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Prenatal Perfluorooctanoic Acid Exposure in CD-1 Mice: Low-Dose Developmental Effects and Internal Dosimetry

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Perfluorooctanoic acid (PFOA) is an environmental contaminant that causes adverse developmental effects in laboratory animals. To investigate the low-dose effects of PFOA on offspring, timed-pregnant CD-1 mice were gavage dosed with PFOA for all or half of gestation. In the full-gestation study, mice were administered 0, 0.3, 1.0, and 3.0 mg PFOA/kg body weight (BW)/day from gestation days (GD) 1–17. In the late-gestation study, mice were administered 0, 0.01, 0.1, and 1.0 mg PFOA/kg BW/day from GD 10–17. Exposure to PFOA significantly ($p < 0.05$) increased offspring relative liver weights in all treatment groups in the full-gestation study and in the 1.0 mg PFOA/kg group in the late-gestation study. In both studies, the offspring of all PFOA-treated dams exhibited significantly stunted mammary epithelial growth as assessed by developmental scoring. At postnatal day 21, mammary glands from the 1.0 mg/kg GD 10–17 group had significantly less longitudinal epithelial growth and fewer terminal end buds compared with controls ($p < 0.05$). Evaluation of internal dosimetry in offspring revealed that PFOA concentrations remained elevated in liver and serum for up to 6 weeks and that brain concentrations were low and undetectable after 4 weeks. These data indicate that PFOA-induced effects on mammary tissue (1) occur at lower doses than effects on liver weight in CD-1 mice, an observation that may be strain specific, and (2) persist until 12 weeks of age following full-gestational exposure. Due to the low-dose sensitivity of mammary glands to PFOA in CD-1 mice, a no observable adverse effect level for mammary developmental delays was not identified in these studies.

Key Words: **perfluorooctanoic acid; mammary gland; development; prenatal; dosimetry.**

Perfluorooctanoic acid (PFOA) is a synthetic eight-carbon perfluorinated compound that is commonly used in the production of fluorotelomers due to its physicochemical properties. PFOA has widespread industrial applications, is persistent in the environment, and is also the final breakdown product of several fluorotelomers (Wang *et al.*, 2005). This surfactant is ubiquitous in the environment and has been detected in sera of animals and humans (Giesy and Kannan, 2002; Olsen *et al.*, 2003). For these reasons, PFOA has become a heavily studied environmental contaminant.

Humans are thought to be exposed to PFOA mainly through ingestion of contaminated water or food products (Begley *et al.*, 2005; Björklund *et al.*, 2009; Gulkowska *et al.*, 2006) and also through residues found in residential dust (Björklund *et al.*, 2009; Strynar and Lindstrom, 2008). PFOA is found in cord blood, human, and rodent milk and, as such, can be passed to developing offspring (Apelberg *et al.*, 2007; Fenton *et al.*, 2009; Hinderliter *et al.*, 2005; Völkel *et al.*, 2008). The most recent U.S. geometric mean serum PFOA concentration is 3.9 ng/ml (2003–2004, Calafat *et al.*, 2007) according to population-based biomonitoring studies; yet, in young children, serum PFOA concentrations are slightly higher (6.1–7.6 ng/ml, least square mean estimates, 2001–2002; Kato *et al.*, 2009). PFOA is a persistent compound as it is slowly eliminated from the body, with an estimated half-life in adult humans of 3.8 years (Olsen *et al.*, 2007). Due to the risk for developmental exposure, its ubiquitous nature, and the presence of this compound at relatively high levels in certain communities

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(Emmett *et al.*, 2006), developmental exposure studies were initiated in the United States and other countries to determine potential adverse health effects of PFOA exposure (Apelberg *et al.*, 2007; C8 Science Panel, 2010; Fei *et al.*, 2007, 2008; Fromme *et al.*, 2010; Hamm *et al.*, 2009; Midasch *et al.*, 2007; Washino *et al.*, 2009). The significance of the findings from many of these studies has been summarized in a recent review article (White *et al.*, 2011).

From previous adult exposure studies in rats, it has been shown that PFOA causes hepatomegaly and a common tumor triad of hepatocellular adenoma, Leydig cell tumors, and pancreatic acinar cell tumors (Biegel *et al.*, 2001; Kennedy *et al.*, 2004). PFOA has also been reported to modulate the immune system of adult mice (Dewitt *et al.*, 2008; Fairley *et al.*, 2007; Yang *et al.*, 2002). Exposure to PFOA causes an array of developmental toxicities in mice. In CD-1 mice, gestational exposure $\geq$ 10 mg/kg PFOA resulted in increased prenatal loss and prolonged gestational length in dams, whereas offspring exhibited reduced birth weight, delayed eye opening, increased postnatal mortality, delayed sexual maturation in females, and precocious sexual maturation in males (Lau *et al.*, 2006). Some of these developmental effects have been reported at lower doses ($\geq$ 0.1 mg/kg) in 129S1/Sv1mj mice (Abbott *et al.*, 2007). Increased body weight (BW) gain accompanied by elevated serum leptin and insulin was reported in adult CD-1 mice prenatally exposed to low doses of PFOA (0.01–1.0 mg/kg BW; Hines *et al.*, 2009). PFOA exposure may also alter neurodevelopment, as changes in motor function have been observed in early life (Onishchenko *et al.*, 2011) and in adulthood (Johansson *et al.*, 2008).

Female rats rapidly eliminate PFOA ($t_{1/2}$ = 2–4 h vs. 4–6 days in males; Lau *et al.*, 2007). This hyperexcretion leads to episodic fetal and neonatal body burdens in developmental exposure studies (Hinderliter *et al.*, 2005). However, gender differences in elimination of PFOA are not observed in humans or mice, and thus, mice have been utilized as the more appropriate rodent model in PFOA studies, particularly in studies involving developmental or female-specific endpoints. As it pertains to the mammary gland, both rats and mice are known to develop in a morphologically similar pattern to the human breast (reviewed in Fenton, 2006). Due to the similarities in relative elimination rates of PFOA and mammary gland development compared with humans, the mouse has been used in our laboratory as the most appropriate rodent model.

The mammary gland appears to be a sensitive tissue to developmental PFOA exposure, although sensitivity may vary across mouse strains. Prenatal exposure to 5 mg PFOA/kg severely stunted mammary gland development in female CD-1 offspring (White *et al.*, 2007), and the current lowest observable adverse effect level (LOAEL) for abnormal mammary gland development in this mouse strain is 3 mg/kg (White *et al.*, 2009). Recent studies by another laboratory have demonstrated that peripubertal exposure to 5 mg/kg or higher PFOA delays mammary gland development in inbred Balb/C mice and either deters (at 10 mg/kg) or accelerates (at 5 mg/kg) mammary development in C57Bl/6 mice, depending on dose (Yang *et al.*, 2009). No significant effects on mammary growth were detected at 0.1 mg/kg in Balb/C or 1 mg/kg in either strain (Yang *et al.*, 2009). Thus, even though cross-foster studies suggest that doses lower than 3 mg/kg may alter mammary gland development in CD-1 mice (White *et al.*, 2009), studies in other strains, under different exposure conditions, do not necessarily support this LOAEL.

Herein, two studies were conducted to further address the issue of low-dose effects of PFOA in the mammary gland of CD-1 mice and the internal dosimetry corresponding to these effects. Specifically, we tested the hypothesis that PFOA exposures that result in human relevant internal doses are also associated with perturbed mammary development in mice. In the first study, with a maximum dose of 3 mg/kg, PFOA was administered to pregnant mice for the entire gestational period (GD 1–17), and we determined effects from birth until adulthood. In a subsequent study, utilizing overlapping and lower doses, PFOA was administered to pregnant mice for only the latter portion of gestation (GD 10–17), as this period of gestation was previously identified as a window of susceptibility for mammary gland developmental abnormalities (White *et al.*, 2007). In the late-gestation study, we evaluated early life time points to better assess the internal dosimetry for these effects in mice. The overarching goal of these two studies was to establish a LOAEL and a no observable adverse effect level (NOAEL) for mammary gland effects following prenatal exposure to PFOA and to determine the corresponding internal dosimetry associated with these effects.

MATERIALS AND METHODS

Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Pregnant dams were housed individually in polypropylene cages and received chow and tap water *ad libitum*; both were known to contain PFOA below the levels of detection. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity.

Chemicals

PFOA (PFOA as its ammonium salt APFO, > 98% pure) was purchased from Fluka Chemical (Steinhiem, Switzerland), and the PFOA lot number was identical to previous studies (White *et al.*, 2007, 2009). PFOA dosing solutions were prepared fresh daily in deionized water, agitated immediately prior to administration, and were given at a volume of 10-μl solution/g BW.

Experimental Design

Full-gestation exposure study. Fifty-two dams arrived at the animal facility on GD 0 and were divided into four treatment groups (n = 13 per treatment). Each group was gavage dosed with deionized water as vehicle or 0.3, 1.0, or 3.0 mg PFOA/kg BW once daily from GD 1 to 17 (shown as mg/kg). Upon parturition, the litters were equalized to 10 pups of equal male and female representation (5:5) when possible. Approximately 15% of the dams were not pregnant, as expected. The desired number of female offspring was not always achieved due to inequities in litter sex ratios.

Late-gestation exposure and early development study. The second study was performed in two blocks (block 1 = 20 dams, block 2 = 32 dams). Mice arrived at the animal facility on GD 9 and were divided into four treatment groups of equal size (block 1 = 5 dams per treatment, block 2 = 8 dams per treatment). Each group was gavage dosed with deionized water as vehicle or 0.01, 0.1, or 1.0 mg PFOA/kg BW once daily (shown as mg/kg) from GD 10 to 17. Approximately 15% of the dams were not pregnant, again unrelated to PFOA exposure. To be consistent with previous work (White *et al*., 2007), on postnatal day (PND) 1, pups within a treatment group were pooled and randomly distributed among the dams of their respective treatment group, resulting in a final litter size of 7–9 pups per dam, with an unequal sex ratio (n = 4–7 females per litter).

Due to the short acclimation periods for both studies (1 day), the dams were observed by the veterinary staff and study personnel for signs of maternal stress, such as lack of weight gain or aggressiveness. None were observed.

Necropsy

For the full-gestation study, 6 pups per treatment group (1–2 per dam) were weighed and then necropsied at PNDs 7, 14, 21, 28, 42, 63, and 84 following decapitation. The trunk blood was collected and centrifuged, and the serum was collected and stored at −80°C. Liver, brain, and one set of fourth and fifth mammary glands were collected, weighed, and stored at −80°C for future RNA analyses. The contralateral fourth and fifth inguinal mammary glands were removed from female pups for preparation as a whole mount. Dams were sacrificed on PND 24, and afterward male and female pups were housed separately. Due to low female pup numbers in the control group, on PND 63, control females were omitted from the necropsy schedule.

In the late-gestational study, female pups from at least three litters per treatment group were weighed, then necropsied on PNDs 1, 4, 7, 14, and 21 following decapitation and the trunk blood was collected, and serum stored as indicated above. On PND 1 only, serum samples were pooled from several pups, both male and female, within a litter to achieve sufficient volume for PFOA analysis. In all further instances, only female endpoints were measured. Liver and a single set of fourth and fifth inguinal mammary glands were collected, weighed, and stored at −80°C for future RNA and protein analyses. The contralateral fourth and fifth inguinal mammary gland tissues were removed to be prepared as whole mounts. Due to lower than expected female pup numbers, PND 4 measurements were not collected for the 0.1 mg/kg treatment group.

Mammary Gland Preparations

For whole-mount preparations, mammary tissues were flattened onto glass slides, fixed in Carnoy's solution, stained with Carmine alum, and cleared in xylene as previously described in Fenton *et al*. (2002). Mammary glands were evaluated by light microscopy (Leica Z16 APO and Leica DFC295 [Camera], Leica Microsystems, Frankfurt, Germany) and assessed an overall developmental score based on a 1–4 scale (1 = poor development, 4 = best development) at each age, similar to methods previously reported (Hilakivi-Clarke *et al*., 1997a,b; Welsch *et al*., 1988). Scores were based on qualitative and quantitative histological characteristics of each developmental time point, including, but not limited to, lateral and longitudinal epithelial growth, change in epithelial growth, appearance of budding from the ductal tree, branching density, and number of differentiating duct ends (Hilakivi-Clarke *et al*., 1997a). Where applicable, at a given time point, mammary glands from both studies were compared on the microscope to ensure consistency in the scoring scale between studies.

To better understand the morphological differences between the developmental scores assessed, in the late-gestational study, several endpoints were quantitatively measured. Longitudinal growth was defined as the distance from the nipple end of the primary duct to the most distal terminal duct. Lateral growth was defined as the distance between the most distal terminal ducts with the collecting duct as an axis. Terminal end buds (TEBs) were defined as densely staining, bulbous structures located at the end of mammary ducts that were at least twice the width of the duct. These endpoints were measured using the Leica Application Suite (Version 3.5.0, Leica Microsystems). Quantitative measurements were recorded by one staff member; scoring was performed by two staff without knowledge of treatment, and the two scores were averaged. Mean scores for treatment groups were calculated for each time point and analyzed for statistical significance. Quantitative criteria were analyzed for statistical significance as well.

Dosimetry

Chromatographic and mass spectrometer conditions. The quantitative analysis of PFOA was performed using an Agilent 1100 High Performance Liquid Chromatograph (HPLC; Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple-quadrupole mass spectrometer (HPLC-MS/MS, Applied Biosystems/MDS Sciex, Foster City, CA).

Blank matrices and quality control. Swiss-Webster mouse serum (with trace hemolysis) was purchased from Pel-Freeze Biologicals (Rogers, AR) to prepare blank and spiked quality control (QC) samples and for calibration curve construction. Liver and brain tissues used to prepare blank and spike QC samples were obtained from nonpregnant control CD-1 animals that were sacrificed upon arrival.

A standard curve was prepared by spiking six solutions with serial dilutions of a 10,000 ng PFOA/µl standard in methanol to obtain final concentrations across the ranges of PFOA expected in the respective matrices to be analyzed. For example, for the late-gestation study, serum samples were analyzed with four separate calibration curves covering 5–50,000 ng PFOA/ml (5–100 ng/ml for control animals; 5–1000 ng/ml for 0.01 mg/kg animals; 100–5000 ng/ml for 0.1 mg/kg animals; 1000–50,000 ng/ml for 1.0 mg/kg animals). For the full-gestation study, serum samples were analyzed with one calibration curve covering 10–40,000 ng PFOA/ml. QC samples were prepared in 25 µl of Pel-Freeze mouse serum by enrichment with PFOA to obtain multiple concentrations. For every treatment group, two QC samples were used, one spiked with a low concentration of PFOA and another spiked with a high concentration of PFOA relative to the range of the calibration curve.

Calibration curves were generated by plotting the ratio of PFOA peak area to $^{13}C_2$-PFOA peak area versus concentration and were fitted to a linear regression equation with $1/x$ weighting. Batch-specific calibration curves were prepared to obtain linear curves in the ranges utilized for each treatment group. A minimum of six standards were used to generate each calibration curve and the coefficients of determination (r^2) values were 0.99 or greater for each analysis. Matrix-matched standard curves and QC pools were used to minimize potential errors associated with matrix enhancement or suppression of analyte signal due to coeluting matrix interferents.

Method accuracy and precision were determined by analyzing the QC sample repeatedly, using the complete analytical method. Accuracy was calculated as the percentage of the concentration measured compared with the theoretical concentration. The precision of the methods was determined by calculating the average relative SD of the replicate analysis of the QC materials.

Preparation of serum samples. Serum samples were prepared as previously described in Reiner *et al*. (2009). Briefly, 25 µl of mouse serum from the collected samples was transferred into 15 ml polypropylene tubes. An appropriate amount of internal standard ($^{13}C_2$-PFOA) was added to achieve the approximate midpoint of the calibration curve of the anticipated sample range. For example, for serum samples expected to be in the 5–100 ng/ml range, the standardized amount of labeled PFOA was added to the sample with 1 ml acetonitrile so that it would fall at the midpoint of the calibration curve (50 ng/ml). Formic acid (0.1M) was added to the serum to denature the proteins at a volume at least four times the serum volume. The samples were vortexed, and the cold (−20°C) acetonitrile internal standard mixture was added to precipitate proteins in a volume of at least 10 times the formic acid volume used. The sample was then vortexed and centrifuged at 2000 × g for 3 min. A 200-µl aliquot of the acetonitrile supernatant was placed in an HPLC vial with 2mM ammonium acetate buffer (pH 6.5; 1:1 vol/vol), and the PFOA concentration was determined using HPLC-MS/MS analysis.

Preparation of liver and brain samples. One gram of thawed tissue was placed in 6 ml of distilled water and ground into a homogenized mixture. Briefly, 50 µl of the liver or brain homogenate was combined with 100 µl of 0.1M formic acid and 1 ml of acetonitrile spiked with $^{13}C_2$-PFOA. The samples were vortexed and centrifuged at 3500 × g for 3 min. A 200-µl aliquot of the acetonitrile supernatant was placed in an HPLC vial with 2mM ammonium acetate buffer (pH 6.5) (1:1), and the PFOA concentration was determined using HPLC-MS/MS analysis.

Sample analysis. Each sample batch of tissue or fluids contained a maximum of 30 unknowns along with at least 10% QC samples. In addition, each sample batch included a minimum of six calibration standards, a matrix blank (containing internal standard and formic acid), and a method blank (containing internal standard, formic acid, and blank mouse serum, liver, or brain). Blanks, calibration curve standards, and QCs were all subjected to the same preparation procedures as the unknown samples. QC samples were intermixed with unknown samples. If a QC sample exceeded the range of acceptable variance from that of the theoretical value, the entire batch was rejected and new aliquots of the samples would be put through the sample preparation procedure again and rerun. Analytical batches were considered to be acceptable if the standard curve and QC samples were ± 20% of the theoretical value. Quantification of some serum samples from the 0.1 mg/kg group from the late-gestation cohort were taken from a batch run in which the high QC value was just out of the range of the 20% acceptance criteria compared with the standard curve. However, the unknown sample values were within the range of the standard curve that was verified by the low QC value, and thus, these values were deemed acceptable. The limit of quantitation (LOQ) is defined as the lowest point on the stand curve with back prediction within ± 30% of the theoretical value. For the serum samples from the full-gestation study, the LOQ ranged from 10 to 20 ng/ml, whereas the LOQ for the liver, brain, and late-gestation serum samples were 35 ng/g, 35 ng/g, and 5 ng/ml, respectively. Analytical values below the LOQ were reported as the calculated value of LOQ/$\sqrt{2}$ for purposes of statistical comparisons.

Statistical Analysis

Dams, or litter, were utilized as the unit of measure for statistical analysis throughout both studies. Thus, when more than one pup was necropsied from one dam, the values were averaged and the mean was used for statistical analysis. Data were evaluated by age and treatment using general linear model ANOVA in SAS 9.1 (SAS Institute, Inc., Cary, NC). In the late-gestation study, data were evaluated for block and treatment effects by ANOVA mixed model analysis. Due to slight variance in litter sizes, litter and litter × treatment effects were evaluated and data were assessed for normal distribution. In cases where a litter × treatment effect existed, data within a treatment group were evaluated for significant outliers calculated by Grubbs' test utilizing GraphPad's QuickCalc outlier online calculator (2005) and were removed from analysis if detected. For all measurements, data are reported as the mean ± SEM. Differences between PFOA-treated groups and controls were determined using Dunnett's *t*-tests. Differences within PFOA-treated groups over time were determined utilizing Tukey's *t*-tests. Differences between the 1.0 mg/kg full-gestation and late-gestation groups were determined using Student's *t*-tests.

In the full-gestation study, due to the lack of female controls at PND 63, no statistical differences were determined for that time point. Due to low male numbers throughout the study, the male data were considered incomplete and were statistically analyzed with low power. They are briefly discussed herein, and the data are provided in the Supplementary tables.

Net pup weights were calculated to determine if PFOA-induced increases in liver weight disguised a BW effect. They were calculated by subtracting liver weight from BW for an individual. Means were then calculated for each dose group. Relative tissue weights were calculated by dividing the absolute tissue weight by the individual BW. For relative tissue weights and dosimetry data, the values were log transformed prior to statistical analysis. For mammary gland endpoints in the late-gestation study, change in epithelial growth was calculated as the difference between the measured length at a given time point from the respective treatment mean values at PND 1. A value of $p \leq 0.05$ was used as the limit to determine statistical significance.

RESULTS

Full-Gestation PFOA Exposure Study

Body weights. A comparison of female and male offspring BWs over time are shown in Supplementary tables 1 and 2. Also shown are net pup weights, following subtraction of liver weights. PFOA (0.1–3.0 mg/kg) did not affect the absolute or net weights of either female or male offspring (Supplementary tables 1 and 2).

Liver weights. Gestational exposure to low doses of PFOA elevated the absolute and relative liver weights in both females and males compared with respective controls (Supplementary tables 1 and 2). Importantly, on PND 7, prenatal PFOA exposure elevated relative liver weights in the 0.3 mg/kg treatment group in both females and males, a dose that is lower than the previously reported LOAEL in CD-1 mice (Lau *et al.*, 2006; Wolf, *et al.*, 2008). This effect had dissipated after PND 7. In the 1.0 mg/kg group, the relative liver weights remained elevated in females on PNDs 7 and 14 ($p < 0.05$) and were also elevated on PNDs 7 and 42 in the males ($p = 0.02$). In the 3.0 mg/kg group, the relative liver weights remained elevated from PNDs 7 to 28 in the females ($p \leq 0.05$) and similarly elevated at PNDs 7, 14, 21, and 42 in the males ($p < 0.001$). These elevated liver weights are indicative of hepatomegaly, a common effect of PFOA exposure. The extent of liver weight elevation illustrates the dose-dependent effect of PFOA for this endpoint.

Brain weights. Gestational PFOA exposure decreased absolute brain weights only at PND 63 in the males of the two highest treatment groups (1.0 and 3.0 mg/kg; $p < 0.05$, Supplementary table 2); yet, there were no statistical differences in the PFOA-treated females at any time point (Supplementary table 1). Also, there were no differences in relative brain weights of either gender. These data suggest that, across dose and time, PFOA exposure has little (male) or no effect (female) on brain weight.

Mammary glands. Mammary glands from PFOA-treated groups exhibited histological characteristics of delayed epithelial growth, similar to those documented in rats neonatally exposed to endocrine-disrupting compounds (Brown *et al.*, 1998; Fenton, 2006; Fenton *et al.*, 2002) and had lower developmental scores compared with controls (Supplementary table 3). Even the lowest treatment group (0.3 mg/kg) had developmental scores that were statistically lower compared with controls at numerous time points (PNDs 14, 21, 42, and 84). Representative mammary gland whole mounts from the control, 0.3 mg/kg, and 1.0 mg/kg treatment groups at PNDs 21 and 84 are shown in Figure 1. In normal mouse mammary

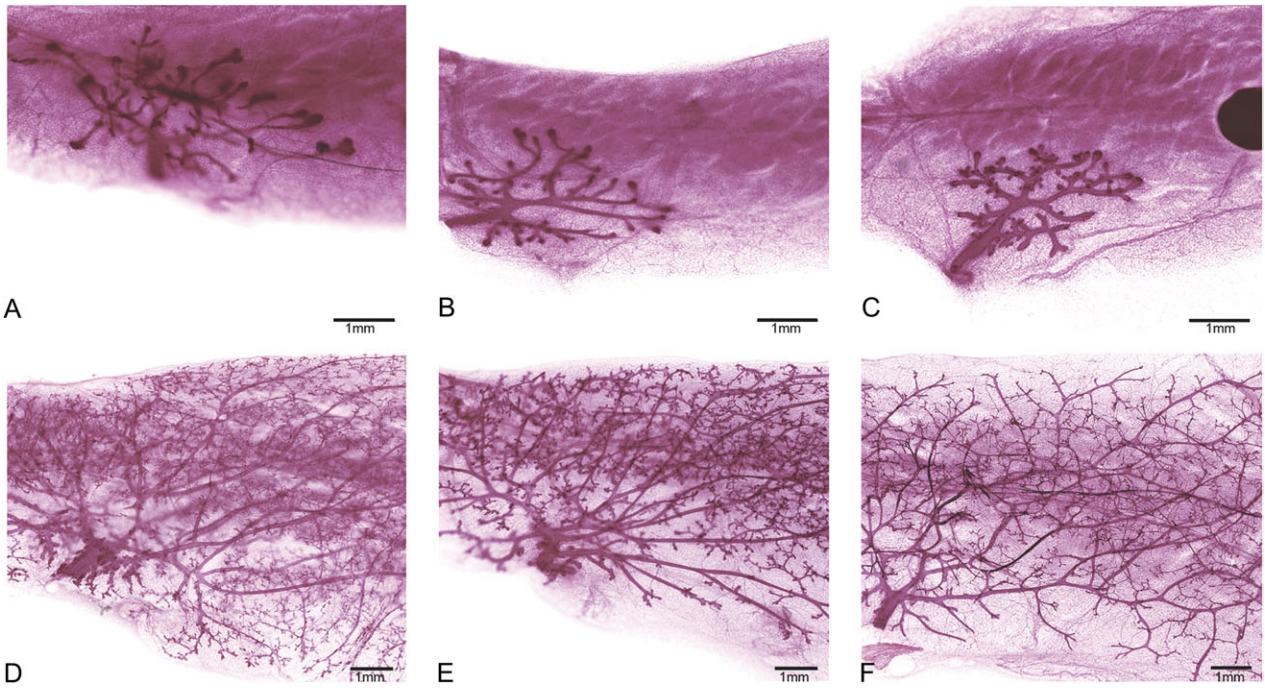

FIG. 1. Female offspring mammary gland whole mounts in the full-gestation study. Control glands from (A) PNDs 21 and (D) 84; 0.3 mg/kg glands from (B) PNDs 21 and (E) 84; 1.0 mg/kg glands from (C) PNDs 21 and (F) 84. Glands pictured are representative of mean score for each treatment group; $n = 3$–5 (PND 21) and 2–4 (PND 84). At PND 21 (top panel), PFOA-exposed glands (B and C) are smaller in overall size, with poor branching patterns and fewer visible TEBs when compared with the control gland (A). At PND 84 (bottom panel), the PFOA-exposed glands (E and F) have poor branching patterns, poor differentiation, and several TEBs. All PFOA-treated mammary glands received significantly lower developmental scores compared with controls at both time points ($p \leq 0.01$).

gland development, from PNDs 1 to 14, the primary duct extends from the nipple area toward the lymph node, branching to fill the mammary fat pad. After PND 14, the mouse mammary gland forms TEBs, which are the precursors of future branching (Watson and Khaled, 2008). As the mammary gland grows, it continues to branch and form additional TEBs until the entire fat pad has been filled. The branches extend toward the ends of the fat pad, and the TEBs disappear as they differentiate into terminal structures around PND 63 (Watson and Khaled, 2008), so that in the adult mouse at PND 84, there are no TEBs. The presence of numerous TEBs at PNDs 63 and 84 in the offspring of the PFOA-treated animals in this study are indicative of substantial mammary gland developmental delays. In the absence of control glands at PND 63, we compared our PFOA-treated glands to historical controls from previous studies (White *et al.*, 2009); thus, these data may necessitate repeating. Nevertheless, epithelial branching in treated glands did not fill the mammary fat pad as completely as the controls, which was highly evident at PND 84 (Fig. 1).

Serum PFOA concentrations. Serum PFOA concentration data from female offspring are summarized in Figure 2 and detailed in the Supplementary table 4, and male offspring data are detailed in Supplementary table 5. As would be expected, the highest concentrations of PFOA were found in the serum of pups in the 3.0 mg/kg group. In the females of the 1.0 and 3.0 mg/kg treatment groups, the mean serum PFOA concentrations peaked at PND 14 and then gradually declined through the remainder of the study. However, in the 0.3 mg/kg group, the highest serum concentrations occurred at PND 7 (Fig. 2, Supplementary table 4). The serum concentrations of all PFOA-treated groups were statistically elevated from PNDs 7 until 42 and in the two highest exposure groups (1.0 and 3.0 mg/kg) until PND 84 in females.

Unlike the adult, in the developing animal, the BW and blood volumes increase rapidly from birth to weaning. The

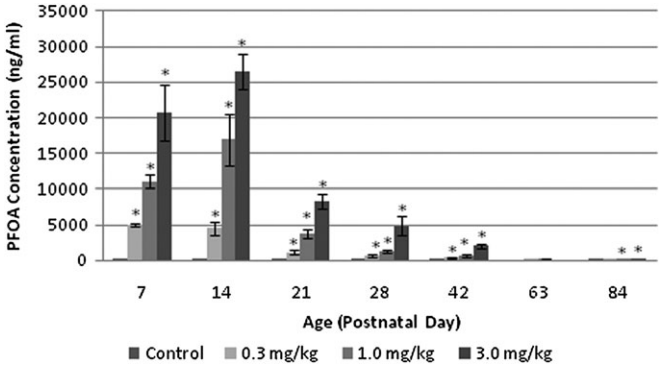

FIG. 2. Serum PFOA concentration in female offspring following the full-gestation exposure. Data presented as the mean ± SEM. Litter $n = 2$–5 (PND 7), 4–6 (PND 14), 3–6 (PND 21), 3–6 (PND 28), 4–6 (PND 42), 2–5 (PND 63), and 1–5 (PND 84). *Significant treatment effect compared with controls ($p \leq 0.01$).

increasing blood volumes and BWs of the offspring were taken into account to estimate the total blood burdens of PFOA in the pups from this study to understand when the body burden peaks. We developed a formula to calculate the estimated total amount of PFOA in the blood per pup based on weight and serum PFOA concentrations. This calculation is based on the assumption that there is approximately 58.5 ml of blood/kg BW in the mouse and that 55% of the whole blood is serum (Hoff, 2000) resulting in the equation: blood burden = [BW (58.5 ml/kg/1000) × serum concentration × 55%]. The total blood burdens were calculated using the analytical values from the HPLC-MS/MS, regardless if values were below the LOQ for each individual sample. Developmental effects are heavily influenced by timing of exposure, in relation to organogenesis, as well as dose. Thus, comparisons of total blood burdens over time may provide insight into the timing or extent of adverse effects of toxicant exposure, particularly in the developing animal. In addition, blood burdens may be more useful for interspecies comparisons "(o)wing to the gender and species difference in elimination" of PFOA and other perfluoroalkyl acids (Lau *et al*., 2007). Evaluation of total blood content of PFOA over time resulted in an inverted U-shaped curve in females (Fig. 3; Supplementary table 4), with a peak at PND 14 for all doses, which happen to occur after lactation peaks and just before pups begin to rely on solid food and water for their sustenance. As with the serum concentrations, all treatment groups had calculated total blood burdens of PFOA that were statistically greater than controls from PNDs 7 to 42.

Liver PFOA concentrations. It should be noted that the livers were not perfused before analysis; therefore, residual amounts of blood were potentially analyzed with these tissues. The concentration of PFOA was greater in liver samples of females in all treated groups compared with controls from PNDs 7 to 42 (Supplementary table 4). The highest liver concentrations of PFOA were found at PND 7, and unlike serum concentrations, the liver concentrations decreased or remained constant from PND 7 to PND 14 (Figure 2; Supplementary table 4). At the two latest time points evaluated, the mean PFOA concentrations in the liver were higher compared with concentrations in the serum (with exception of 1.0 mg/kg at 84 days). This trend has been observed in other studies (Kudo *et al*., 2007) and suggests that the liver may be a more sensitive biomarker than serum for long-term PFOA exposure estimates.

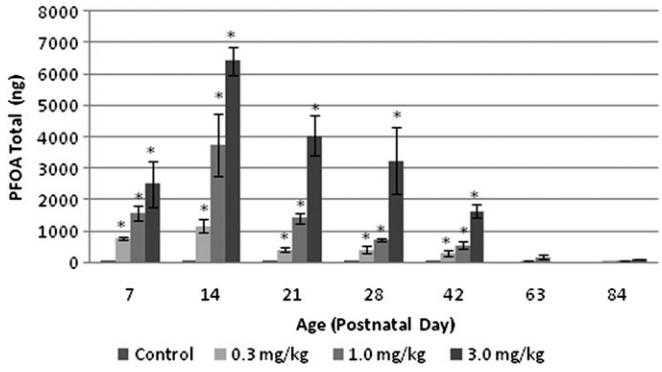

FIG. 3. Calculated PFOA blood burdens in female offspring following full-gestation exposure. Data presented as the mean ± SEM. Calculated blood burden is determined by the equation [BW (58.5 ml/kg/1000) × serum × 55%] for litters, and mean values are presented for each treatment group. Litter n = 2–5 (PND 7), 4–6 (PND 14), 3–6 (PND 21), 3–6 (PND 28), 4–6 (PND 42), 2–3 (PND 63), and 1–5 (PND 84). *Significant treatment effect compared with controls ($p \leq 0.01$).

Brain PFOA concentrations. As with the livers, the brain samples were not perfused before analysis of PFOA content. PFOA was present in the brain on PND 7 in all treatment groups but at substantially (10- to 30-fold) lower levels than in serum or liver. Judging from these diminishing trends, our data demonstrated that, relative to serum and liver, PFOA is more readily eliminated from the brain. Brain PFOA concentrations in the females remained statistically elevated compared with controls on PND 7 and PND 14 in the 0.3 and 1.0 mg/kg treatment group and from PND 7 until PND 28 in the 3.0 mg/kg group (Supplementary table 4).

Late-Gestation PFOA Exposure Study

Body weights. No significant differences in BW or net BWs were observed at any time point in the PFOA-exposed groups when compared with controls (Supplementary table 6). No differences in BW were expected at these exposure levels based on previous data from Lau *et al*. (2006).

Liver weights. Absolute liver weights in the highest treatment group (1.0 mg/kg) were significantly increased compared with controls from PND 4 through PND 7 ($p < 0.05$, Supplementary table 6). The relative liver weights were also significantly increased in this treatment group from PND 4 through 14 ($p < 0.05$, Supplementary table 6). There was no effect at the 0.1 mg/kg dose or lower. The effects in the 1.0 mg/kg group were independent of BW and demonstrate that liver weight effects are dose and exposure length dependent; the LOAEL for hepatomegaly from late-gestation exposure was 1.0 mg PFOA/kg, whereas 0.3 mg PFOA/kg was adequate to induce significant hepatomegaly in the full-gestation exposure.

Mammary glands. Upon visual observation, mammary glands of PFOA-exposed mice displayed aberrant morphology and thus were assessed lower developmental scores compared with controls (Fig. 4). It should be noted that statistical differences found in a single quantitative endpoint did not necessarily determine aberrant development; rather, all quantitative and qualitative measurements were collectively utilized to determine overall developmental mammary gland scores. At PND 14, the longitudinal epithelial growth of the mammary glands from the 0.1 and 1.0 mg/kg groups was reduced compared with controls by 14.4% ($p = 0.04$) and 37.3% ($p = 0.01$), respectively, and the change in longitudinal growth from

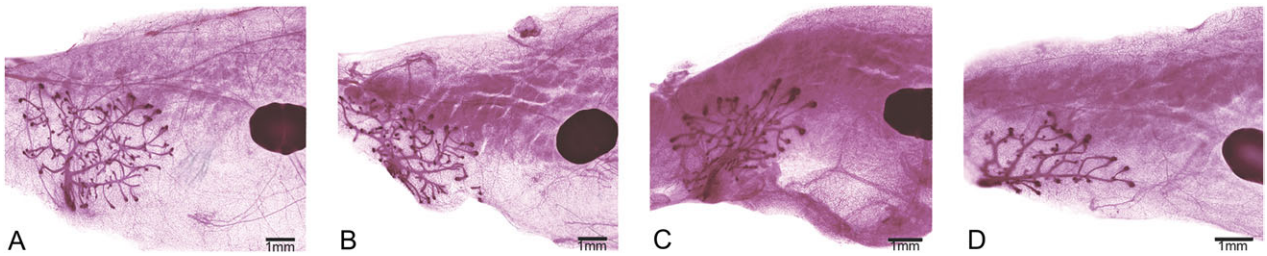

FIG. 4. Female offspring mammary gland whole mounts at PND 21 in the late-gestation study from (A) controls, (B) 0.01 mg/kg, (C) 0.1 mg/kg, and (D) 1.0 mg/kg. Glands pictured are representative of the mean score for each treatment group; $n = 3–5$. PFOA-exposed glands were smaller in size, displayed poor branching patterns, and had fewer TEBs relative to controls. All PFOA-treated mammary glands received significantly lower developmental scores compared with controls ($p < 0.05$).

PNDs 1 to 14 was reduced by 27.4% ($p = 0.005$) and 56.5% ($p = 0.002$), respectively. Developmental delays were most evident at PND 21, and all treated groups exhibited statistically lower developmental scores compared with controls ($p < 0.02$; Table 1). Developmental scores and quantitative endpoints at PND 21 are presented in Table 1; mammary gland whole mounts from PND 21 are presented in Figure 4. At PND 21, the pups in the highest dose group (1.0 mg/kg) displayed the most impaired mammary gland growth, with low mammary gland scores, poor longitudinal epithelial growth, and fewer TEBs ($p < 0.05$; Table 1). Taking all developmental criteria into consideration, the lowest dose at which mammary gland developmental abnormalities were visible was 0.01 mg PFOA/kg.

Serum PFOA concentrations. Serum PFOA concentrations in all exposed litters were statistically higher than controls at all time points and in a dose-dependent manner (Fig. 5). The highest serum concentrations were found at PND 1 and gradually declined until the end of the study. Even at the lowest dose (0.01 mg/kg), the mean serum PFOA concentration was not reduced to the levels of controls before the conclusion of the study at PND 21.

As with the full-gestational cohort, we also assessed the total PFOA blood content per pup as an indicator of body burden. In all treatment groups, the total blood PFOA content did not change from PND 7 to PND 14 and then declined substantially thereafter (Fig. 6; Supplementary table 7). PFOA blood burdens among the treated groups were statistically elevated at all time points measured ($p < 0.05$).

To better understand the developmental toxicity of PFOA, we compared the dosimetry data from the 1.0 mg/kg females of the full-gestation study to those of the late-gestation study. These comparisons are highlighted in Figure 7 and also in Supplementary tables 4 and 7 and revealed unexpected similarities in PFOA serum dosimetry. However, it should be noted that slight differences in timing of necropsies or litter size may have some influence on the serum concentrations of offspring, and thus, these comparisons have been cautiously interpreted. There were no differences detected in the serum or calculated blood burdens at PND 7 of the two study groups, although the pups in the full-gestation study received 10 additional days of exposure. Large differences were expected at this point. The full-gestation 1.0 mg/kg females had mean serum and calculated blood burdens that were nearly twofold greater than those of the late-gestation study at PND 14 and PND 21, as expected.

DISCUSSION

The present studies demonstrate that exposure to PFOA throughout gestation (GD 1–17) or during the latter half of gestation (GD 10–17) at doses 10- to 30-fold lower than previously investigated are sufficient to produce abnormal mammary gland development in CD-1 mice. In both studies,

TABLE 1
Late-Gestation Female Mammary Gland Measurements at PND 21

	Developmental score	Longitudinal growth (μm)	Lateral growth (μm)	Δ Longitudinal growth (μm)	Δ Lateral growth (μm)	TEBs	Terminal ends (TEs)
Control (*n*)	3.3 ± 0.3 (5)	4321 ± 306 (5)	5941 ± 280 (5)	3394 ± 306 (5)	4358 ± 280 (5)	40 ± 4 (5)	81 ± 12 (5)
0.01 mg/kg (*n*)	2.2 ± 0.2* (4)	3803 ± 386 (4)	5420 ± 326 (4)	3087 ± 386 (4)	3899 ± 326 (4)	33 ± 4 (4)	61 ± 8 (4)
0.1 mg/kg (*n*)	1.8 ± 0.3** (3)	3615 ± 320 (3)	4822 ± 672 (3)	2370 ± 320 (3)	3035 ± 672 (3)	24 ± 4* (3)	58 ± 4 (3)
1.0 mg/kg (*n*)	1.6 ± 0.1*** (5)	2775 ± 285** (5)	4822 ± 313 (5)	1553 ± 301** (5)	3380 ± 313 (5)	15 ± 2***(5)	47 ± 11 (5)

Note. Mammary gland measurements from late-gestation female offspring at PND 21. Data presented as the mean ± SEM.
Significant effects compared with controls, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

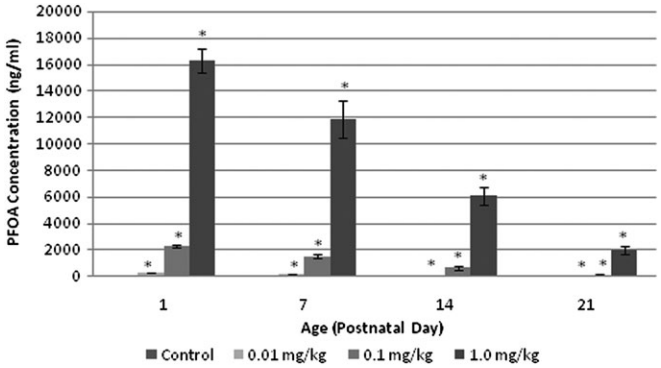

FIG. 5. Serum PFOA concentration of female offspring from the late-gestation study. Data presented as the mean ± SEM. Litter $n = 2$–7 (PND 1), 2 (PND 4), 5–11 (PND 7), 7–11 (PND 14), and 7–11 (PND 21). *Significant treatment effect compared with controls ($p < 0.001$).

low doses of PFOA elevated relative liver weights and stunted mammary gland development. Full-gestational exposure to $\geq$ 0.3 mg/kg PFOA resulted in reduced mammary gland developmental scores compared with controls at perinatal (PNDs 14 and 21), peripubertal (PND 42), and adult (PND 84) time points. Moreover, exposure to 30-fold lower doses of PFOA (0.01 mg/kg) during GD 10–17 suppressed mammary gland development as well. These data suggest that prenatal exposure to PFOA may alter mammary gland development in CD-1 mice at doses lower than investigated here. Additionally, effects on mammary tissue were observed at doses of PFOA lower than those required to exert an effect on the liver and the mammary effects persisted longer. These findings implied that in CD-1 mice, the mammary gland was more sensitive to prenatal PFOA exposure than was the liver.

In a previous study by Yang *et al.* (2009), peripubertal exposures to PFOA in Balb/C and C57Bl/6 mice resulted in mammary gland growth effects at doses $\geq$ 5 mg/kg but not at

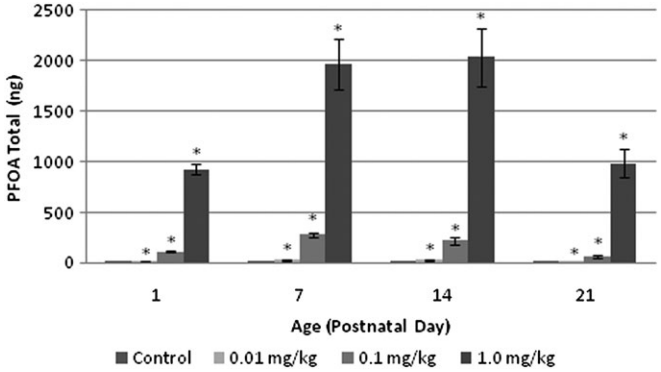

FIG. 6. Calculated PFOA blood burdens in offspring from the late-gestation study. Data presented as the mean ± SEM. Calculated blood burden is determined by the equation [BW (58.5 ml/kg/1000) × serum × 55%] for litters/individual pups, and mean values are presented for each treatment group. Litter $n = 2$–7 (PND 1), 2 (PND 4), 5–11 (PND 7), 7–11 (PND 14), and 7–11 (PND 21). *Significant treatment effect compared with controls ($p < 0.001$).

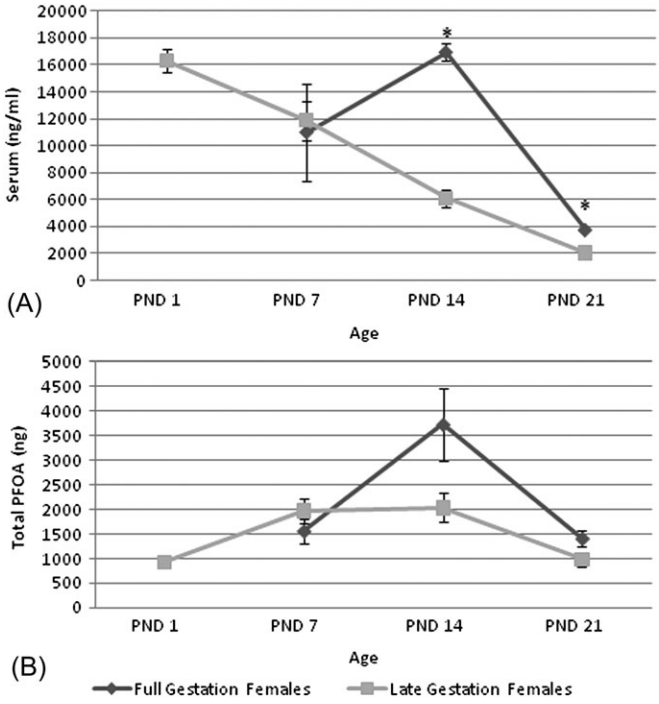

FIG. 7. Comparison of 1.0 mg/kg treatment group dosimetry in full- and late-gestation study. (A) Serum PFOA concentrations and (B) calculated PFOA blood burdens of female offspring from the 1.0 mg/kg treatment group from the full- and late-gestation studies. Data presented as the mean ± SEM. Litter $n = 7$ (PND 1), 5–11 (PND 7), 6–11 (PND 14), and 5–11 (PND 21). *Significant differences by ANOVA ($p < 0.001$).

$\leq$ 1 mg/kg, even though elevated liver weights were observed at 1 mg/kg. Thus, the mammary gland effects presented from our studies appear to be the result of an increased sensitivity in the CD-1 mouse strain. We postulate that intraspecies differences in effects are more likely due to the timing of exposure, as there are strain differences in timing of puberty (Nelson *et al.*, 1990); yet, mammary gland morphology remains fairly consistent during stages of development (i.e., puberty and pregnancy). Further research is needed to determine if the sensitivity is attributed to timing of exposure or the mouse strain utilized or if there are other novel mechanisms underlying this apparent sensitivity in CD-1 mice.

The developmental scoring method utilized in these studies, which incorporated both qualitative and quantitative endpoints, accurately predicted the long-term mammary gland developmental delays seen in PND 84 full-gestation–treated animals. Following full-gestational PFOA exposure, several TEBs remained in PFOA-treated mammary glands at PND 84. Other studies have shown that the extended presence of TEBs, in general, can lead to long-term adverse effects on the gland, including a higher risk for mammary tumor formation following exposure to carcinogens (Russo and Russo, 1978) and altered lactation (Rayner *et al.*, 2005). Although it is unclear whether there are lasting adult effects on the mammary gland due to late-gestation exposure, evaluation of mammary

tissues indicated that prenatal exposure to doses as low as 0.01 mg PFOA/kg can also lead to developmental delays. Thus, we did not identify an NOAEL for PFOA-induced mammary gland developmental effects in CD-1 mice.

In the full-gestational study, relative liver weights were elevated in the 0.3 mg/kg group, which is lower than the previously reported liver LOAEL of 1.0 mg/kg in this mouse strain (1.0 mg/kg was the lowest dose utilized; Lau *et al*., 2006; Wolf *et al*., 2008). Another recent study found that prenatal exposure to 0.3 mg/kg of PFOA elevated absolute liver weights independent of BW differences (Onishchenko *et al*., 2011). Importantly, in the late-gestation study, we used exposure periods that were half the length previously used; yet, this study produced comparable effects on liver weights at the same doses reported previously (Lau *et al*., 2006). This warrants strength to the supposition that the LOAEL for PFOA-induced liver hepatomegaly in CD-1 mice is lower than 1.0 mg/kg and that the NOAEL is approximately 0.1 mg/kg.

PFOA did not appear to affect brain weights in our studies. However, the presence of PFOA in the tissue warrants further investigation, as it may impart other effects to the brain. A recent study (Johansson *et al*., 2008) showed that low-level developmental exposure to PFOA produced behavioral effects in mice that extended into adulthood. Onishchenko *et al*. (2011) reported that prenatal exposure to 0.3 mg/kg affected activity levels in mice independent of brain weight changes. The presence of PFOA in the neonatal brain, coupled with its absence after four weeks of age, suggests that PFOA passes through the fetal mouse blood-brain barrier but is not able to pass through the fully functional barrier that is normally formed by the time of birth (Bauer *et al*., 1993).

Theoretically, animals dosed for longer periods are expected to exhibit higher serum concentrations than those exposed for shorter periods. However, as seen in Figure 7, there are similarities in the serum PFOA concentration of 1.0 mg/kg females from the full-gestation study compared with those of the late-gestation study at PND 7. Other studies have found that there are comparable serum concentrations in adult CD-1 mice following differing lengths of exposure (Lau *et al*., 2006). It is postulated that these similarities in PFOA serum concentrations are attributable to differences in clearance rates relative to exposure length. In a study by Lou *et al*. (2009), with repeated low doses of PFOA, net urinary elimination rates of PFOA were found to be high, potentially due to saturation of renal resorption. However, in the same study, urinary elimination rates were found to be low when resorption saturation was not reached. Thus, it is speculated that the longer exposure parameters for the full-gestation study offspring resulted in higher urinary elimination rates and consequently higher blood clearance rates, which may have reduced the serum PFOA concentration to comparable levels of the offspring exposed during late gestation. Additionally, it is important to consider the functional capacity of the developing urinary system in pups at this age relative to their ability to eliminate toxicants.

A temporal comparison revealed that the mean serum PFOA concentration and calculated blood burden of the 1.0 mg/kg group female offspring were greatest at PND 14 in the full-gestation study, whereas in the 1.0 mg/kg female offspring of the late-gestation study, the mean serum concentration was greatest at PND 1 and the calculated PFOA blood burden was greatest from PNDs 7 to 14 (Fig. 7, Supplementary tables 4 and 7). A similar trend to that observed in the late-gestation females was also seen in mice given a single prenatal dose of PFOA with analytically measured, not calculated, body burdens (Fenton *et al*., 2009).

After parturition, offspring of PFOA-treated lactating dams experience extended exposure via milk consumption (Fenton *et al*., 2009). In addition to the PFOA that is transported into milk from the blood supply, the grooming habits of rodents further contribute to milk-borne exposure; PFOA present in urine of PNDs 1–10 offspring is consumed by dams and subsequently recirculated back into the maternal system (Rodriguez *et al*., 2009). Due to the relationship between maternal grooming habits and pup exposure, pup urinary excretion rates can also influence the availability of PFOA for recycling by the dam. Therefore, higher urinary excretion rates in pups may indeed account for greater serum PFOA concentrations and blood burdens at the second week of age in those exposed throughout gestation compared with those exposed from GD 10 to 17.

Importantly, serum PFOA concentrations found in the 0.01 mg/kg group are lower than those measured in young children living in areas highly contaminated with PFOA, such as the Ohio River Valley in West Virginia (Emmett *et al*., 2006). Emmett *et al*. (2006) reported that the 2004 mean serum PFOA concentrations were near 600 ng/ml in children aged 2–5 years from the Ohio River Valley area. Approximately 2 years later, and after some exposure intervention, Frisbee *et al*. (2009) reported mean serum concentration of 77.6 and 59.9 ng/ml in children from the C8 Health Project of age < 12 and 12–19 years, respectively. The late-gestation study conducted here observed mice only until weaning, which would be equivalent to 2–3 years of age in humans. Due to the elimination rates of PFOA in female mice ($t_{1/2}$ ~16 days, Lou *et al*., 2009), it can be assumed that serum PFOA levels of pups in the 0.1 mg/kg treatment group would have decreased after PND 21 to levels approaching, if not lower, than those reported in children by Frisbee *et al*. (2009). Importantly, from the full-gestation study mammary glands, we observed that the developmental effects were not transient and in fact were apparent at adulthood, although PFOA exposure had ceased 12 weeks earlier. These findings are of great concern considering that children are likely to be exposed to PFOA prenatally, as well as throughout life. Therefore, it is important to determine the human relevance of the observed endpoints in relation to internal dosimetry to establish a benchmark dose for the PFOA mammary gland effects.

It is also critical to establish a mode of action (MOA) for the developmental mammary gland growth effects following

PFOA exposure to determine whether this MOA is biologically relevant to humans. The liver toxicity and general developmental effects of PFOA are believed to be mediated by activation of peroxisome proliferator–activated receptor-alpha (PPAR-α) (Abbott *et al*., 2007; Rosen *et al*., 2009; Wolf *et al*., 2008); yet, there are data to suggest that PFOA-induced mammary gland effects are mediated by other pathways (Zhao *et al*., 2010). Previous studies using PPAR-α knockout (KO) mice reported normal lactation after exposure to PFOA (Zhao *et al*., 2010). Changes in serum progesterone reported in the study of Zhao *et al*. (2010) suggest that stimulatory, and potentially inhibitory, mammary gland effects may be mediated through endocrine disruption. PFOA may indirectly affect branching morphogenesis through modulation of progesterone synthesis (Zhao *et al*., 2010), and other endocrine-disrupting effects of PFOA have been reported (White *et al*., 2011). In the future, we plan to further compare mammary gland developmental effects in PPAR-α wild-type and KO mice after prenatal PFOA exposure to determine whether PPAR-α activation is involved in this outcome.

In summary, an NOAEL was not achieved in either study for PFOA-induced mammary gland effects in CD-1 mice, as altered mammary gland development was observed in offspring of dams treated with the lowest PFOA dose utilized in each study. As these are the lowest doses of PFOA tested in CD-1 mice thus far, additional studies are necessary to determine an NOAEL, as well as to establish the human relevance of PFOA-induced mammary gland effects.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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