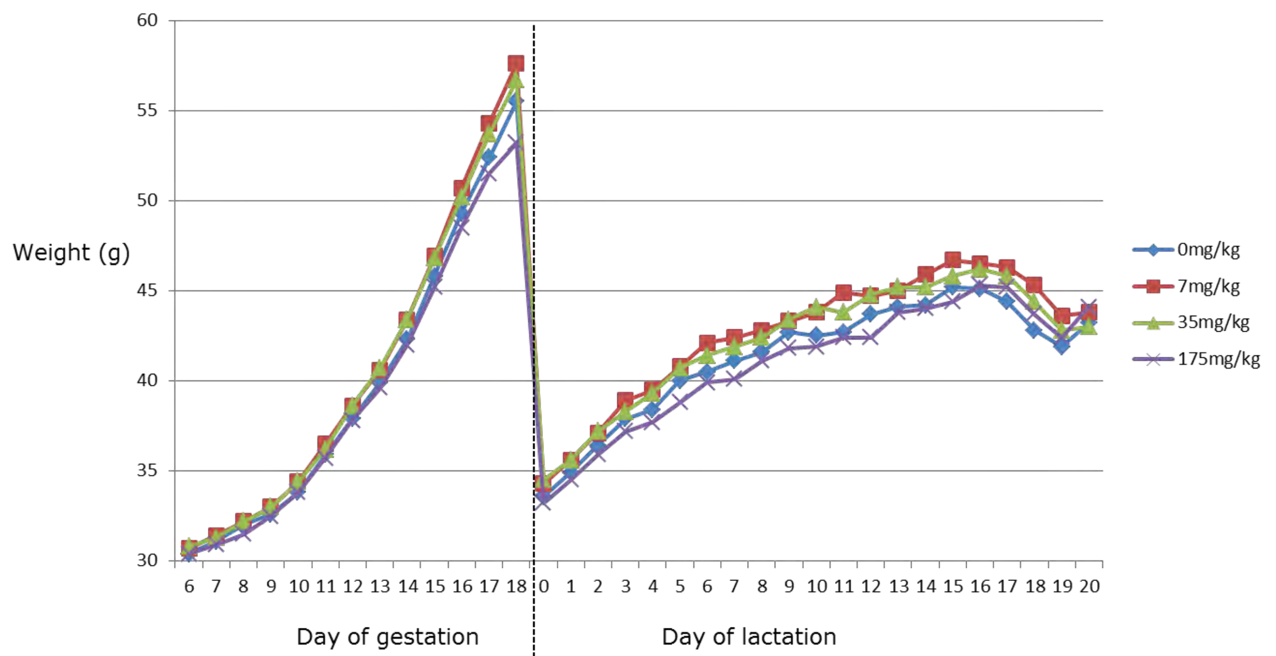

Figure 5

Maternal body weights (phase II) : F0 generation female mice.

Mean maternal body weight for mice during the gestation period and during the lactation period dosing with APFHx. Body weight of maternal mice treated with 0, 7, 35, and 175mg/kg/day APFHx. All values were comparable among the 4 dosage groups and did not differ significantly.

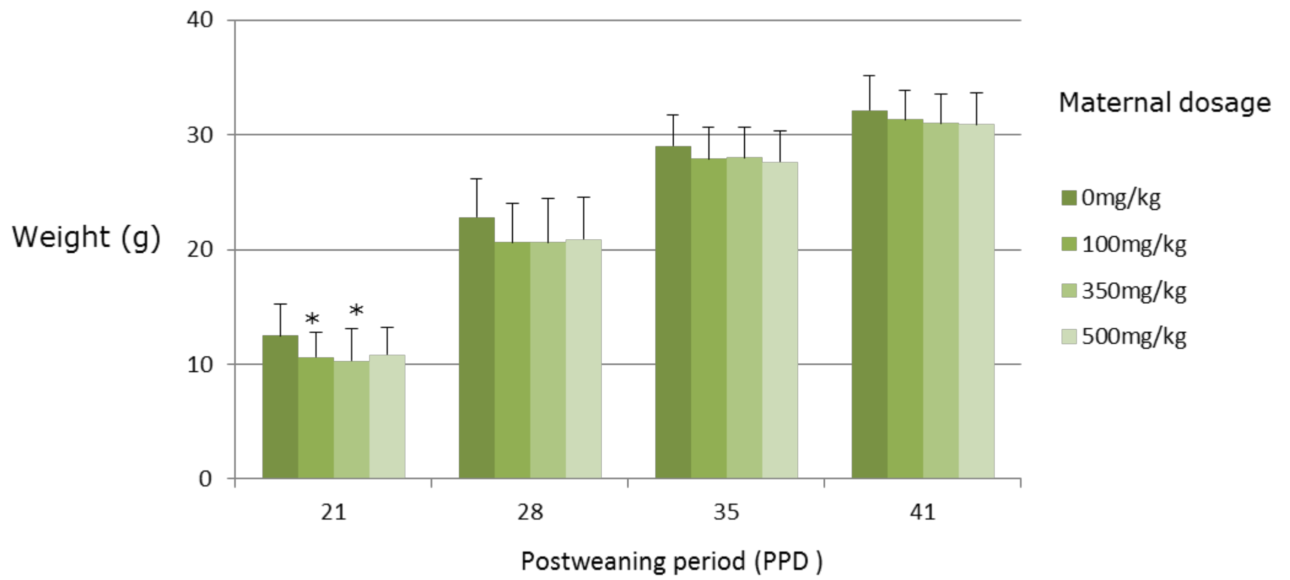

Figure 6

Body weights (phase I) : F1 generation male mice.

Mean F1 generation male body weight for mice during the postweaning period (PPD 21-41). Body weight of maternal dosages with 0, 100, 350, and 500mg/kg/day APFHx.

The only significant difference that occurred among the groups were significantly reduced (*: $p \leq 0.05$) body weights on PPD 21 in the 100 and 350 mg/kg/day dosage groups compared to the control group value. No other significant differences occurred among the groups during the postweaning period (PPD 21 to 41).

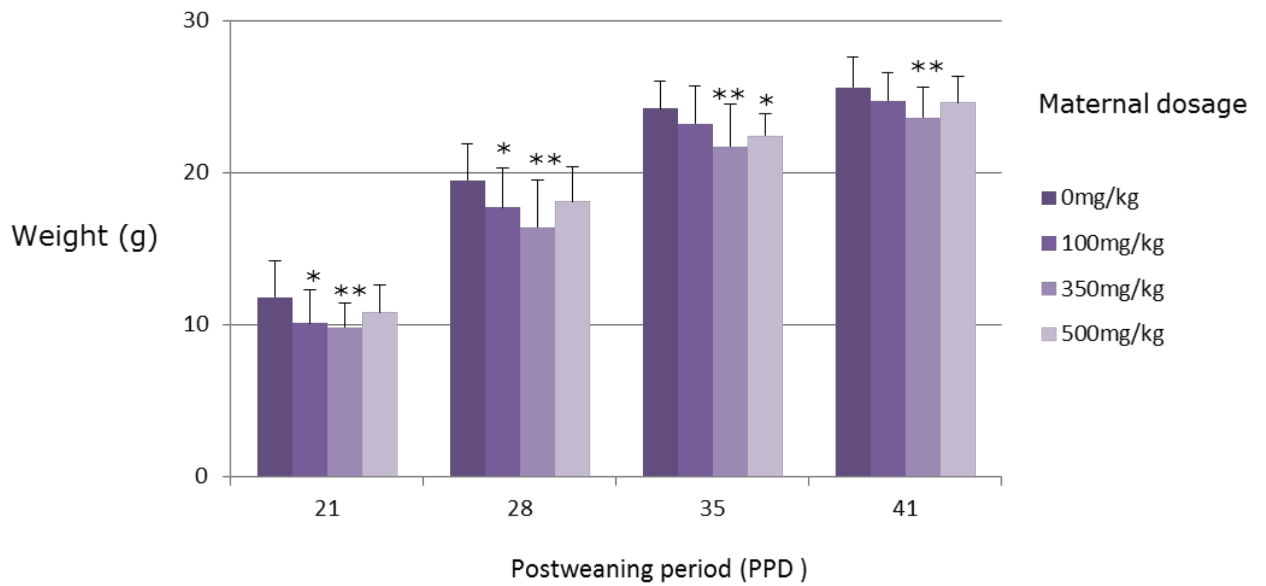

Figure 7

Mean F1 generation female body weight for mice during the postweaning period (PPD 21-41). Body weight of maternal dosages with 0, 100, 350, and 500mg/kg/day APFHx.

Body weights were significantly reduced (*: $p \leq 0.05$ to **:$p \leq 0.01$) in the F1 generation female mice on PPDs 21 and 28 in the 100 and 350 mg/kg/day dosage group and on PPDs 35 and 41 (350 mg/kg/day only); and body weights were significantly reduced (**:$p \leq 0.01$) in the 500 mg/kg/day dosage group on PPD 35 compared to the control group values.

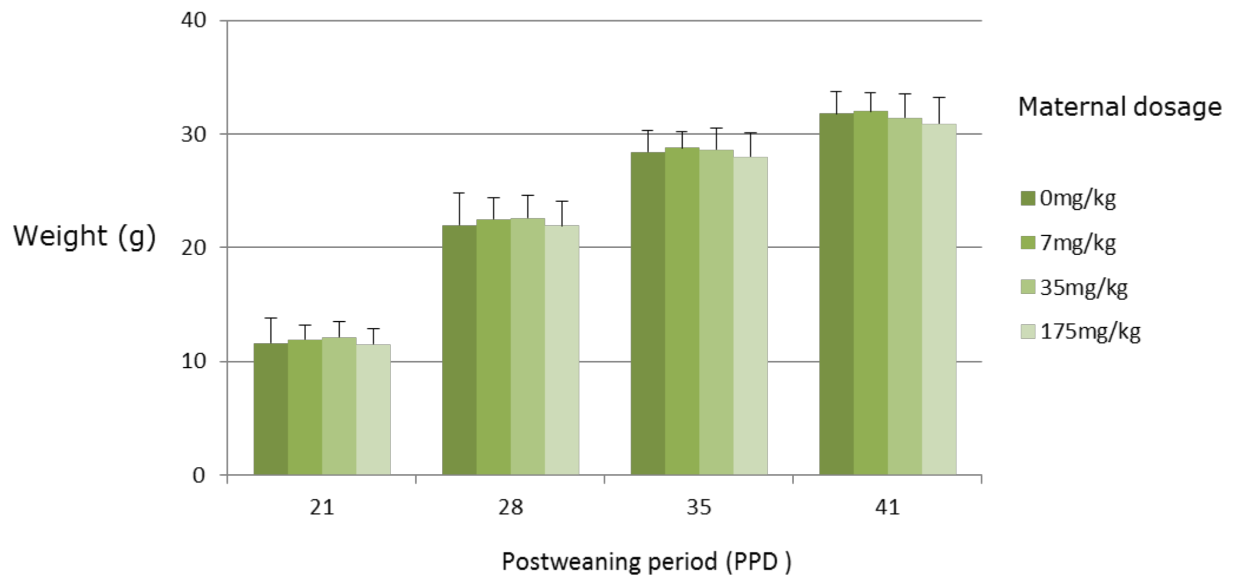

Figure 8

Mean F1 generation male body weight for mice during the postweaning period (PPD 21-41).

Body weight of maternal dosages with 0, 7, 35, and 175mg/kg/day APFHx.

No significant differences occurred among the groups during the postweaning period.

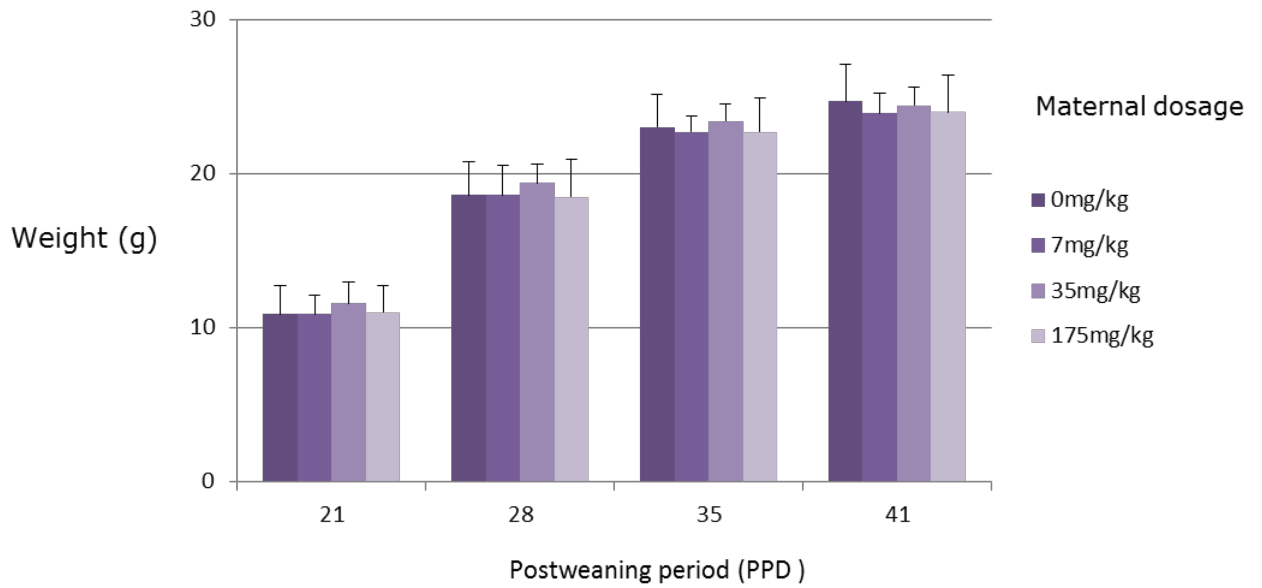

Figure 9

Body weights (phase II) : F1 generation female mice.

Mean F1 generation female body weight for mice during the postweaning period (PPD 21-41).

Body weight of maternal dosages with 0, 7, 35, and 175mg/kg/day APFHx.

No significant differences occurred among the groups during the postweaning period.

General discussion

Among rodents, rats showed little change in blood lipids. This suggests that the secondary effect of the chemical on immunocompetent cells was absent in rats.

Our investigation on rats and mice showed that APFHx administered as a single dose of PFHxA was rapidly eliminated. Its body distribution in rats and mice was similar. Further, its elimination showed the same pattern in the repeated-dose study.

This manuscript described the dose-dependent observations of maternal and developmental toxicity of PFHxA in mice, after oral gavage exposure to the dams during pregnancy. PFHxA is rapidly eliminated, which may contribute to lower toxicity for developmental and reproductive parameters in mammalian species.

1. In rats, treatment with APFO elevated ß-oxidation but did not affect any parameter of blood lipids suggesting that the phenomenon of peroxisome proliferation in the liver varies greatly among different species.

2. Excretion was rapid, with mean recoveries of over 90% of the administered dose (mean values >95% of the ultimately recovered material) at 24 hours after dosing, irrespective of gender or species.

3. The major route of elimination was via the urine, followed by the feces.

4. The maternal NOAEL for APFHx is 100 mg/kg/d, the NOAEL in the F1 generation is also 100 mg/kg/day.
5. None of the effects observed in the pups preweaning at any dose level persisted into the postweaning period.

Finally we show that APFHx is rapidly eliminated and have lower toxicity for developmental and reproductive parameters. We think that APFHx could be used in the fluorochemical industries instead of APFO.

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