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Gestational and lactational exposure to potassium perfluorooctanesulfonate (K^+PFOS) in rats: Toxicokinetics, thyroid hormone status, and related gene expression

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ABSTRACT

Perfluorooctanesulfonate (PFOS), a persistent and accumulative compound, is widely distributed in humans and wildlife. Human exposure can occur early in development, as evidenced by the detection of PFOS in umbilical cord blood and breast milk. As part of a developmental neurotoxicology study for which developmental endpoints, including those related to the developing nervous system, have been reported separately, groups of 25 pregnant Sprague Dawley rats were given daily oral doses of either vehicle control or potassium PFOS (K^+PFOS) at 0.1, 0.3, and 1.0 mg/kg-d from gestation day (GD) 0 (day positive for mating) through postnatal day (PND) 20. An additional 10 pregnant females per treatment group were treated through GD 19 and sacrificed on GD 20 in order to obtain maternal and fetal serum and tissue samples at the end of gestation. The present paper reports the results of samples of serum, liver, brain, and thyroid glands taken at various times to evaluate: (1) serum, liver, and brain PFOS concentrations by LC–MS/MS to establish the relationship between PFOS concentrations and study outcomes; (2) serum thyrotropin (TSH) concentrations by RIA; (3) thyroid follicular cell proliferation index by Ki-67 immunohistochemical staining; (4) thyroid follicle epithelial cell height and colloidal area by histomorphometric analysis; (5) selected liver mRNA transcripts by quantitative RT-PCR. PFOS concentrations in dam and pup serum, liver, and brain increased across treatment groups in approximate proportion to the proportional increases in maternal K^+PFOS dose, and sex differences in PFOS concentrations were not apparent in pups on PND 21. In pups from K^+PFOS maternal dose groups on PND 72, serum PFOS had decreased to about 3 and 11% of PND 21 concentrations in males and females, respectively, and liver PFOS had decreased to about 17% of PND 21 concentrations in both sexes. Liver PFOS concentrations were approximately 0.6–0.8 times serum PFOS in GD 20 fetuses, and increased to about 2–4 times serum concentrations on PND 4 and 21. GD 20 fetal and PND 4 pup brain PFOS concentrations were approximately 33% of the corresponding serum concentrations, dropping to approximately 10% by PND 21, in contrast to dam brain PFOS concentrations, which were approximately 4–9% of serum PFOS concentrations. Compared to controls, Cyp2b2 mRNA was increased (2.8-fold) in the 1.0 mg/kg-d treatment-group dams on GD 20. In male pups on PND 21, Cyp4A1, ACoA, and Cyp2b2 were increased 2.1-, 1.5-, and 1.8-fold, respectively, and Cyp7A1 was decreased 3.5-fold. Serum TSH and thyroid follicular morphology were not altered by K^+PFOS treatment. The mean number of proliferating thyroid follicular cells was increased 2.1-fold over control in GD 20 female fetuses from 1.0 mg/kg-d-treated dams, yet the highest individual count was similar to that of controls (116 versus 113 in controls).

1. Introduction

Perfluorooctanesulfonate (PFOS, $C_8F_{17}SO_3^-$) has been found to be widely distributed in samples from humans and wildlife [1–3]. PFOS is exceptionally resistant to environmental and metabolic degradation [4,5], and has been shown to bioconcentrate and biomagnify in the marine food web [6]. Due to evidence of widespread presence, persistence, and accumulation of PFOS in the environ-

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ment, 3M Company, the major manufacturer of PFOS and other compounds formed from perfluorooctanesulfonyl fluoride that may potentially degrade to form PFOS, announced on 16 May 2000 that 3M Company would discontinue manufacture of these materials by the end of 2002. There has been interest in the environmental and health properties of PFOS at an international level [7].

Children are known to be exposed to PFOS [8,9], and these exposures can occur from gestational and lactational transfer of PFOS [10–17]. As reviewed by Olsen et al. [18 this issue], there are several groups of investigators that have studied the association of PFOS concentrations in human maternal and/or umbilical cord blood to birth outcomes, and one recent study followed developmental landmarks in infants through approximately 18 months of age [19].

The developmental toxicity of PFOS has been studied extensively in laboratory rats and mice [20–32], and one developmental study in rabbits has been reported [27]. In the developmental and reproductive studies with rats and mice, it has been demonstrated that neonates can be exposed to PFOS from *in utero* and lactational exposures [20,22]. Effects on gestation length, birth weight, postnatal growth and developmental delays, and neonatal survival have been noted [20,22–25,32]. Structural anomalies have been noted in fetal rats and mice at doses that affect maternal weight gain and food and/or water consumption [26,27] and include cleft palate, anasarca, ventricular septal defect, and enlargement of the right atrium. Structural defects were not observed in rabbits at maternally toxic dose levels [27]. Increased motor activity and decreased habituation have been noted in developmental neurotoxicological evaluations in rats and mice [28,29,31]; however, learning and memory do not appear to be affected [20,22,31].

Neonatal mortality and delayed postnatal growth in rat and mouse pups exposed to PFOS *in utero* are pronounced effects in these laboratory species that occur at doses not affecting the maternal rodents in an obvious manner. In exploring the potential etiology of these effects in laboratory rats and mice, several investigations have focused on lung development [20,23,24], cholesterol metabolism [25], the potential role of activation of the nuclear receptor peroxisome proliferator activated receptor α (PPARα) [32 this issue], and thyroid hormone status [22,25,26,31]. At the present time, neonatal mortality resulting from *in utero* exposure of rats to PFOS is hypothesized to result from alterations in lung function at birth, perhaps through a direct interaction of PFOS with components of pulmonary surfactant [23,33]. Alterations in cholesterol metabolism did not appear to relate to altered developmental outcomes [25]. Recent studies suggest that PFOS, unlike the eight-carbon perfluorinated carboxylate, perfluorooctanoate (PFOA), may not act via PPARα activation to produce the majority of observed developmental effects [32,34].

Thyroid hormones have numerous important roles in development [35–38] and are critical for mammalian brain development and maturation in that they control expression of genes involved in myelination, cell differentiation, migration, and signaling [35,36]. PFOS exposure has been associated with maternal and offspring hypothyroxinemia without a compensatory elevation of TSH in laboratory rats [22,25,26]. Although hypothyroxinemia was noted in a dose-dependent manner in pregnant mice on gestation day (GD) 6 by Thibodeaux et al. [26], it was not present on GD 12 or 18 in their study or in pregnant mice on GD 18 in a study reported by Fuentes et al. [39]. Neither was hypothyroxinemia observed in neonatal mice from PFOS-treated dams [22]. The ability of the pituitary to respond to hypothalamic thyrotropin-releasing hormone to release TSH in response to decreased thyroid hormone production after treatment with propylthiouracil was not altered in rats by co-treatment with PFOS [40]. The activity of choline acetyltransferase, an enzyme sensitive to thyroid hormone status, was marginally but statistically-significantly reduced in rat pups from PFOS-treated dams; however, activity in the hippocampus was unaffected [22]. PFOS-exposed rats appear to maintain a euthyroid state despite significant reductions in serum total thyroid hormones, likely due to competition for binding sites between PFOS and thyroid hormones in rat serum, leading to an adequate supply of free hormone while reducing the concentration of hormone carried on serum binding proteins [40–42].

The PFOS-induced hypothyroxinemia reported in previous developmental studies [22,25,26] led us to evaluate thyroid status and histomorphological factors associated with thyroid follicles during the course of a developmental neurotoxicological study in rats. The maternal, birth outcome, litter, and developmental neurotoxicity endpoints have been reported separately in a companion article to this [31 this issue]. Herein, we report the results of thyroid parameters, in addition to concentrations of PFOS in samples of serum, liver, and brain taken at various times during the study as well as and the results of quantitative evaluation of a select set of liver mRNA transcripts associated with liver hypertrophic modes of action, thyroid hormone and cholesterol metabolism, and liver cell proliferation.

2. Methods

2.1. Study design

The work presented in this article represents the analysis of biological samples collected during the course of a developmental neurotoxicity study involving gestational and lactational exposure to potassium PFOS (K^+PFOS, CASRN 2795-39-3, Lot number 217 (86.9% pure) 3M Company, St. Paul, MN) in rats. The details of treatment as well as results of birth outcomes, litter parameters, and neurological investigations have been reported in a companion article [31 this issue]. Briefly, groups of 25 pregnant Sprague Dawley rats (maternal rats) were given daily oral doses of either vehicle control (0.5% Tween 20 in water) or K^+PFOS (solublized in 0.5% Tween 20 in water due to limited water solubility of K^+PFOS) at 0.1, 0.3, and 1.0 mg/kg-d GD 0 (day positive for mating) through postnatal day (PND) 20. An additional 10 pregnant females per treatment group were treated through GD 19 and sacrificed on GD 20 in order to obtain maternal and fetal serum and tissue samples at the end of gestation. Pups were allowed to nurse until PND 21 and evaluated for developmental neurotoxicity landmarks specified per protocol throughout PND 72. Rats were euthanized by carbon dioxide asphyxiation. There was no evidence of treatment-related effect on postnatal growth and survival.

Aliquots of the dosing solutions were analyzed for concentration, stability, and homogeneity. The LC–MS/MS analyses indicated that the dosing solution aliquots were 97–100% of the target concentration and all were stable and homogeneous.

All experiments involving live animals were performed in laboratory accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and all procedures were reviewed and approved by facility's Institutional Animal Care and Use Committees (IACUC). Animal care and procedures were followed according to the US Department of Health and Human Services guide for the care and the use of laboratory animal guideline [43].

2.2. Determination of PFOS concentrations in serum, liver, and brain

Serum samples for PFOS analysis were obtained from dams on GD 20, PND 4, and PND 21, fetuses (pooled by litter) on GD 20, pups (pooled by litter) on PND 4, and individual male and female offspring on PND 21 and 72. Samples of livers for PFOS analysis were obtained from dams on GD 20, fetuses and pups (pooled by litter) on GD 20 and PND 4, respectively, and individual offspring on PND 21 and 72. Samples of brains for PFOS analysis were obtained from dams on GD 20, fetuses and pups (pooled by litter) on GD 20 and PND 4, respectively, and individual offspring on PND 21. Samples of serum, liver, and brain were snap frozen and remained frozen at approximately −80 °C until processing for analysis.

New Zealand newborn calf serum (Invitrogen, Carlsbad, CA) and liver and brain homogenates obtained from naïve Sprague Dawley rats (male and female, 10–12 weeks old) were used as the blank matrices to prepare the appropriate matrix-matched PFOS standard curves.

Due to limited sample volume and size, serum and liver samples from GD 20 fetus and PND 4 pups were pooled by litter. PFOS concentrations were determined by LC–MS/MS as described in Chang et al. [41]. Briefly, liver samples were homogenized in water (1 part liver and 4 parts water, w/w) with IKA® WERKE Ultra-Turrax T25 homogenizer at 20,000 rpm for ∼1.5 min followed by sonication in a water bath sonicator (30 min). After adding $^{18}O_2$-PFOS internal standard, 100 μL serum samples and/or liver homogenate aliquots were further treated with 1 mL of 1.0N formic acid, 100 μL of saturated ammonium sulfate (serum samples only), and 300 μL of water followed by solid phase extraction (SPE).

For the determination of PFOS concentrations in brain collected on GD 20 (maternal rats and their pooled fetuses), PND 4 (pups, pooled by litter), and PND 21 (male

and female pups), approximately 0.2 g of brain was weighed and homogenized with deionized water in a clean polypropylene tube. The ratio between brain and water was 1:4 (w/w). After homogenization, 100 μL of the whole homogenate was further digested with 100 μL 1.0 N KOH. The mixture was vortexed for 2 h at room temperature, followed by the addition of 1 mL 1.0N formic acid, 400 μL of saturated ammonium sulfate, and 5 mL of acetonitrile. The solution was mixed using a mechanical shaker for 30 min at room temperature followed by centrifugation (2500 × *g*, 5 min). The organic layer was transferred to a clean polypropylene tube and evaporated. One milliliter of 1.0N formic acid and 100 μL saturated ammonium sulfate were added to the remaining aqueous solution followed by vortex and SPE.

SPE extractions were based on 100 μL of sample matrix and utilized Waters Oasis® hydrophilic–lipophilic balance (HLB) 3 mL columns with column conditioning, column loading, column wash, and column elution performed as described in Ehresman et al. [44]. The instruments used for analysis of PFOS were API 4000 mass spectrometer (for serum and liver samples) and API 5000 mass spectrometer (for brain samples). Both instruments were from Applied Biosystems/MDS-Sciex Instrument Corporation and were configured with Turbo Ion Spray (pneumatically assisted electrospray ionization source) in negative-ion mode. A Mac-Mod ACE ® C-18, 5 μm, 75 mm × 2.1 mm i.d. HPLC column with a flow rate of 0.25 mL/min was used for PFOS analysis. The gradient condition for the mobile phase started with 30% acetonitrile and 70% 2 mM ammonium acetate and ramped to 90% acetonitrile and 10% 2 mM ammonium acetate in 5.5 min. All source parameters were optimized under these conditions according to manufacturer's guidelines. Mass spectroscopy transition ions (in atomic mass units, or amu) monitored were 499 → 80 for PFOS and 503 → 84 for the internal standard, $^{18}O_2$-labeled PFOS.

2.3. Serum TSH measurements

Serum samples collected on GD 20 (maternal rats and fetuses (pooled by litter), PND 4 (maternal rats and pups (pooled by litter)), and PND 21 (maternal rats and male and female pups) were analyzed for TSH levels using the Biotrak™ rat thyroid stimulating hormone ^{125}I assay system (Amersham Pharmacia Biotech, Piscataway, NJ). The assay is based on the competition between unlabelled TSH (present in the serum samples) and a fixed quantity of ^{125}I-TSH for a limited number of TSH antibody binding sites. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody, measured by gamma counter, was inversely proportional to the concentration of TSH present in the serum.

2.4. Thyroid histology and morphometry

On PND 4 and PND 21, thyroids from 1 pup/(sex litter) from 10 randomly selected litters in each treatment group were collected and preserved in 10% neutral-buffered formalin. The thyroids were then embedded in paraffin, processed via standard histological procedures, sectioned, and stained with hematoxylin and eosin (H&E). Offspring thyroids obtained from the control and highest maternal dose (1.0 mg/kg-d) group were evaluated microscopically first. Because there were no significant toxicological findings in the offspring thyroids obtained from the highest maternal dose group compared to controls, further evaluation of the thyroids from the lower maternal dose groups was not performed. The histopathological examination of H&E-stained thyroid sections also included a simple morphometric analysis, consisting of measurement of thyroid follicular epithelial cell height and dimensions of colloid contained within thyroid follicles.

For thyroid follicular epithelial cell height measurements, digital images of thyroid glands were captured using an Olympus BX-51 microscope equipped with an Olympus DP-70 digital camera. Linear measurements of epithelial cell height were made using Image Pro® Plus version 5.1 software (Media Cybernetics, Silver Spring, MD). For each thyroid slide, 5 follicles were chosen for the follicular epithelial cell height measurement; and in each follicle, 3 cells (located approximately at 12, 4, and 8 o'clock positions) were measured for the cell heights. Measurements extended from the deep aspect of nuclear membranes up to the luminal surface of selected follicular epithelial cells. The mean and standard deviation were calculated for all 15 measurements on each thyroid slide, and the group mean and standard deviation was calculated for each treatment group.

For thyroid follicular area measurements, digital images for each thyroid slide were captured as described above and Image Pro® Plus software was used for measurements. Long and short axis measurements were performed on the colloid mass of the 10 largest follicles that appeared in each digital image. The mean and standard error were calculated for all 10 measurements on each thyroid slide, and the group mean and standard error was calculated for each treatment group.

2.5. Thyroid proliferation assay

Ki-67 immunohistochemical staining was performed on thyroid glands obtained from GD 20 fetuses for the evaluation of any PFOS-related alterations in cellular proliferation. GD 20 control male and female fetal thyroids (n = 6 and 7, respectively) and 1.0 mg/kg-d dose group male and female fetal thyroids (n = 6 and 5, respectively) were preserved in 10% neutral-buffered formalin and embedded in paraffin followed by sectioning. The sections were then evaluated for cellular proliferation via Ki-67 immunohistochemical staining using monoclonal mouse anti-rat Ki-67 antigen (DAKO Corporation, Carpinteria, CA) and a standard avidin–biotin (ABC) staining procedure. All procedures were performed according to the manufacturer's guidelines.

2.6. Quantitative RT-PCR

To evaluate whether PFOS treatment elicited any changes in hepatic gene expression, liver samples from dams and fetuses (pooled by litter) on GD 20 and male pups on PND 21 were analyzed by quantitative real-time PCR for selected mRNA transcripts. Genes associated with various nuclear receptors, thyroid hormones, and hepatic conjugation enzymes were examined (Table 1). The samples that underwent RT-PCR transcript profiling on selective genes were livers (preserved in RNALater®, Applied Biosystems) from control and highest-dose group (1.0 mg/kg-d) obtained on GD 20 (maternal rats and fetuses) and PND 21 (male pups only).

To isolate mRNA, approximately 25 mg liver tissue was homogenized in 600 μL of lysis buffer using a ground glass on glass Duall® tissue grinder. Samples were further homogenized by passing 5 times through a 27 gauge needle using a sterile 1-mL syringe. The liver homogenate was loaded onto Qiagen RNeasy® spin columns followed by DNAse digestion according to the manufactures protocol using Qiagen RNAse free DNAse set. The RNA was eluted in 35 μL of RNase free water and quantified by measuring the absorbance at 260 nm using the Nanodrop™ ND1000 spectrophotometer.

2.6.1. RT-PCR instrumentation

mRNA sequences for target genes were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and gene specific primers were designed using IDT PrimerQuest℠ (www.idtdna.com). DNA standards for RT-PCR quantitation were made by PCR of a reverse transcription reaction using random primers, specific primers, or oligo dT primers with Qiagen's HotStarTaq Master Mix (Qiagen, Valencia, CA). After verification by gel electrophoresis, the standards were further purified using Qiagen's QIAquick™ chromatography columns. The standards were quantitated by measuring absorbance at 260 nm. The identities and the corresponding primer sequences used in this study are listed in Table 1. Reverse transcription with the Qiagen's Omniscript RT kit was performed using random nonamer primers and

Table 1
Primer sequences.

Gene	Upper primer	Lower primer	Length (bp)	GenBank Reference
18s rRNA	CGC CGC TAG AGG TGA AAT TCT T	CAG TCG GCA TCG TTT ATG GTC	149	M11188
ACoA	TGG AGA GCC CTC AGC TAT GG	CGT TTC ACC GCC TCG TAA G	338	NM_017340
PCNA	GTG AAC CTC ACC AGC ATG TCC AAA	ACA GCT GTA CTC CTG TTC TGG GAT	198	NM_022381
Malic Enzyme	AGG CCT CTT TAT CAG TAT CCA C	CCA TCC CGT TAC AAC CAA	140	AH002199
Dio1	TCT GCC TGA GAG GCT CTA TGT GAT	TTT CCA GAA CAG CTC GGA CTT CTT	104	NM_021653
Por	AAG ACA TGG ACG TAG CCA AGG TGT	TGA GAT GTC CAA CTA CAT GTG CAT	173	NM_031576.1
ApoA1	GAT GAA AGC TGC AGT GTT GGC TGT	GTT CAG CTG TTT GCC CAA AGT GGA	199	NM_012738.1
Cyp1a1	TGA CCT CTT TGG AGC TGG GTT TGA	ATG TCG GAA GGT CTC CAG GAT GAA	199	NM_012540
Cyp3a1	TTT CGC CCA GAA AGG TTC AGC AAG	AGA GCA AAC CTC ATG CCA ATG CAG	107	NM_173144
Cyp4a1	ACC TCT TTC ACT CCC GTG TG	GTG TGT GGC CAG AGC AGA GA	344	M57718
Cyp7a1	TTT CTA CAT GCC CTT CGG ATC A	TTC GCT TCT TCC AAC CAC GTA T	238	NM_012942
Cyp2b2	TCC AGA CAC CTT CAA TCC TGA GCA	GAC AAA TGC GCT TTC CTG TGG AGA	98	XM_001062335
Ugt1A common	GCC TTC CCA GTG TTA GTC ATT	CTT GGT TTG GGA ACA CAA TAG	200	U20551
Ugt1a1	AGG AAG TAC CCT GTG CCA TTC CAA	TCT TGA TCA AAG ACA CTC CGC CCA	80	U20551
Ugt1a6	AAC GCG GAC ACG ACA TTG T	AAC TGG CAG CAA AGT GGT TGT T	156	U20551
Ugt2b	GTG CAC TGG AGG AAG TCA TAG ACA	TAG GCT GGT CAT GGT GAA TCC TTG	81	XM_001062335

1.5 μg of total RNA according to the manufacturer's protocol. Quantitative PCR was performed with the LightCycler® using the Sybr® Green Master Mix Kit (Roche, Indianapolis, IN). PCR was carried out on samples, along with a 10-fold serial dilution of a target specific DNA standard, for selected transcripts using target sequence specific primers. Target specific mRNA expression was normalized against 18s rRNA, and the results were reported as transcript specific mRNA copies per 18s rRNA copies.

2.7. Statistical analysis

Following ANOVA, Dunnett's tests were used to compare data resulting from PFOS treatment to control values using SAS or JMP® 5.1 – Windows – Release 5.1.2 (SAS Institute, Inc., Cary, NC). All data are expressed as means ± standard error (S.E.). Individual means were calculated for all endpoints with the exception of GD 20 and PND 4 fetal serum, liver, and brains samples, which were pooled by litter and are presented as litter means.

3. Results

3.1. PFOS concentrations in serum, liver, and brain

Presented in Table 2 are mean PFOS concentrations (±S.E.) for serum, liver, and brains. The mean percent ratio of liver and brain PFOS concentrations to serum PFOS concentration for each dose group are presented in Table 3.

From GD 20 through PND 21, the PFOS concentrations in maternal rat serum, liver, and brain correlated well with the daily K^+PFOS doses given to the maternal rats. While the maternal liver-to-serum PFOS concentration ratios ranged from 1.8 to 4.9, the corresponding maternal brain-to-serum PFOS concentration ratios ranged from 0.04 to 0.09.

From GD 20 to PND 72, the PFOS concentrations in fetal and pup serum, liver, and brain correlated well with the daily litter-matched maternal K^+PFOS doses. Even though none of the rat fetuses and pups were dosed directly with K^+PFOS, *in utero* exposure to PFOS was evident in fetal litters from K^+PFOS-treated dams. Because cross-fostering of neonates from control dams with K^+PFOS-treated dams was not incorporated into our design, it is not possible to comment quantitatively on the extent to which lactation contributed to exposure after birth in this study. Mean fetal serum PFOS concentrations were higher than those of dams on GD 20. In both maternal rats and their offspring, liver PFOS concentrations were higher than the respective serum PFOS concentrations at all times, and brain PFOS concentrations were always lower than time-matched serum concentrations.

There did not appear to be a sex difference in serum, liver, or brain PFOS concentrations between male and female offspring through PND 21. On PND 72, liver PFOS concentrations remained comparable between male and female offspring by maternal K^+PFOS-treatment group; however, female offspring had higher serum PFOS concentrations than respective treatment-group males.

3.2. Serum TSH measurements

Mean maternal serum TSH concentration data are presented in Fig. 1, and fetal and pup TSH concentration data are presented in Fig. 2. Mean serum TSH values of maternal rats and offspring from K^+PFOS-treated groups were not significantly different from controls on GD 20, PND 4, and PND 21.

Table 2
Mean PFOS concentrations (±standard error) in serum, liver, and brain in dams (dam), fetus (fetus), and pups (pup, male = M, female = F). None of the offspring directly received K^+PFOS doses.

Time	Dose group	Serum [PFOS] (μg/mL)		Liver[a] [PFOS] (μg/g)		Brain[a] [PFOS] (μg/g)	
		Dam	Fetus[b]	Dam	Fetus[b]	Dam	Fetus[b]
GD 20	Control[c]	<LLOQ[d]	0.009 ± 0.001	<LLOQ[e]	<LLOQ	<LLOQ[f]	<LLOQ
	0.1 mg/kg-d[g]	1.722 ± 0.068	3.906 ± 0.096	8.349 ± 0.344	3.205 ± 0.217	0.151 ± 0.012	1.233 ± 0.067
	0.3 mg/kg-d[h]	6.245 ± 0.901	10.446 ± 0.291	21.725 ± 0.721	5.814 ± 0.245	0.368 ± 0.043	3.126 ± 0.238
	1.0 mg/kg-d[i]	26.630 ± 3.943	31.463 ± 1.032	48.875 ± 72.733	20.025 ± 2.021	0.999 ± 0.083	12.984 ± 1.122
		Dam	Pup[b]	Dam	Pup[b]	Dam	Pup[b]
PND 4	Control	0.008 ± 0.000	<LLOQ	NS[j]	<LLOQ	NS	<LLOQ
	0.1 mg/kg-d	3.307 ± 0.080	2.236 ± 0.070	NS	9.463 ± 0.512	NS	0.680 ± 0.033
	0.3 mg/kg-d	10.449 ± 0.234	6.960 ± 0.163	NS	20.130 ± 0.963	NS	1.910 ± 0.074
	1.0 mg/kg-d	34.320 ± 31.154	22.440 ± 0.723	NS	50.180 ± 1.124	NS	6.683 ± 0.428

Time	Dose group	Serum [PFOS] (μg/mL)			Liver[a] [PFOS] (μg/g)			Brain[a] [PFOS] (μg/g)		
		Dam	M Pup	F Pup	Dam	M Pup	F Pup	Dam	M Pup	F Pup
PND 21	Control	0.007 ± 0.000	<LLOQ	<LLOQ	NS	<LLOQ	<LLOQ	NS	<LLOQ	<LLOQ
	0.1 mg/kg-d	3.159 ± 0.081	1.729 ± 0.079	1.771 ± 0.076	NS	5.980 ± 0.614	5.278 ± 0.174	NS	0.220 ± 0.014	0.229 ± 0.011
	0.3 mg/kg-d	8.981 ± 0.275	5.048 ± 0.108	5.246 ± 0.138	NS	14.780 ± 0.832	13.550 ± 0.298	NS	0.649 ± 0.053	0.735 ± 0.039
	1.0 mg/kg-d	30.480 ± 1.294	18.611 ± 1.011	18.010 ± 0.744	NS	44.890 ± 2.637	41.230 ± 2.295	NS	2.619 ± 0.165	2.700 ± 0.187
PND 72	Control	NA[k]	<LLOQ	<LLOQ	NA	<LLOQ	<LLOQ	NA	NS	NS
	0.1 mg/kg-d	NA	0.042 ± 0.004	0.207 ± 0.042	NA	0.981 ± 0.091	0.801 ± 0.082	NA	NS	NS
	0.3 mg/kg-d	NA	0.120 ± 0.009	0.556 ± 0.062	NA	2.464 ± 0.073	2.252 ± 0.095	NA	NS	NS
	1.0 mg/kg-d	NA	0.560 ± 0.105	1.993 ± 0.293	NA	7.170 ± 0.382	7.204 ± 0.414	NA	NS	NS

[a] Non-perfused samples.
[b] Samples pooled by litters.
[c] Represents dams receiving vehicle control solution, 0.5% Tween 20 in water.
[d] Lower limit of quantitation (LLOQ) for serum is 0.010 μg/mL.
[e] Lower limit of quantitation (LLOQ) for liver is 0.050 μg/g.
[f] Lower limit of quantitation (LLOQ) for brain is 0.025 μg/g.
[g] Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day.
[h] Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day.
[i] Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day.
[j] NS = no sample collected.
[k] NA = not applicable; all dams were sacrificed on PND 21.

Table 3
Mean liver [PFOS]-to-serum [PFOS] and brain [PFOS]-to-serum [PFOS] ratios in dams (dam), fetuses (fetus, pooled by litter), pups (pup, male = M, female = F). None of the offspring directly received K⁺PFOS doses.

Time	Dose group	$[PFOS]_{liver}:[PFOS]_{serum}$		$[PFOS]_{brain}:[PFOS]_{serum}$	
		Dam	Fetus[a]	Dam	Fetus
GD 20	Control[b]	NA[c]	NA	NA	NA
	0.1 mg/kg-d[d]	4.85	0.82	0.09	0.32
	0.3 mg/kg-d[e]	3.46	0.56	0.06	0.30
	1.0 mg/kg-d[f]	1.84	0.64	0.04	0.41

Time	Dose group	$[PFOS]_{liver}:[PFOS]_{serum}$		$[PFOS]_{brain}:[PFOS]_{serum}$	
		Dam	Pup[a]	Dam	Pup
PND 4	Control	–[g]	–	–	NA
	0.1 mg/kg-d	–	4.23	–	0.30
	0.3 mg/kg-d	–	2.89	–	0.27
	1.0 mg/kg-d	–	2.24	–	0.30

Time	Dose group	$[PFOS]_{liver}:[PFOS]_{serum}$			$[PFOS]_{brain}:[PFOS]_{serum}$		
		Dam	M Pup	F Pup	Dam	M Pup	F Pup
PND 21	Control	–	NA	NA	–	NA	NA
	0.1 mg/kg-d	–	3.46	2.98	–	0.13	0.13
	0.3 mg/kg-d	–	2.93	2.58	–	0.13	0.14
	1.0 mg/kg-d	–	2.41	2.29	–	0.14	0.15
		Dam	M Pup	F Pup	Dam	M Pup	F Pup
PND 72	Control	–	NA	NA	–	–	–
	0.1 mg/kg-d	–	23.62	3.88	–	–	–
	0.3 mg/kg-d	–	20.53	4.05	–	–	–
	1.0 mg/kg-d	–	12.79	3.61	–	–	–

[a] Samples pooled by litters.
[b] Represents dams receiving vehicle control solution, 0.5% Tween 20 in water.
[c] NA = not applicable; ratio cannot be calculated as PFOS concentrations in serum and liver were below lower limit of quantitation (LLOQ).
[d] Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day.
[e] Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day.
[f] Represents dams rats receiving 1.0 mg of potassium PFOS per kg body weight per day.
[g] "–" means no sample collected hence no ratio can be calculated.

3.3. Thyroid histology and morphometry

Thyroid histology findings in the offspring are summarized in Table 4. Only control and 1.0 mg/kg-d maternal dose group fetal and pup thyroids obtained from GD 20, PND 4, and PND 21 were examined. Subjective histopathologic evaluation revealed no K⁺PFOS treatment-related histologic changes, including the number of follicles present and the distribution of follicle sizes, in the H&E-stained thyroid sections obtained from GD 20 fetuses, PND 4 pups, and PND 21 pups in the 1.0 mg/kg-d maternal dose group when compared to controls.

Mean thyroid follicular epithelial cell heights and thyroid follicular colloid area morphometric data for pups are presented in Figs. 3 and 4, respectfully. There were no K⁺PFOS-treatment related

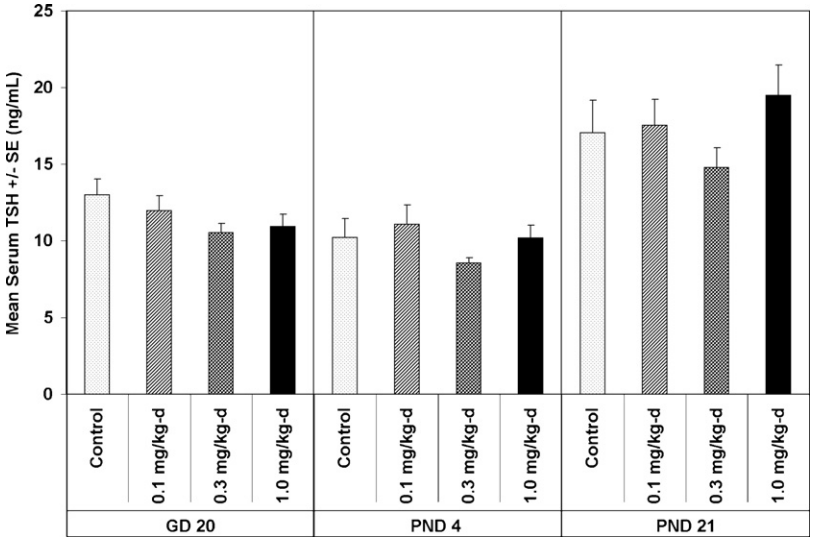

Fig. 1. Mean serum TSH concentrations in dams obtained on GD 20, PND 4, and PND 21. Dams were treated with either vehicle control (0.5% Tween® 20, dotted bars: □), 0.1 mg/kg-d K⁺PFOS (left-striped bars: ▨), 0.3 mg/kg-d K⁺PFOS (checkered bars: ▩), or 1.0 mg/kg-d K⁺PFOS (solid bars: ■). TSH was measured via Biotrak™ rat thyroid stimulating hormone ^{125}I assay system (Amersham Pharmacia Biotech, Piscataway, NJ). Each bar represents the mean serum TSH determinations and error bars represent standard errors. There were no differences in maternal serum TSH levels between PFOS-treatment groups and the control.

Table 4
Thyroid histology in fetuses and pups from control and 1.0 mg/kg-d K^+PFOS maternal dose groups only. None of the pups directly received K^+PFOS doses.

Males				
	Control[a]	0.1 mg/kg-d[b]	0.3 mg/kg-d[c]	1.0 mg/kg-d[d]
GD 20				
Total # of thyroid examined	10	0[e]	0[e]	6
Decreased colloid				
Moderate	5	–[f]	–	4
Severe	5	–	–	2
Cellular luminal debris				
Minimal	1	–	–	0
Mitotic figures				
Minimal	6	–	–	6
Mild	3	–	–	0
PND 4				
Total # of thyroid examined	10	–	–	10
Decreased colloid				
Minimal	4	–	–	3
Cellular luminal debris				
Minimal	3	–	–	0
Mitotic figures				
Minimal	5	–	–	4
PND 21				
Total # of thyroid examined	10	–	–	10
Cellular luminal debris				
Minimal	0	–	–	1
Mitotic figures				
Minimal	5	–	–	3
Ultimobranchial cyst				
Present	4	–	–	0
Ectopic thymus				
Present	1	–	–	0
Females				
	Control	0.1 mg/kg-d	0.3 mg/kg-d	1.0 mg/kg-d
GD 20				
Total # of thyroid examined	9	0[e]	0[e]	6
Decreased colloid				
Moderate	2	–	–	3
Severe	7	–	–	3
Mitotic figures				
Minimal	4	–	–	1
Mild	5	–	–	5
Congestion				
Moderate	1	–	–	0
PND 4				
Total # of thyroid examined	10	–	–	8
Decreased colloid				
Minimal	2	–	–	2
Mitotic figures				
Minimal	7	–	–	3
Mild	1	–	–	0
PND 21				
Total # of thyroid examined	10	–	–	10
Cellular luminal debris				
Minimal	1	–	–	4
Mitotic figures				
Minimal	5	–	–	5

[a] Represents dams receiving vehicle control solution, 0.5% Tween 20 in water.
[b] Represents dams receiving 0.1 mg of potassium PFOS per kg body weight per day.
[c] Represents dams receiving 0.3 mg of potassium PFOS per kg body weight per day.
[d] Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day.
[e] The thyroids were collected and processed, but not evaluated microscopically.
[f] "–" means no histology evaluation available.

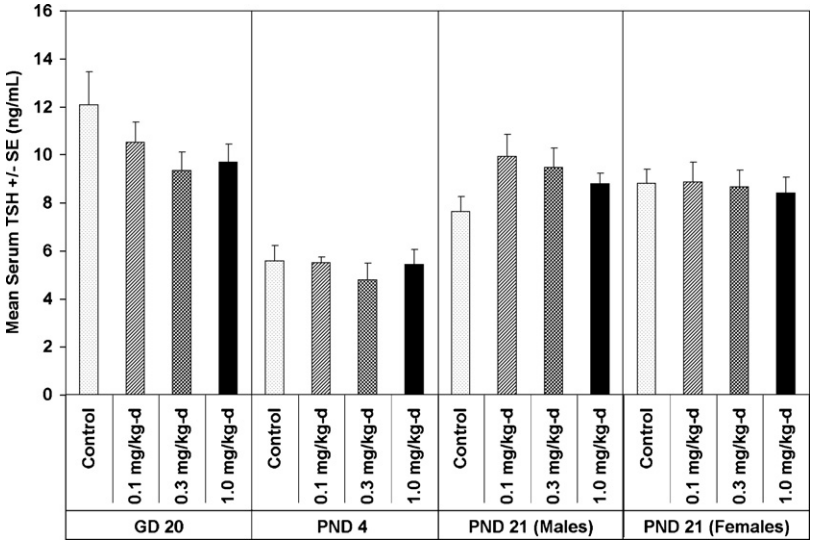

Fig. 2. Mean serum TSH concentrations in fetal rats obtained on GD 20 (pooled by litters), PND 4 (pooled by litters), and PND 21. Rats were the offspring from dams treated with either vehicle control (0.5% Tween® 20, dotted bars: ▫), 0.1 mg/kg-d K⁺PFOS (left-striped bars: ▨), 0.3 mg/kg-d K⁺PFOS (checkered bars: ▩), or 1.0 mg/kg-d K + PFOS (solid bars: ■). None of the offspring received K⁺PFOS treatments directly. TSH was measured via Biotrak™ rat thyroid stimulating hormone ^{125}I assay system (Amersham Pharmacia Biotech, Piscataway, NJ). Each bar represents the mean serum TSH determinations and error bars represent standard errors. There were no differences in offspring serum TSH levels between PFOS-treatment groups and the control.

alterations in thyroid follicular colloid area on PND 4 and PND 21. Follicular epithelial cell height was similar in all groups on PND 4. On PND 21, mean thyroid follicular epithelial cell height from the 1.0 mg/kg-d maternal dose group male pups was significantly ($p < 0.01$) higher than that of the control group. This difference was suspected to be spurious due to the extraordinarily low value in the male control group compared to the female control group on PND 21.

3.4. Thyroid proliferation assay

Ki-67 proliferation assay data for GD 20 fetal thyroids are presented in Table 5. The mean number (±S.E.) of Ki-67-positive thyroid follicular epithelial cells in female fetal thyroids from the 1.0 mg/kg-d dose group was 2.1-fold higher than the control group and was statistically significant. The range of values in the female controls was quite wide (4–113, $N = 7$) compared to the range in female fetal thyroids from the 1.0 mg/kg-d dose group (64–116, $N = 5$).

3.5. Quantitative RT-PCR

The mRNA transcript data for the control and 1.0 mg/kg-d dose groups are summarized as mean ± S.E. in Table 6 for the GD 20 dams, GD 20 fetuses, and PND 21 male pups. Mean Cyp2b2 levels for dams and their male pups in the 1.0 mg/kg-d maternal dose group were higher than the control group values on GD 20 and PND 21. Additionally, on PND 21, mean ACoA and Cyp4a1 levels for male

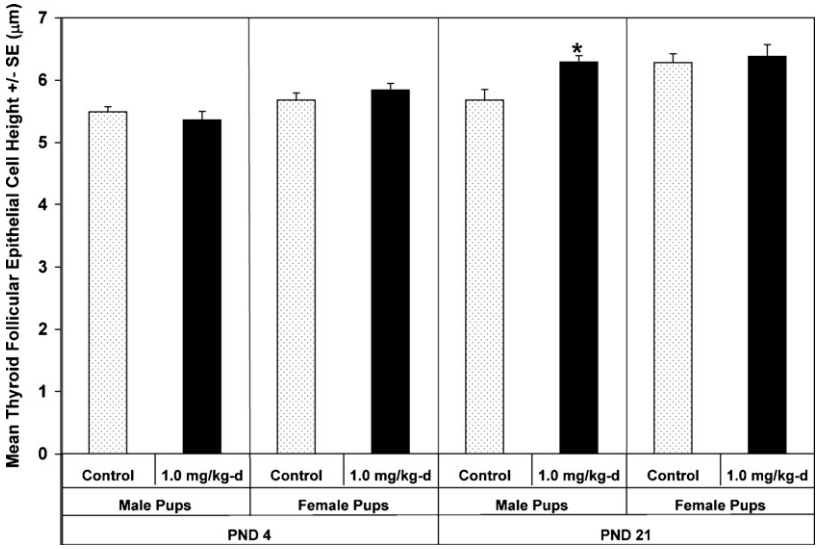

Fig. 3. Mean thyroid follicular epithelial cell heights in offspring on PND 4 and PND 21. Only control and 1.0 mg/kg-d K⁺PFOS dose groups were evaluated. Control group is represented with dotted bars (▫) while 1.0 mg/kg-d K⁺PFOS group is represented with solid bars (■). Each bar represents the mean and error bars represent standard errors. Asterisk (*) denotes significant difference from control ($p < 0.05$). Follicular epithelial cell height was similar in all groups on PND 4. On PND 21, mean thyroid follicular epithelial cell height from the 1.0 mg/kg-d maternal dose group male pups was significantly higher than that of the control group. This difference was suspected to be spurious due to the extraordinarily low value in the concurrent control group compared to other groups on PND 21 in this study.

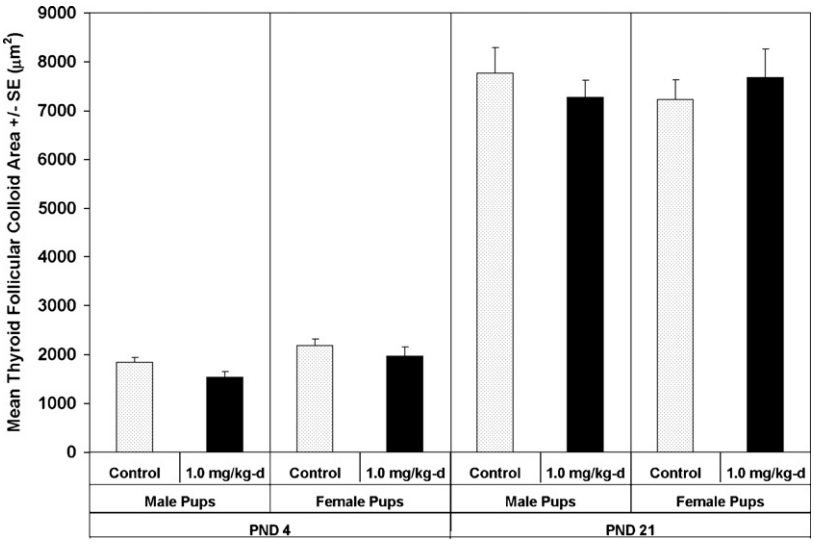

Fig. 4. Mean thyroid follicular colloid area in offspring on PND 4 and PND 21. Only control and 1.0 mg/kg-d K⁺PFOS dose groups were evaluated. Control group is represented with dotted bars (□) while 1.0 mg/kg-d K⁺PFOS group is represented with solid bars (■). Each bar represents the mean and error bars represent standard errors. There were no K⁺PFOS-treatment related alterations in thyroid follicular colloid area on PND 4 and PND 21.

Table 5
Individual and mean (±standard error) thyroid follicular epithelial cell counts based on positive staining for Ki-67 as evidence of cell proliferation on GD 20 male and female fetal rats.

	Males		Females	
	Control[a]	1 mg/kg-d[b]	Control	1 mg/kg-d
Individual counts	18	10	4	64
	22	14	22	83
	25	24	23	88
	36	29	34	89
	70	36	39	116
	70	80	57	
			113	
Number of thyroids	6	6	7	5
Mean Ki-67-positive cells	40 ± 10	32 ± 10	42 ± 13	88 ± 8*

[a] Fetal rats from dams receiving vehicle control solution (0.5% Tween 20 in water).
[b] Fetal rats from dams receiving 1 mg of potassium PFOS per kg body weight per day.
* Values are statistically significant from control ($p < 0.05$).

pups in the 1.0 mg/kg-d maternal dose group were higher than the control group, while the mean Cyp7a1 level was lower than the control group. There were no other statistically significant differences among other transcripts evaluated.

4. Discussion

Because of the evidence for exposure of children to PFOS beginning as early as *in utero*, there has been an extensive research effort in evaluating potential developmental effects in laboratory animals and humans [13,19–22,45–47]. However, there have been limited data available on the potential association of PFOS exposure with developmental neurotoxicity. In a companion paper to this, Butenhoff et al. [31 this issue] reported the results of a developmental neurotoxicity study with PFOS per current test guidelines. Maternal rats were given K⁺PFOS by oral gavage through gestation and lactation, and pups were evaluated for various indices of neurological development through PND 72. Outcomes of the companion study included slight but statistically significant effects on maternal food consumption and weight gain in the highest maternal dose group (1.0 mg/kg-d) and a transient decrease in habituation in male offspring from the same maternal dose group on PND 17. During the conduct of that study, samples were obtained on GD 20, PND 4, PND 21, and PND 72 in order to study: (1) the concentrations of PFOS in serum, liver, and brain during the study; (2) thyroid hormone status and thyroid tissue histology; (3) quantitative expression of a selected liver mRNA transcripts in dams and their fetuses on GD 20 and male pups on PND 21. The results of analyses of those samples have been the focus of this article.

Human biomonitoring studies have shown that neonates can be exposed to PFOS effectively through *in utero* exposure. Inoue et al. [16] reported that PFOS was detected in all of 15 paired human maternal serum and neonatal cord blood serum samples from Japan obtained in 2003, with neonatal and maternal serum PFOS ranging from 1.6 to 5.3 ng/mL and 4.7 to 17.6 ng/mL, respectively. Midasch et al. [17] reported detectable PFOS among 11 paired maternal and cord blood plasma samples obtained in 2003 in Germany, with maternal plasma PFOS median concentration of 13.0 and 7.3 ng/mL in cord blood. Monroy et al. [15] evaluated PFOS concentrations in 101 maternal samples paired with 105 cord blood at birth (includes 4 sets of twins), with mean PFOS concentrations of 16.2 and 7.3 ng/mL, respectively. Apelberg et al. [47] found that PFOS concentrations in 293 cord blood sera samples obtained in Baltimore, MD in 2004–2005 had a geometric mean of 4.9 ng/mL, indicating that children are exposed to PFOS beginning *in utero* from placental transfer.

Lactational exposure to PFOS in humans is also possible. Tao et al. [14] reported a mean milk PFOS concentration of 0.131 ng/mL among the 45 samples obtained from nursing mothers in Massachusetts in 2004. So et al. [11] also examined 19 human milk samples in China and reported a mean PFOS concentration of 0.121 ng/mL. Kärrman et al. [10] found that mean PFOS concentration in milk is 0.201 ng/mL and that was about 1% of the respective maternal serum PFOS concentration (based on 12 samples obtained in Sweden). All together, the comparative data between laboratory studies and human biomonitoring reports should be taken into consideration carefully when performing risk assessment, as the difference in body burden also delineates the outcome.

Concentrations of PFOS measured in samples of serum, liver, and brain from rat dams, fetuses, and pups across maternal K⁺PFOS treatment levels were generally in proportion to the relative differences between maternal dose levels. Thus, internal dose was in proportion to administered maternal dose.

Table 6
Mean mRNA transcript levels (expressed as mean number of mRNA copies per 18s rRNA ± standard error from $n = 10$ liver samples per group) in dams and fetus obtained on GD 20, as well as male pups on PND 21 from control and 1.0 mg/kg-d K⁺PFOS maternal dose groups. Dam liver samples were not collected on PND 21. None of the offspring directly received K⁺PFOS doses.

Transcript	Dose group	GD 20		PND 21
		Dam	Fetus	Male pups
ACoA	Control[a]	5.55E−05 ± 1.08E−05	1.51E−05 ± 1.53E−06	6.81E−05 ± 8.19E−06
	1.0 mg/kg-d[b]	6.41E−05 ± 5.57E−06	1.65E−05 ± 5.10E−06	1.04E−04 ± 7.54E−06*
PCNA	Control	2.00E−06 ± 1.37E−07	4.25E−05 ± 4.12E−06	8.16E−06 ± 6.51E−07
	1.0 mg/kg-d	1.97E−06 ± 1.12E−07	3.80E−05 ± 3.96E−06	8.18E−06 ± 1.25E−06
ME	Control	3.98E−06 ± 6.90E−07	6.68E−07 ± 5.59E−08	4.38E−06 ± 8.27E−07
	1.0 mg/kg-d	5.22E−06 ± 9.02E−07	6.71E−07 ± 1.28E−07	4.69E−06 ± 1.59E−06
Diol 1	Control	4.18E−06 ± 1.35E−06	0.00E00 ± 00[c]	8.52E−06 ± 7.31E−07
	1.0 mg/kg-d	3.89E−06 ± 7.95E−07	0.00E00 ± 00	8.62E−06 ± 6.60E−07
Por	Control	3.32E−06 ± 6.08E−07	2.85E−06 ± 2.88E−07	1.09E−05 ± 1.28E−06
	1.0 mg/kg-d	3.47E−06 ± 4.39E−07	2.62E−06 ± 1.87E−07	1.49E−05 ± 2.90E−06
ApoA1	Control	9.07E−04 ± 7.70E−05	7.41E−04 ± 6.74E−05	5.51E−04 ± 6.71E−05
	1.0 mg/kg-d	9.94E−04 ± 1.18E−04	7.62E−04 ± 2.86E−05	4.67E−04 ± 4.29E−05
Cyp1a1	Control	2.09E−05 ± 1.57E−06	3.14E−07 ± 3.76E−08	5.12E−05 ± 5.81E−06
	1.0 mg/kg-d	2.06E−05 ± 1.46E−06	3.91E−07 ± 7.15E−08	6.27E−05 ± 8.89E−06
Cyp3a1	Control	2.10E−05 ± 3.89E−06	3.98E−06 ± 2.61E−06	1.31E−04 ± 1.36E−05
	1.0 mg/kg-d	2.94E−05 ± 3.67E−06	2.53E−06 ± 2.07E−06	1.80E−04 ± 1.76E−05
Cyp4a1	Control	5.42E−06 ± 8.66E−07	2.60E−07 ± 7.87E−08	3.71E−06 ± 2.56E−07
	1.0 mg/kg-d	7.79E−06 ± 1.20E−06	7.79E−07 ± 2.53E−07	7.66E−06 ± 1.02E−06*
Cyp7a1	Control	5.82E−06 ± 1.02E−06	4.60E−06 ± 9.97E−07	2.79E−05 ± 4.21E−06
	1.0 mg/kg-d	5.88E−06 ± 1.67E−06	4.45E−06 ± 1.22E−06	8.06E−06 ± 1.61E−06*
Cyp2b2	Control	2.58E−06 ± 6.01E−07	0.00E00 ± 00[d]	2.22E−05 ± 3.75E−06
	1.0 mg/kg-d	7.30E−06 ± 1.54E−07*	0.00E00 ± 00	3.90E−05 ± 5.46E−06*
Ugt1A common	Control	9.55E−07 ± 8.54E−08	4.41E−06 ± 8.52E−07	4.37E−06 ± 3.25E−07
	1.0 mg/kg-d	1.36E−06 ± 1.60E−07	1.30E−05 ± 3.70E−06	5.23E−06 ± 9.29E−07
Ugt1a1	Control	2.37E−01 ± 3.31E−02	1.55E−01 ± 4.43E−02	5.58E−01 ± 1.84E−02
	1.0 mg/kg-d	2.66E−01 ± 2.82E−02	1.81E−01 ± 7.03E−02	6.42E−01 ± 7.47E−02
Ugt1a6	Control	2.97E−06 ± 9.69E−07	6.47E−05 ± 7.34E−06	1.44E−05 ± 2.10E−06
	1.0 mg/kg-d	2.15E−06 ± 3.47E−07	5.43E−05 ± 6.75E−06	1.27E−05 ± 4.24E−06
Ugt2b	Control	1.40E−04 ± 2.46E−05	2.20E−05 ± 2.43E−06	4.67E−05 ± 7.36E−06
	1.0 mg/kg-d	9.45E−05 ± 1.86E−05	2.55E−05 ± 1.51E−06	5.39E−05 ± 2.86E−06

[a] Represents dams receiving vehicle control solution, 0.5% Tween 20 in water.
[b] Represents dams receiving 1.0 mg of potassium PFOS per kg body weight per day.
[c] Represents values that were too low for quantitation.
* Values are statistically significant from control ($p < 0.05$).

Based on paired maternal and fetal samples obtained on GD 20, significant placental transfer of PFOS from rat dams to their developing fetuses occurred. Fetal serum PFOS concentrations were 1.2–2.3 times greater than maternal serum PFOS on GD 20, and fetal brain PFOS concentrations were approximately 10 times maternal brain PFOS. The latter observation likely reflects the fact that the "blood–brain barrier" in GD 20 fetal rats has not been established. However, maternal liver PFOS concentrations were 2.4–3.7 times greater than those of GD 20 fetuses.

Compared to GD 20 fetal serum PFOS concentrations, pup serum PFOS concentrations on PND 4 were lesser by about one-third to one-half. On PND 21, pup serum PFOS concentrations had decreased slightly from PND 4 levels, even though pups had gained approximately 8 times their PND 4 weight. When body weight and corresponding volume expansion increases are considered, this observation suggests a significant contribution from lactational transfer. Although the present study did not incorporate a cross-fostering design, Luebker et al. [20] have provided evidence of lactation exposure in pups born to control rats fostered by dams treated with 1.6 mg/kg-d K⁺PFOS, and subsequent analysis of two paired maternal serum and milk samples from K⁺PFOS-treated dams from the Luebker et al. study by Kuklenyik et al. [48] showed that milk PFOS concentrations were 11 and 51% of the respective maternal serum PFOS concentrations.

Based on data from Table 4 of the companion article [31 this issue], on PND 21, compared to controls, there was no difference in mean body weights of pups by sex in any of the treatment groups when compared to sex-matched controls, and we found no significant within-group difference between male and female pups in PND 21 serum, liver, and brain PFOS concentrations that are reported herein. Mean serum PFOS on PND 72 was less than on PND 21 in male and female offspring from all K⁺PFOS-treated groups. However, on PND 72, male serum PFOS was approximately a fifth to a third that of female serum PFOS, and female body weights were approximately two-thirds of male body weights. These observations suggest that a sex difference in serum PFOS elimination occurred between PND 21 and PND 72, perhaps associated with sexual maturation during this period, with males eliminating PFOS from serum at a somewhat higher rate.

PFOS appeared to concentrate in the livers of dams and pups, with liver PFOS concentrations at least twice serum PFOS concentrations in pups. However, liver concentrations were 60–80% of serum concentrations in fetuses on GD 20. PND 4 pup liver PFOS concentrations by maternal dose group were approximately 2–3

times greater than respective GD 20 fetal liver PFOS concentrations, yet serum PFOS concentrations in pups on PND 4 were less by 30–40% as compared to GD 20 fetal serum PFOS concentrations in the same respective dose groups. The increase in liver concentration in pups on PND 4 may represent redistribution from serum to liver after birth, perhaps as a result of enterohepatic recirculation [5]. Despite the marked difference between males and females in serum PFOS concentrations on PND 72, liver concentrations were essentially the same in both sexes, having decreased by approximately 85% from PND 21 values.

The observations from the pharmacokinetic data described above suggest that differences in urinary elimination may develop between males and females during sexual maturation and that enterohepatic recirculation and fecal elimination may be similar between genders after sexual maturation. Johnson et al. [5] provided evidence that enterohepatic recirculation and fecal elimination of PFOS in rats were factors in PFOS pharmacokinetics. This study may be the first observation of a sex difference in serum PFOS elimination in rats, with male rats apparently eliminating PFOS from serum at a greater rate than females. It is interesting to note that the eight-carbon perfluorinated carboxylate, PFOA, exhibits a sex difference in renal elimination in rats [49,50]; however, in the case of PFOA, the elimination rate in females is much greater than in males. This sex difference in PFOA elimination appears to develop during sexual maturation [51], likely the result of differences in the expression of organic anion transporters [52].

The concentration of PFOS in non-perfused brain tissue was generally lower than that in serum. On GD 20, the average PFOS concentrations for the non-perfused brain tissues of dams were between 4 and 9% of the corresponding serum PFOS concentrations. Fetal brain concentrations at GD 20 were higher than those in respective dose group dams by a factor of approximately 10, and were 30–41% of the respective fetal serum PFOS concentrations. Although it is not possible to know how much of the PFOS in brain tissue was associated with blood versus that amount associated with tissue uptake, these data suggest that brain uptake may have been higher in fetal rats, perhaps due to the undeveloped state of the "blood–brain barrier" on GD 20.

The "blood–brain barrier" represents the complex tight junctions between endothelial cells of brain capillaries. It is physiologically defined by low permeability for small hydrophilic molecules [53]. In rats, the "blood–brain barrier" is not fully developed until PND 24, hence embryonic and early neonatal brain capillaries are more permeable for substances [54]. This could be why highest levels of PFOS were detected in neonatal brains on GD 20. It is conceivable that the volume expansion (i.e., brain growth) could contribute to the lowering of brain PFOS concentrations seen postnatally (even with lactational exposure). Brain PFOS concentrations were less than corresponding serum PFOS concentrations in all dose groups. Compared to liver-to-serum ratio (which increased during the postnatal period), the corresponding brain-to-serum ratio were all less than 1. The only other published data on brain concentrations in PFOS-exposed rats comes from a study by Austin et al. [55] in which female rats were given daily i.p. injections of 1 or 10 mg/kg-d K^+PFOS for 14 days. Brain region concentrations were analyzed for PFOS concentration (it was not noted whether or not the brains were perfused). At the 1 mg/kg-d dose, concentrations of PFOS in the various brain regions were a fraction (approximately 2–4%) of the serum concentration. Therefore, PFOS does not appear to concentrate in brain tissue relative to serum concentrations.

Thyroid hormones have numerous important roles in development [35–38]. Although PFOS-induced hypothyroxinemia has not been observed in pregnant and neonatal mice [22,26,39] (with the exception of the 20 mg/kg dose on GD 6 (not GD 12 and 18) in Thibodeaux et al. [26]), PFOS exposure has been associated with maternal and offspring hypothyroxinemia characterized by decreased serum total thyroxine and/or triiodothyronine without a major compensatory elevation of TSH in laboratory rats [22,25,26,40]. Although significant reductions in serum free thyroxine were reported by Thibodeaux et al. and Lau et al. [22,26] using analog methods, PFOS-induced reductions in serum free thyroxine and free triiodothyronine hormones were not observed when measured by equilibrium dialysis [25,40,41]. PFOS-exposed adult and neonatal rats appear to maintain an euthyroid state despite significant reductions in serum total thyroid hormones [25,40]. This is believed to be due to competition for binding sites between PFOS and thyroid hormones in rat serum, leading to an adequate supply of free hormone while reducing the concentration of hormone carried on serum binding proteins [41]. Lau et al. [22] measured choline acetyltransferase (ChAT) activity, an enzyme sensitive to thyroid hormone status, in prefrontal cortex and hippocampus of rat pups exposed *in utero* at maternal doses of 3 mg/kg-d PFOS given orally from GD 2 to GD 21. At this dose, marked hypothyroxinemia occurred without a compensatory change in TSH. Free thyroxine was decreased; however, an indicated above, an analog assay was used that has since been shown to be prone to negative bias in the presence of PFOS [41]. These investigators also evaluated learning and memory using a T-maze. Hippocampal activity of ChAT and T-maze performance were unaltered when compared to controls. ChAT activity in the pre-frontal cortex was slightly (but with statistical significance) decreased. Luebker et al. [20] included an evaluation of learning and memory using a modified M-maze in rat pups whose mothers were given daily oral doses of 0.1 and 0.4 mg/kg-d K^+PFOS beginning 6 weeks before mating, and during mating, gestation, and lactation. A second study in which the same dosing protocol was used [25] produced marked hypothyroxinemia in PND 5 pups without a clinically meaningful change in free thyroxine when measured by equilibrium dialysis or TSH (based on data from higher doses). There was no effect on learning or memory. In the companion paper to this, there were also no effects on learning or memory [31 this issue].

Production and release of metabolically active thyroid hormones by the thyroid gland is regulated by pituitary secretion of TSH and hypothalamic secretion of TRH. This regulatory process for controlling circulating thyroid hormone concentrations constitutes what is commonly referred to as the hypothalamic–pituitary–thyroid (H–P–T) axis. In this system, serum free thyroxine and free triiodothyronine function as primary or secondary feedback signals, acting on the hypothalamus and/or pituitary when there is an imbalance in their circulating concentrations. Although measurement of free thyroid hormones in serum can provide information on thyroid status, the clinical diagnosis of primary hypothyroidism is based mainly on a substantial elevation of TSH in response to reduced free thyroid hormones [42]. In a study designed to evaluate the potential effect of PFOS on the H–P–T axis [40], the goiterogen, propylthiouracil (PTU), was administered to rats with and without concurrent PFOS treatment. Rats treated with only PFOS experienced hypothyroxinemia without a change in TSH as compared to controls. PTU reduced serum thyroid hormones to a greater extent than PFOS in rats given only PTU, and serum TSH was increased significantly over control concentrations. PFOS given in conjunction with PTU did not alter the PTU-induced response to TSH. In addition, the TRH-induced release of TSH in excised, cultured pituitaries from rats exposed to PFOS or PFOS and PTU in combination was not affected by PFOS treatment.

In addition to TSH measurement, thyroid hormone status can also be evaluated by determining the follicular epithelial cell height and colloid area in histologic sections of thyroid gland. The thyroid gland consists of multiple, closely approximated follicles, each of which consists of a central accumulation of colloid surrounded by a single layer of follicular epithelial cells. The colloid consists of a homogeneous mixture of proteins, including thyroglobulin,

iodoproteins, and serum proteins, and serves as a repository of thyroglobulin. Under conditions that require an increase in thyroid function, there is an increase in the rate of colloid resorption, resulting in a decrease in colloid cross-sectional area in histologic sections, and an increase in the synthesis activities of follicular epithelial cells, the latter resulting in an increase in the cytoplasmic volume (height) of epithelial cells. Measurement of epithelial cell height and colloid area is easily accomplished from routine histological sections, and provides insight into the level of activity of the thyroid gland.

This study reported herein revealed no evidence of PFOS treatment-related alterations in TSH values for maternal rats and their offspring. PFOS treatment did not elicit any histological alterations of the thyroid glands in fetuses (GD 20) and pups (PND 21). Morphometric analysis on the thyroid revealed no PFOS treatment-related changes in follicular colloid area in pups on PND 4 and PND 21 when compared to control. There was a higher mean thyroid follicular epithelial cell height in male pups from the 1.0 mg/kg-d maternal dose group on PND 21, but this was suspected to be a spurious observation, as an unusually low value in the concurrent control group was obtained in this study when compared to the laboratory's historical control values.

Evaluation of cell proliferation in GD 20 fetal thyroid follicular epithelial cells from fetuses of the control and high-dose groups by Ki-67 immunohistochemistry produced revealed a statistically significant 2.1-fold increase in high-dose group female fetuses. The range of values in the female controls was quite wide (range 4–113, $N = 7$), and the highest value for the control females approximated the high end of the range in female thyroids from the 1.0 mg/kg-d dose group (range 64–116, $N = 5$). In GD 20 fetuses from dams treated with K^+PFOS, TSH was not increased, and, in PND 4 and PND 21 female pups, morphometric analysis of thyroid follicles did not reveal effects due to gestational and lactational exposure to K^+PFOS. Furthermore, adult females given 20 ppm K + PFOS in their diet for 2 years (equivalent to approximately 1.5 mg/kg-d) did not exhibit an increase in proliferative lesions of the thyroid [56]. Therefore, interpretation of the toxicological significance of fetal cell proliferation data is problematic without further study of fetal rats due to the range of control values and limited number of samples analyzed.

Several previous studies have reported data that suggest that peroxisome proliferator activated receptor alpha (PPARα) agonists increase hepatic responses to thyroid hormone in rats by effective competition for thyroid hormone carrier protein binding sites. PPARα agonists are typically amphiphilic acids and include endogenous fatty acids, hypolipidemic drugs, acetylsalicylic acid, and perfluorinated alkyls, such as PFOA and PFOS [57–60]. The thyromimetic effects by PPARα agonists in rat liver is believed to be due to transcriptional activation of genes regulated by thyroid hormone, either directly or indirectly, such as liver malic enzyme (ME), deiodinase 1 (Diol), apolipoprotein A1 (Apoa1); P450 oxidoreductase (Por), and the UDP glucuronyl transferae (Ugt) family of proteins [61–65].

For the control and 1.0 mg/kg-d dose group, the mRNA transcript levels of these enzymes were evaluated in this study as a secondary measure of concordance of the thyroid hormone responses in both dams (GD 20) and their male offspring (GD 20 and PND 21). While both maternal and fetal tissues were examined on GD 20, only male pups from PND 21 were included because in a 2-year dietary feeding study, there was an increased incidence of thyroid follicular cell adenoma observed in male rats fed with K^+PFOS diet for 1 year followed by control diet for another year [56]. Oral administration of K^+PFOS to pregnant rats through gestation did not appear to elicit any changes in the hepatic genes mediated by thyroid hormones.

PFOS has been shown to induce hepatomegaly in rodents and monkeys [32,66,67]. Liver enlargement in rodents can occur through the induction of the nuclear hormone receptors such as PPARα, pregnane X receptor (PXR), or constitutive androstane receptor (CAR). Chemicals such as Wyeth 14,643, dexamethasone, and phenobarbital are prototypical agonists used to study activation of PPARα, CAR, and PXR receptors, respectively. Wy14,643 induces hepatomegaly that was characterized by peroxisome proliferation and selective induction of Cyp4a1 and Cyp7a1 [68–70]. Dexamethasone produces hepatomegaly as a consequence of extensive periportal fat accumulation and induction of Cyp3a1 [71,72]. Phenobarbital induces hepatomegaly by simulating cell proliferation and the induction of Cyp2b2 [73,74] and other cell proliferation markers such as proliferating cell nuclear antigen (PCNA).

Compared to control, the RT-PCR analyses on all the transcripts evaluated suggest induction of hepatic CAR in 1.0 mg/kg-d dose-group maternal rats on GD 20 based on a 2.8-fold increase in Cyp2b2; as well as increased expression for the PPARα-regulated genes, ACoA (increased by 1.5-fold) and Cyp4a1 (increased by 2.1-fold), and the CAR regulated gene Cyp2b2 (increased by 1.8-fold) in male pups from the 1.0 mg/kg-d maternal dose group on PND 21. Interestingly, decreased expression of Cyp7a1 (decreased by 0.3-fold) was observed in PND 21 male pups, even though ACoA and Cyp4A1 were increased. Transcripts with a potential relationship to thyroid status (ME, Por, Dio1, Ugt1A family, and ApoA1) were unaffected in dams and offspring following maternal K^+PFOS treatment.

5. Conclusion

Data reported herein demonstrate that K^+PFOS administration to maternal rats during gestation and lactation: (1) leads to significant placental transfer and potentially significant lactational transfer and (2) has no clear adverse effect on thyroid status (morphology, hormone homeostasis, proliferation, and liver gene expression). In addition, sex difference in serum elimination of PFOS (but not liver or brain elimination) appears to develop in association with sexual maturation, with males eliminating PFOS from serum at a somewhat greater rate than females.

Samples reported on herein were obtained during the course of a developmental neurotoxicity study (reported as a companion article [31 this issue]) in which the only developmental effect observed were increased motor activity and decreased habituation in males from the highest maternal dose group (1.0 mg/kg-d) on PND 17. At this dose, there was also a slight but statistically significant effect on maternal weight and food consumption. Maternal and fetal serum concentrations in the 1.0 mg/kg-d dose group proximal to birth (GD 20) were approximately 27 and 31 μg/mL. No adverse maternal or developmental outcomes were noted at 0.3 mg/kg-d, and maternal and fetal serum concentrations on GD 20 were approximately 6 and 10 μg/mL, respectively. Based on the most recent (2003–2004 sampling period) report on PFOS concentrations in human serum of a representative sample of females across the United States, geometric mean serum PFOS was 0.0184 μg/mL and 95th percentile was 0.0457 μg/mL. Thus, when serum PFOS in this sample of United States females is compared to those in GD 20 dams from no observed adverse effect level of the study, margins of exposures of 333 and 130 exist at the geometric mean and 95th percentile for United States females, respectively.

Conflict of interest

John L. Butenhoff, Shu-Ching Chang, and David J. Ehresman are employees of 3M Company, a former manufacturer of PFOS and the company supporting the work reported on in the article. Kendall B. Wallace and James A. Bjork, from University of Minnesota, Duluth, conducted parts of the work with an unrestricted gift grant from 3M. George A. Parker and Donald G. Stump do not have compet-

ing interests other than employment in contract research facilities (Biotechnics, LLC and Wil Research, Inc., respectively) conducting parts of the work.

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