Developmental Toxicity of Perfluorooctanoic Acid in the CD-1 Mouse after Cross-Foster and Restricted Gestational Exposures

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Perfluorooctanoic acid (PFOA) is a persistent pollutant and is detectable in human serum (5 ng/ml in the general population of the Unites States). PFOA is used in the production of fluoropolymers which have applications in the manufacture of a variety of industrial and commercial products (e.g., textiles, house wares, electronics). PFOA is developmentally toxic and in mice affects growth, development, and viability of offspring. This study segregates the contributions of gestational and lactational exposures and considers the impact of restricting exposure to specific gestational periods. Pregnant CD-1 mice were dosed on gestation days (GD) 1–17 with 0, 3, or 5 mg PFOA/kg body weight, and pups were fostered at birth to give seven treatment groups: unexposed controls, pups exposed in utero (3U and 5U), lactationally (3L and 5L), or in utero + lactationally (3U + L and 5U + L). In the restricted exposure (RE) study, pregnant mice received 5 mg PFOA/kg from GD7–17, 10–17, 13–17, or 15–17 or 20 mg on GD15–17. In all PFOA-treated groups, dam weight gain, number of implantations, and live litter size were not adversely affected and relative liver weight increased. Treatment with 5 mg/kg on GD1–17 increased the incidence of whole litter loss and pups in surviving litters had reduced birth weights, but effects on pup survival from birth to weaning were only affected in 5U + L litters. In utero exposure (5U), in the absence of lactational exposure, was sufficient to produce postnatal body weight deficits and developmental delay in the pups. In the RE study, birth weight and survival were reduced by 20 mg/kg on GD15–17. Birth weight was also reduced by 5 mg/kg on GD7–17 and 10–17. Although all PFOA-exposed pups had deficits in postnatal weight gain, only those exposed on GD7–17 and 10–17 also showed developmental delay in eye opening and hair growth. In conclusion, the postnatal developmental effects of PFOA are due to gestational exposure. Exposure earlier in gestation produced stronger responses, but further study is needed to determine if this is a function of higher total dose or if there is a developmentally sensitive period.

Key Words: perfluorooctanoic acid; developmental toxicity; cross-foster; prenatal sensitivity.

Perfluorooctanoic acid (PFOA) is a member of the perfluorooctyl acid (PFAA) family of compounds. These chemicals have a carbon backbone with hydrogen replaced by fluorine and include a functional group, such as a carboxylic or sulfonic acid. The fluorine content renders PFAAs stable, inert, oleophobic and hydrophobic, and resistant to high temperatures (Key et al., 1997; Prescher et al., 1985). These properties make PFAAs useful for a variety of commercial and industrial applications, including use as flame retardants, paint additives, surfactants, and as water and stain repellants for use on clothing, upholstery, carpets, and as coatings on paper products for food containers.

PFOA and other fluorochemicals persist in the environment and have been detected in wildlife and humans (Giesy and Kannan, 2002; Hansen et al., 2002; Harada et al., 2004; Hoff et al., 2003; Kannan et al., 2002, 2005; Kubwabo et al., 2004; Olsen et al., 2003a,b). In the general population of the United States, the average concentration of PFOA in serum was estimated from 645 adult blood donors as 5 ng/ml, ranging from 1.9 to 52.3 ng/ml (Olsen et al., 2003b). Studies of a population residing near a production facility in West Virginia detected levels ranging from 298 to 370 ng/ml, and the predominant exposure route for this population was considered to be the community water supply (Emmett et al., 2006). In that study, the individuals with substantial occupational exposure had serum levels ranging from 422 to 999 ng/ml, with a median of 775 ng/ml. These compounds bioaccumulate, are readily absorbed into the body, but poorly eliminated (Burril et al., 2002; Johnson and Ober, 1979; Johnson et al., 1984; Seacat et al., 2002; Van den
Heuvel et al., 1992). PFOA is estimated to have a half-life (t₁/₂) of 16–19 days in mice and 4–9 days in male rats (Kemper and Jepson, 2003; Kudo et al., 2002; Lau et al., 2005; Van den Heuvel et al., 1991). The t₁/₂ in humans for PFOA is estimated at 3.8 years (Ehresman et al., 2005; Olsen et al., 2005). The toxic effects of PFOA and other PFAAs have been recently reviewed, and in mammals, they include liver hypertrophy, body weight reduction, carcinogenicity, reproductive toxicity, reduction in serum cholesterol, triglycerides, and thyroid hormone levels (Kennedy et al., 2004; Kudo and Kawashima, 2003; Lau et al., 2004).

The reproductive toxicity of a related PFAA, perfluorooctanesulfonate (PFOS), has been examined in several species. In teratology studies conducted in rabbits (Case et al., 2001; Gortner, 1982), rats (Grasty et al., 2003, 2005; Lau et al., 2003; Luebker et al., 2005a,b; Thibodeaux et al., 2003), and mice (Lau et al., 2003; Thibodeaux et al., 2003), gestational exposure to PFOS decreased prenatal and postnatal survival of offspring, and developmental effects included reduced fetal body weight, increased liver weight, cleft palate, edema, delayed maturation of the lung, delays in ossification of bones, and cardiac abnormalities. PFOS also produced dose-dependent effects on neonatal survival and retarded the growth and development of neonates in rats exposed from gestational day (GD) 2–21 to doses ranging from 1–10 mg/kg/day and mice exposed on GD1–18 to 1–20 mg PFOS/kg/day (Lau et al., 2003), and these effects were also reported in a two-generation study in rats exposed to doses ranging from 0.1 to 3.2 mg PFOS/kg/day (Luebker et al., 2005a,b). In the studies by Lau et al. (2003), rat or mouse neonates exposed to PFOS in utero died within hours after birth, and cross-fostering of the exposed neonatal rats did not improve survival. Further studies in the rat with exposure to 25 or 50 mg PFOS/kg/day on GD19 and 20, or to 25 mg/kg/day across various 4-day gestational intervals, demonstrated that the neonatal lethality could be produced by treatment restricted to the late gestational period and suggested that impaired lung function could be involved (Grasty et al., 2003, 2005).

The developmental toxicity of PFOA is similar to that of PFOS and has also been evaluated in the rat with a two-generational study using doses ranging from 1 to 30 mg PFOA/kg/day (Butenhoff et al., 2004) and in the mouse with gestational exposures ranging from 1 to 40 mg PFOA/kg/day from GD1–17 (Lau et al., 2006). In his study, Lau et al. (2006) examined the developmental toxicity of PFOA in the CD-1 mouse with exposures on GD1–17 ranging from 1 to 40 mg PFOA. Although the number of embryos implanted per litter was not affected at any dose, exposure to 5 mg/kg or higher increased the incidence of full-litter resorptions, prenatal losses were significant in the 20 mg/kg group, and no litters survived at 40 mg/kg. At 5 mg/kg or higher, postnatal survival was reduced, and growth retardation and developmental delay were observed.

PFOA readily crosses the placenta and is secreted in milk. In the rat, PFOA and PFOS have been detected in placenta, fetus, amniotic fluid, and milk, and these chemicals have also been found in human breast milk (Hinderliter et al., 2005; Kennedy et al., 2004; Kuklenyik et al., 2004; So et al., 2006). Thus, in the study of Lau et al. (2006), pups born to and nursing on PFOA-dosed dams were exposed in utero and throughout lactation. At exposures of 5 mg/kg/day or higher, Lau et al. found that fetal weights were unaffected but that neonatal body weight gain was significantly decreased. However, body weights of PFOA-exposed pups were not different from controls by 6.5 weeks of age. These findings raise the question of whether factors other than gestational exposure alone contributed to the body weight deficits. For example, could exposure during lactation alone be sufficient, could effects on milk quality or quality be involved, was suckling behavior affected, or were the maternal behaviors of exposed dams involved? The present study uses a cross-foster design to address some of these questions, particularly regarding the contribution of gestational versus lactational exposures to the postnatal body weight deficits, neonatal lethality, and developmental delay. Additionally, the present study was designed to evaluate weight gain after weaning in more detail than was done previously, by monitoring body weight on a weekly basis and examining the potential for different responses in male and female pups. Considering the evidence that PFOS can produce early neonatal lethality with only a brief exposure late in gestation in the rat, it was also of interest to determine if PFOA had a specific gestational window for producing some or all of its developmental effects. Thus, the present study also included a restricted exposure (RE) protocol to evaluate the impact of dosing at specific gestational periods. The doses in the cross-foster and RE studies were based on the outcomes reported by Lau et al. (2006) with selection of 3 mg/kg/day, a dose expected to affect body weight but not survival and 5 mg/kg/day, a dose expected to affect both survival and weight gain.

MATERIALS AND METHODS

**Perfluorooctanoic Acid**

PFOA (ammonium salt; >98% pure) was purchased from Fluka Chemical (Steinheim, Switzerland). NMR analysis kindly provided by 3M Company (St Paul, MN) indicated that approximately 98.9% of the chemical was straight-chain isomers, and the remaining 1.1% was branched isomers. For all studies, PFOA was dissolved in deionized water and prepared fresh daily.

**Animals**

All animal studies were conducted in accordance with the guidelines established by the U.S. Environmental Protection Agency’s Office of Research and Development/National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee. Procedures and facilities were consistent with the recommendations of the 1996 National Research Council’s “Guide for the Care and Use of Laboratory Animals,” the Animal Welfare Act, and Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Raleigh, NC), where females were bred overnight, and the sperm-positive females, considered to be at GD0, were shipped to our facility on the same day. Upon arrival, mice were housed individually in polypropylene cages with Alpha-dri (Shepherd Specialty Papers, Kalamazoo, MI) bedding and provided pellet chow (LabDiet 5001, PMI Nutrition
International LLC, Brentwood, MO) and tap water ad libitum. Animal facilities were controlled for temperature (20–24°C) and relative humidity (40–60%) and kept under a 12-h light-dark cycle.

Cross-Foster Study

The study was performed in two blocks, spaced 4 weeks apart, with 56 mice per block. Upon arrival at the animal facility, mice were weighed and randomly assigned to one of the three treatment groups, vehicle control which received deionized water (n = 48), 3 mg PFOA/kg body weight (n = 28) or 5 mg PFOA/kg body weight (n = 36). On GD1–17, mice were weighed and dosed by gavage at 10 ml/kg volume. On GD18–19, mice were monitored at frequent intervals until parturition (2, 4, and 10 P.M. and 2, 6, and 10 A.M.), and the date and time of birth, number of live and dead pups, and number of pups of each sex were recorded and litters were weighed by sex. Each of the scheduled monitoring periods, litters of similar ages were fostered to yield the following seven exposure groups: (1) control pups fostered to control dams (control), (2) control pups fostered to dams dosed during gestation with 3 mg PFOA/kg (3U, lactational exposure), (3) control pups fostered to dams dosed during gestation with 5 mg PFOA/kg (5U, lactational exposure), (4) pups exposed in utero to 3 mg PFOA/kg fostered to control dams (3U, in utero exposure), (5) pups exposed in utero to 5 mg PFOA/kg fostered to control dams (5U, in utero exposure), (6) pups exposed in utero to 3 mg PFOA/kg fostered to dams dosed during gestation with 3 mg PFOA/kg (3U + L, in utero and lactational exposure), and (7) pups exposed in utero to 5 mg PFOA/kg fostered to dams dosed during gestation with 5 mg PFOA/kg (5U + L, in utero and lactational exposure). All pups were either assigned to foster litters or killed for collection of blood and liver. No pups remained with their original birth mother. Foster litters included 10 pups with equal representation of males and females (where possible).

RE Study

Seventy timed-pregnant CD-1 mice were received on the day after mating and were housed, fed, and weighed as described for the cross-foster study. Mice were assigned to treatment groups and dosed orally by gavage as follows: vehicle control dosed with deionized water at 10 ml/kg on GD7–17 (n = 12); 5 mg PFOA/kg on GD7–17 (n = 14), GD10–17 (n = 14), GD13–17 (n = 12), or GD15–17 (n = 12) or 20 mg PFOA/kg on GD15–17 (n = 6). On GD18–19, mice were monitored at frequent intervals until parturition (2, 4, and 8 P.M. and 2, 6, and 10 A.M.), and the date and time of birth, number of live and dead pups, and number of pups of each sex were recorded and litters were weighed by sex. Litters were culled to 10 pups with equal representation of male and female (where possible).

Postnatal observations and necropsy. In both the cross-foster and RE studies, litters were observed daily and weighed on postnatal day (PND) 1, 2, 3, 4, 7, 10, 14, 17, and 22. Eye opening was monitored beginning on PND10. Hair growth was recorded with a Nikon D70 digital camera on PND8 (RE study), 11 (cross-foster study), and 17 (both studies). On PND22, pups were weighed and weaned, and males and females were housed separately. After weaning, one male and one female pup (selected randomly from each litter) and all dams were weighed and killed. Blood samples were collected, and serum was prepared and stored frozen for PFOA analysis. Livers were removed and weighed. Uteri were removed from the adult females and stained with 2% calf serum, were analyzed with the samples to ensure the accuracy and reliability of the data (Kuklenyik et al., 2005).

RESULTS

Cross-Foster Study

Maternal weight, reproductive outcomes, and pup birth weight (Table 1). PFOA had no detrimental effect on maternal weight (Table 1), although mean weight was elevated in the 3-mg PFOA/kg group and average weight gain was elevated in the 3- and 5-mg PFOA-exposed groups. PFOA exposure did not affect the number of uterine implantation sites or the number of live pups per litter. There was an increase in the incidence of whole litter loss (WLL) in the 5-mg/kg group (dams were considered to have WLL if there were uterine implantation sites, but no live pups were found with the dam on or after the expected parturition date). The mean number of implantation sites in uteri of the five dams with WLL was 9.2, but the average weight gain from GD1–17 was not significantly different from nonpregnant females (3.4 ± 0.5 g for non-pregnant controls vs. 2.9 ± 0.9 g for WLL dams). It appeared that the dams in the 5-mg/kg group with WLL resorbed their examined for effects of PFOA on mammary gland development (see White et al., 2006) and the serum samples were collected from female mice killed at intervals appropriate for that evaluation. All serum samples were stored frozen in polypropylene vials, shipped on dry ice to the Centers for Disease Control and Prevention’s National Center for Environmental Health laboratory, and then kept at −40°C until analysis. Measurement of the concentrations of PFOA in serum was performed through a multiple reaction monitoring experiment using online solid-phase extraction (SPE) coupled to reversed-phase high-performance liquid chromatography (HPLC)-tandem mass spectrometry as described by Kuklenyik et al. (2005). Necessary dilution of the serum samples was performed in two steps. First, at least 10 μl serum was diluted to 1 ml with water in a 2-ml eppendorf tube, then a second dilution was performed by aliquoting the appropriate amount of the dilute into an autosampler vial, adding blank calf serum and 0.1M formic acid, and injected into a commercial column switching system allowing for concentration of PFOA on a C18 SPE column. The column was automatically positioned in front of a C8 analytical HPLC column for chromatographic identification of PFOA. Detection and quantification utilized negative-ion TurbolonSpray ionization, a variant of electrospray ionization, tandem mass spectrometry. The isotope-labeled internal standard used for quantification was 13C2-PFOA. Quality control materials, prepared in calf serum, were analyzed with the samples to ensure the accuracy and reliability of the data (Kuklenyik et al., 2005).

Data analysis. Data are presented as means and standard errors. Pup data were analyzed on a gender basis. Percentage data were arcsine transformed. Statistical significance of treatment effect was determined for each outcome variable using analysis of variance (ANOVA) that included block effect. When a significant treatment effect was detected, each treatment group was tested for difference from the control group using Dunnett’s test or a pairwise t-test with a Bonferroni adjustment for multiple comparisons. Linear regression analysis was used to test for trends in pup birth weight and PND22 relative liver weight with respect to total administered dose. Serum PFOA concentration data were analyzed using log10 (transformation) to calculate means and standard errors, and ANOVA was performed followed by pairwise t-test with Bonferroni adjustments for multiple comparisons, where appropriate. Effects of block, litter, sex, pup age, and treatment were examined in mixed effects linear models. Analysis of pup data was done separately for each sex, and for female pups, the PFOA levels at each age were compared after adjusting for block and treatment.
Exposure to PFOA at either 3 or 5 mg/kg throughout gestation was associated with significant deficits in pup survival and development. While the mean body weight of surviving pups on PND1 was not different between the control and PFOA groups (excludes dams with WLL), PFOA reduced the survival of pups to weaning in all exposure groups. The incidence of pup death in the 3U and 5U groups was significantly reduced only in the 5U group (Fig. 1). The incidence of pup death in the 3U and 5U groups was significantly different from controls by PND4, and only 65% of the pups were alive on PND22. Survival in all groups did not differ from the controls after weaning.

Eye opening and growth of body hair were used as landmarks of postnatal developmental progress. The mean day of eye opening (i.e., the day that both eyes were fully open) was 14.8 ± 0.07 in control pups and was significantly delayed (p < 0.05) in the 3U, 5U, and 5U + L pups (15.8 ± 0.2, 15.9 ± 0.1, and 15.9 ± 0.4, respectively). In general, as shown in Figure 2A, the delay was progressively more severe across dose and exposure type (3 < 5 mg/kg; L < U < L + U). The emergence of body hair was also delayed in PFOA-exposed pups in 5U, 3U + L, and 5U + L litters. On PND11, pups in the control litters had appeared on pups from the 5U, 3U + L, and 5U + L groups. The developmental delay resolved with time, as the PFOA-exposed pups had open eyes and grew body hair by PND17.

PFOA exposure produced body weight deficits in both male and female pups from PND1 to 22 (Table 3). Both males and females of the 5U, 5U + L, and 5U + L groups had reduced body weights throughout that period (with the exception of 3U + L in utero on PND1). In utero exposure to 3 mg PFOA/kg had less effect on pup body weight than 5 mg/kg. Similarly, lactational exposure had less effect than in utero in lactational exposure. The average weight gain from PND1–22, regardless of sex, was significantly reduced in the 3U, 5U, and 5U + L groups.

Males recovered from the PFOA-induced body weight deficits within a week of weaning with the exception of the 5U + L pups, which recovered to control levels by PND36 (Fig. 3A). However, female offspring of the 5U and 5U + L groups displayed persistent deficits in body weight throughout the postnatal period (Fig. 3B).
groups continued to show body weight deficits as late as PND85 (Fig. 3B). After PND85, males of the 3U group showed increased body weight relative to controls, while females were no longer different from controls. Body weights were monitored out to 35 weeks of age (data after PND92 not shown). At 35 weeks of age, the males in the 3U group continued to have significantly elevated body weights (*p < 0.05), while males of all other groups and females in all exposure groups did not differ from the controls.

Serum levels of PFOA in dam and pups (Table 4). The mean serum concentrations of PFOA of the adult females at weaning (23 days after the last administered dose) showed a dose-related increase. The control dams that received foster litters that had been exposed in utero (3U and 5U groups) were exposed to PFOA through maternal grooming behavior and ingestion of their pups’ urine and feces. At weaning, the mean concentrations of PFOA in these control dams were substantial but significantly lower than any of the dams that were dosed during gestation.

PFOA in the pups’ serum was evaluated at weaning (3 weeks of age) for one male and one female pup from each litter and in female pups at 6 and 9 weeks of age. In male and female pups, the mean serum concentration of PFOA was highest in the 5U + L group, and the second highest levels were in the 3U + L group. It is worth noting that the mean serum concentration of PFOA in 3-week-old pups at weaning is very similar for those exposed in utero and those exposed via milk only (3U vs. 3L, 5U vs. 5L, not significantly different). One possible explanation for this may be that the levels in the groups exposed in utero decreased with elimination during the 3 weeks from birth to weaning.

FIG. 1. Survival of the pups from birth to weaning (PND1–22) was reduced only in the litters with both gestational and lactational exposures to 5 mg PFOA/kg. The increased postnatal lethality was significant beginning on PND4. 3L, 3U, 3U + L: dams dosed from GD1–17 with 3 mg PFOA/kg/day and pups fostered to have exposure during lactational, in utero, or both, respectively. 5L, 5U, 5U + L: dams dosed from GD1–17 with 5 mg PFOA/kg/day and pups fostered to have exposure during lactation, in utero, or both, respectively. a = *p < 0.001 versus control on same PND.
while the control pups fostered to exposed dams acquired an increasing body burden of PFOA via the milk during that period. In the female pups, the levels of PFOA decreased from 3 to 9 weeks of age but remained significantly elevated at 9 weeks compared to controls.

**RE Study**

Maternal weight, reproductive outcomes, and pup birth weight and survival (Table 5). PFOA did not adversely affect maternal weight or weight gain during pregnancy, although females in the groups dosed earlier in gestation gained significantly more weight than the controls. PFOA exposure did not affect the number of uterine implantation sites, percentage of litter loss, or number of pups per litter at birth. Pup weight at birth was significantly decreased for male, but not female, pups in litters exposed to 5 mg PFOA/kg from GD7–17 and 10–17 and to 20 mg PFOA/kg on GD15–17. The percent of pups surviving from PND1–22 was significantly decreased only in the litters exposed to 20 mg PFOA/kg on GD15–17 (mean time to death was 6.9 ± 0.7 days).

**FIG. 2.** The incidence of eye opening from PND12–19 is shown for pups in the cross-foster study (A) and the restricted exposure study (B). (A) The delay in opening of both eyes observed in pups exposed to PFOA was more severe in pups exposed during both gestation and lactation and in pups exposed to 5 mg PFOA/kg in utero. Pups exposed to 3 mg/kg showed lesser effects than those exposed to 5 mg/kg, and the pups exposed only during lactation opened their eyes on a schedule similar to the controls. (B) Pups exposed to 5 mg PFOA/kg during progressively later stages of gestation and subsequently throughout lactation also showed delayed eye opening with more severe responses as the exposures began earlier in gestation. 3L, 3U, 3U + L: dams dosed from GD1–17 with 3 mg PFOA/kg/day and pups fostered to have exposure during lactational, in utero, or both, respectively. 5L, 5U, 5U + L: dams dosed from GD1–17 with 5 mg PFOA/kg/day and pups fostered to have exposure during lactation, in utero, or both, respectively.

**FIG. 3.** Body weights of male and female pups after weaning on PND22 were monitored for each of the exposure groups. (A) Body weights of males exposed to 5 mg PFOA/kg both in utero and during lactation were reduced on PND29. On PND92, males exposed to 3 mg PFOA/kg only in utero had significantly increased body weights. (B) Female weight deficits persisted in the groups exposed to 5 mg PFOA/kg either in utero or in utero plus during lactation, and the significance levels on each day are indicated in the inset table. 3L, 3U, 3U + L: dams dosed from GD1–17 with 3 mg PFOA/kg/day and pups fostered to have exposure during lactational, in utero, or both, respectively. 5L, 5U, 5U + L: dams dosed from GD1–17 with 5 mg PFOA/kg/day and pups fostered to have exposure during lactation, in utero, or both, respectively.
### TABLE 4
Serum Levels of PFOA in Cross-Foster Dams and Male Pups at Weaning (3 weeks) and Female Pups at 3, 6, and 9 Weeks of Age

<table>
<thead>
<tr>
<th></th>
<th>Dams at weaning</th>
<th>Male pups at 3 weeks</th>
<th>Female pups at 3 weeks</th>
<th>Female pups at 6 weeks</th>
<th>Female pups at 9 weeks</th>
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<tr>
<td></td>
<td>N</td>
<td>PFOA (ng/ml)</td>
<td>N</td>
<td>PFOA (ng/ml)</td>
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<tr>
<td>Control dam with</td>
<td>13</td>
<td>24 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
<td>19 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
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<td>control pups</td>
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<td>Control dam with</td>
<td>11</td>
<td>10,047 ± 1169&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11</td>
<td>9562 ± 1067</td>
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<td>3 mg pups (3U)</td>
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<td>Control dam with</td>
<td>13</td>
<td>13,797 ± 1439&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12</td>
<td>11,548 ± 1398</td>
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<td>5 mg pups (5U)</td>
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<td>3 mg/kg dam with</td>
<td>11</td>
<td>23,645 ± 1979</td>
<td>11</td>
<td>8371 ± 530</td>
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<td>29,470 ± 2554</td>
<td>12</td>
<td>18,074 ± 2614</td>
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<td>5 mg/kg dam with</td>
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<td>35,231 ± 3756</td>
<td>13</td>
<td>10,388 ± 1306</td>
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<td>control pups (5L)</td>
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<td>5 mg/kg dam with</td>
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<td>36,900 ± 4749</td>
<td>12</td>
<td>24,948 ± 4291&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11</td>
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<tr>
<td>5 mg pups (5U + L)</td>
<td></td>
<td></td>
<td></td>
<td>22,114 ± 3677&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9</td>
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Note. Means ± SEM. Treatment groups: dams dosed on GD1–17 with 3 or 5 mg PFOA/kg/day. Pup exposure was L = lactational, U = in utero, and U + L = in utero + lactational.
<sup>a</sup>Female pups: mean of all treatments within time period: 3 > 6 > 9 weeks, p < 0.001.
<sup>b</sup>All groups: control < all PFOA-exposed groups, p < 0.001.
<sup>c</sup>Dams: 3U < 3L, 5L, 5U + L, 5U, p < 0.001.
<sup>d</sup>Dams: 5U < 3L, 5U + L, 3L, p < 0.001; 3U < 5L, p < 0.05.
<sup>e</sup>Male pups: 3U + L < 3L, 5L, 5U, p < 0.01; 3U + L > 3L, 5L, p < 0.05.
<sup>f</sup>Female pups 6 weeks: 3U + L < 3L, 5L, 5U, p < 0.01; 3U + L > 3L, 5L, p < 0.001.
<sup>g</sup>Female pups 6 weeks: 5L > 3U, p < 0.01; 5L > 5U, p < 0.05.
<sup>h</sup>Female pups 6 weeks: 5U + L > 3L, 3U, 5U, p < 0.001; 5U + L > 3U + L, p < 0.05.
<sup>i</sup>Female pups 3 weeks: 5U + L > 3L, 5U, p < 0.01; 5U + L > 3L, 5U, p < 0.05.
<sup>j</sup>Female pups 6 weeks: 5U + L > 3L, 5U, p < 0.001; 5U + L > 3U + L, p < 0.01.
<sup>k</sup>Female pups 9 weeks: 5U + L > 3L, 5U, 3U + L, p < 0.05; 5U + L > 3U, p < 0.01.

### TABLE 5
Restricted Exposure: Effects of PFOA on Maternal Weight, Reproductive Outcomes, and Pup Survival

<table>
<thead>
<tr>
<th>PFOA dose and gestational dosing period</th>
<th>Control GD7–17</th>
<th>5 mg/kg GD7–17</th>
<th>5 mg/kg GD10–17</th>
<th>5 mg/kg GD13–17</th>
<th>5 mg/kg GD15–17</th>
<th>20 mg/kg GD15–17</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pregnant females&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Maternal weight (g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.4 ± 3.7</td>
<td>55.1 ± 1.5</td>
<td>57.5* ± 1.1</td>
<td>51.3 ± 2.6</td>
<td>50.9 ± 1.7</td>
<td>54.8 ± 2.6</td>
</tr>
<tr>
<td>Maternal weight gain (g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.1 ± 2.8</td>
<td>29.0* ± 1.1</td>
<td>31.1** ± 1.0</td>
<td>24.2 ± 2.3</td>
<td>25.1 ± 1.5</td>
<td>27.3 ± 2.4</td>
</tr>
<tr>
<td>Litters (no. with live pups)</td>
<td>7</td>
<td>13</td>
<td>13</td>
<td>10</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Implants (no. per litter)</td>
<td>11.4 ± 1.8</td>
<td>13.4 ± 0.5</td>
<td>13.8 ± 0.4</td>
<td>10.8 ± 1.3</td>
<td>11.8 ± 1.1</td>
<td>14.0 ± 0.6</td>
</tr>
<tr>
<td>No. of pups per litter&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.4 ± 2.0</td>
<td>11.5 ± 0.8</td>
<td>12.3 ± 0.6</td>
<td>9.8 ± 1.5</td>
<td>10.5 ± 1.1</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>Litter loss (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19.8 ± 11.7</td>
<td>14.7 ± 4.0</td>
<td>10.9 ± 2.6</td>
<td>21.9 ± 10.8</td>
<td>10.6 ± 3.4</td>
<td>14.2 ± 4.0</td>
</tr>
<tr>
<td>Male birth weight (g)</td>
<td>1.7 ± 0.1</td>
<td>1.5** ± 0.04</td>
<td>1.5 ± 0.02</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.04</td>
<td>1.4* ± 0.04</td>
</tr>
<tr>
<td>Female birth weight (g)</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.03</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.03</td>
</tr>
<tr>
<td>Pup survival PND1–22 (%)</td>
<td>100</td>
<td>87.7 ± 4.1</td>
<td>94.6 ± 3.3</td>
<td>100</td>
<td>100</td>
<td>23.3** ± 23.3</td>
</tr>
</tbody>
</table>

Note. Means (dams) or litter means (pups) ± SEMs.
<sup>a</sup>Pregnancy verified by the presence of uterine implantation sites.
<sup>b</sup>Excludes not pregnant; maternal weight on GD17 and weight gain from GD1–17.
<sup>c</sup>Number of pups per litter: mean no. of live + dead at birth.
<sup>d</sup>Percent litter loss = (no. of implants – no. of live pups)/(no. of implants) × 100; includes litters with no live pups.
<sup>e</sup>p < 0.05 and **p < 0.01.
Effects of PFOA on absolute and relative liver weight. Relative liver weights were significantly increased in PFOA-exposed dams at weaning (23 days after the last administered dose), with the exception of dams exposed to 5 mg/kg on GD15–17 (Fig. 4A). When considering total administered dose, there was a trend for increasing relative liver weight with increasing dose ($p < 0.001$). PFOA exposure also increased the relative liver weights of male and female pups in all exposure groups (Fig. 4B and 4C).

Pup development and growth. Eye opening and body hair growth were delayed in pups exposed to 5 mg PFOA/kg on GD7–17 and 10–17. The mean day at which both eyes were fully open was significantly delayed in pups exposed to 5 mg PFOA/kg on GD7–17 and 10–17 compared to control (16.5 ± 0.2, 16.0 ± 0.3, and 14.6 ± 0.1, respectively, $p < 0.01$). The delay was progressively more severe as the exposure began earlier, and thus the total dose increased (Fig. 2B). On PND8, body hair growth was evident in the controls, but the pups in the GD7–17 and 10–17 litters remained hairless. These effects resolved over time, and by GD17, PFOA-exposed pups had open eyes and body hair.

PFOA exposure significantly reduced male and female pup body weights on PND1–22 (Fig. 5). The specific days and degree of statistical significance for body weight deficits differed slightly between groups, across time, and for male and female pups, as shown in the tables beneath each graph. After weaning (Fig. 6), the male pups continued to exhibit significant deficits in body weight in the groups exposed on GD7–17 and 10–17. Male body weights recovered to control levels after 10 weeks (PND71) for the GD10–17 group and after 11 weeks (PND78) for the GD7–17 pups. The female pup body weights after weaning were not significantly different from control, except for the group exposed on GD10–17, which was only different on PND29. After 23 weeks (PND161) of age, the weights of females in the GD13–17 group increased above control ($p < 0.05$) (data not shown).

Serum levels of PFOA in dam and pups (Table 6). The group of dams exposed to 20 mg/kg on GD15–17 had the highest mean levels of PFOA at weaning (23 days after the last administered dose). The lowest PFOA serum concentration was in dams dosed with 5 mg/kg on GD15–17. In PFOA-exposed pups, the mean serum levels increased with longer durations of dosing, and the highest levels at weaning were found in those exposed GD7–17. Serum levels were only measured at PND22 for male pups, but in female pups, the levels were determined at 22, 29, and 32 days of age and serum PFOA decreased across time.

**DISCUSSION**

The present study showed that *in utero* exposure to PFOA (in the absence of lactational exposure) was sufficient to produce...
developmental delay and body weight deficits in neonatal mice. In the cross-foster study, gestational exposure was critical to the failure to gain weight, as growth deficits in the group exposed in utero alone were similar to those in the group exposed both in utero and during lactation. Only those pups exposed to PFOA in utero (U or U + L) exhibited developmental delay and reduced birth weight and postnatal growth deficits. This outcome suggests that during gestation, PFOA is altering growth regulation in the developing fetus and that the alteration may persist as the effects on growth continue during the postnatal period.

Lactational exposure alone did not produce significant developmental delay or neonatal mortality. The pups of the 5L group had only transient decreases in body weight on PND2–4 and at weaning, even though these pups had serum
PFOA levels at weaning that were comparable to the 5U pups. PFOA is eliminated in milk by lactating rodents and thus control pups fostered to a PFOA-exposed dam would be exposed during lactation. The amounts of PFOA transferred in milk are expected to be low, as in a repeated dosing study in which pregnant rats were dosed daily throughout pregnancy and lactation, the amount of PFOA in milk was approximately 10 times less than the steady-state concentrations in maternal plasma (Hinderliter et al., 2005). However, even though the levels in milk may be low, exposure continues throughout lactation for the 3L and 5L pups, and at weaning, the pups had accumulated PFOA to levels comparable to that found in the pups exposed in utero only (3L vs. 3U, 5L vs. 5U, not statistically different). Postnatal pharmacokinetics might be expected to differ between the groups exposed in utero and those exposed via lactation alone, and this may also play a role in the different responses of these groups. The lack of response in the 3L and 5L pups also suggests that exposure to PFOA throughout gestation did not affect maternal behavior, the ability of the dams to lactate, or in some manner alter the quality or quantity of the milk. If these dam-dependent mechanisms were occurring, then control pups fostered to a treated dam would be expected to have growth deficits and this was not the case.

PFOA exposure during the earliest stages of gestation (GD1–6) is not required to produce the developmental toxicity observed in this study, and exposure to higher doses late in gestation (GD15–17) can be sufficient to affect postnatal weight gain. Restricting exposure to successively later developmental periods during the second and third week of gestation was effective in reducing body weight and delaying eye opening and body hair growth. The severity of these responses was greater in groups exposed earlier and longer (GD7–17 and 11–17) versus later and for shorter times (GD13–17 and 15–17). It is not possible to attribute this to a developmentally sensitive period as the mean serum levels of PFOA were significantly higher in the pups exposed for longer periods and the effects may simply be due to higher total dose. However, although the serum mean levels in the GD10–17 and GD13–17 groups were similar, the GD10–17 (but not the GD13–17) exposure reduced male pup birth weight, delayed eye opening, emergence of body hair, and produced postnatal growth deficits that persisted after weaning.

PFOA exposure resulted in persistent effects on body weight in the female offspring. In the cross-foster study, male pups of the 5U group recovered to control levels within a week of weaning and the 5U+L males by 36 days, but the female pups in these groups continued to have weight deficits out to 85 days of age. These recovery findings are similar to those reported in a previous study (Lau et al., 2006), in which body weights of males and females exposed to 5 mg/kg/day on GD1–17 (comparable to the 5U+L group of the present study) were evaluated at 6.5 and 13 weeks. The previous study did not monitor body weight as early or as frequently as the present study and did not provide information for in utero only exposure and thus would not detect some of the effects which are reported in the present study. It is not clear why there was an apparent sex difference in the persistence of the weight deficits. Additional research may be needed to establish whether a differential response in postweaning recovery of body weight actually exists between male and female mice exposed to PFOA in utero.

Although in utero exposure alone appeared to be sufficient to produce effects on body weight and developmental delay, this was not the case for neonatal lethality, as only the 5U+L group had a significant increase in the incidence of postnatal

### Table 6

<table>
<thead>
<tr>
<th>PFOA dose and gestational period</th>
<th>Dams PND22</th>
<th>Male pups PND22</th>
<th>Female pups PND22&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Female pups PND29</th>
<th>Female pups PND32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GD7–17</td>
<td>N</td>
<td>PFOA (ng/ml)</td>
<td>N</td>
<td>PFOA (ng/ml)</td>
<td>N</td>
</tr>
<tr>
<td>5 mg/kg GD7–17</td>
<td>12</td>
<td>69 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>32 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>5 mg/kg GD7–17</td>
<td>14</td>
<td>24,843 ± 1840</td>
<td>10</td>
<td>8680 ± 1091&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>5 mg/kg GD10–17</td>
<td>14</td>
<td>25,643 ± 1686</td>
<td>12</td>
<td>6495 ± 297</td>
<td>13</td>
</tr>
<tr>
<td>5 mg/kg GD13–17</td>
<td>11</td>
<td>20,259 ± 2627</td>
<td>10</td>
<td>5364 ± 673</td>
<td>10</td>
</tr>
<tr>
<td>5 mg/kg GD15–17</td>
<td>12</td>
<td>16,104 ± 2312&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>4771 ± 762</td>
<td>9</td>
</tr>
<tr>
<td>5 mg/kg GD15–17</td>
<td>12</td>
<td>53,460 ± 11,024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>20 mg/kg GD15–17</td>
<td>5</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>18 ± 6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>10 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>PND22 Male pups</td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Female pups PND29</td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Female pups PND32</td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

Note. Means ± SEMs.

<sup>a</sup>Female pups: mean of all treatments within PND: 22 > 29 and 32, p < 0.001.
<sup>b</sup>Control < all PFOA-exposed groups, p < 0.001.
<sup>c</sup>Male pups: 5 mg/kg GD7–17 > 15–17, p < 0.01.
<sup>d</sup>PND22: GD7–17 > 10–17, 13–17, 15–17, p < 0.001.
<sup>e</sup>Dams: 5 mg/kg GD15–17 < 7–17, 10–17, p < 0.05.
<sup>f</sup>PND22: GD15–17 < 10–17 and 13–17, p < 0.001 and p < 0.05, respectively.
<sup>g</sup>PND32: GD15–17 < 7–17, 10–17, 13–17, p < 0.001.
<sup>h</sup>Dams: 5 mg/kg GD15–17 > all 5 mg/kg exposure groups, p < 0.05.
pup deaths. This may be a function of body burden as the highest serum levels in dams and pups were in that group. However, it is difficult to use that information to interpret/explain the early postnatal lethality as the 5U and 5U + L pups would be expected to have similar body burdens at birth. Although the 5U + L pups would have ongoing exposure via milk, deaths in that group were in the first week after birth which limits the duration of lactational exposure. From the RE study, it is also apparent that exposure late in gestation is sufficient to induce early postnatal lethality and that this was dose dependent as lethality was seen after 20 mg PFOA/kg GD15–17 but not after 5 mg/kg on GD15–17. This neonatal lethality appears similar to effects reported for PFOS in developing rat fetuses, where dose-dependent lethality occurred within the first week after birth, and restricting exposure to the late gestational period was sufficient for induction of the lethality (Grasty et al., 2003; Lau et al., 2003; Thibodeaux et al., 2003).

PFOA exposure increased relative liver weight in dams and pups. Exposure late in gestation (GD15–17) and lactational exposure in the absence of gestational exposure were sufficient to produce liver enlargement detectable at weaning in the pups. In general, the increased relative liver weights appeared closely related to the PFOA serum levels in dams and pups. Liver hypertrophy is a well-documented response to PFOA and PFOS in many species, and these compounds activate the peroxisome proliferator–activated receptor (PPAR) pathway, inducing peroxisome proliferation, changing liver enzymatic activities, affecting lipid metabolism and transport, and decreasing cholesterol and triglyceride (Kennedy et al., 2004; Peraza et al., 2006; Sohlenius et al., 1994). The weight gain deficits that we observed in PFOA-exposed pups could also be related to PFOA activation of PPAR. The PPARα pathway regulates lipid homeostasis, and if this pathway was involved in the response of the PFOA-exposed mouse fetuses, altered regulation of lipid catabolism and metabolism may be involved in the growth deficits of the pups. Studies underway in our laboratory with the PPARα KO mouse will be useful in delineating the role of PPARα in the mode of action for developmental toxicity. However, other factors may also contribute to the failure of pups to thrive, and effects on maternal-fetal behaviors and interactions could also be involved. White et al. (2006) report morphological changes in the mammary glands of PFOA-exposed dams and suggest that delayed development of these glands could be attributed to poor suckling behaviors of the PFOA-exposed pups. Suckling is known to stimulate mammary gland development and milk production, and it is possible that the poor weight gain in PFOA-exposed pups is related to a failure to stimulate the mammary gland. These issues will require further evaluation.

In conclusion, the present study demonstrated that exposure to 5 mg/kg PFOA in utero is sufficient to produce developmental toxicity and that lactational exposure alone is not a major contributor. This study also showed that PFOA exposure early in gestation is not required and that exposure late in gestation can be sufficient to increase liver weight, reduce survival, induce developmental delay, and produce deficits in postnatal weight gain.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr Jack Reidy at the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention in Atlanta for their technical expertise and assistance in the evaluation of the levels of PFOA in serum.

REFERENCES


