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Serum perfluorooctanoic acid (PFOA) concentrations in normal and hyperlipidemic female hamsters dosed orally with ammonium perfluorooctanoate (APFO) for up to 30 days

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ABSTRACT

In epidemiology studies, the presence of perfluorooctanoate (PFOA) in human blood has been associated with higher serum cholesterol concentrations. A possible explanation for these results is that elevated serum cholesterol might reduce clearance of PFOA. In this study, female hamsters, which transport and regulate cholesterol in a manner similar to humans, were fed normal diet or diet supplemented with 0.05% cholesterol and 10% coconut oil (high-fat diet) resulting in hyperlipidemia throughout the study in supplemented animals. Hamsters on either a normal and high-fat diet were given oral doses of 0.1, 1.0, or 10 mg APFO/kg for 30 days. Serum PFOA concentrations evaluated 24 h after 1, 10, 20, and 30 doses of APFO were not altered in hyperlipidemic hamsters compared to those fed normal diet. For a given dose group, serum concentrations of PFOA were highest following the 10 doses (except for the 10 mg/kg group where concentrations were the highest after the first dose) and were lowest after 20 and 30 doses. Under the condition of this study, higher serum lipids did not affect the absorption and clearance of serum PFOA. Serum PFOA concentrations declined over the course of the study despite continued daily dosing with APFO. This does not support the hypothesis that higher serum lipids might increase the retention of PFOA in the body.

1. Introduction

Perfluorooctanoate (PFOA, $CF_3(CF_2)_6COO^-$, CAS No. 335-67-1) is a perfluorinated carboxylate ion that has been used, generally as the ammonium salt (APFO, CAS No. 3825-26-1), as a surface-active agent in the production of various fluoropolymers. Because of its chemical stability against metabolic and environmental degradation and long elimination half-life in humans (approximately 3 years) [1,2], PFOA is found widely distributed in the environment [3–9] and in samples of blood from non-occupationally exposed populations [10–12]. Recent reports indicate that serum PFOA concentration in the general population averages less than 5 ng/mL and appears to have been decreasing since 1999–2000 [11–13].

A positive association of PFOA with serum cholesterol has been observed in occupational studies [14–18,19], in an exposed community [20–22], and in one general population study [23]. These associations between serum PFOA and cholesterol concentrations varied in statistical significance. Of these studies, all were cross sectional with the exception of 3 studies with multiple measurements per subject: Sakr et al. [19] that had multiple PFOA and

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cholesterol measurements in approximately 450 workers; Costa et al. [14] that had multiple measurements in 56 workers; and Olsen et al. [17] that had at least 2 measurements for each of 174 workers.

The relationship between cholesterol and PFOA exposure expressed as the change in cholesterol concentration (mg/dL) per change in PFOA concentration (mg/mL) is based on the assumption of linearity which is not always seen in the studies reported. The change in cholesterol as related to exposure was reviewed by Steenland et al. [22] and expressed as a change in cholesterol per mg/mL change in serum PFOA. An increase of 1–5 mg/dL cholesterol was reported in occupationally exposed workers with mean PFOA concentrations of approximately 500–1000 ng/mL [18,19]. An increase of approximately 10 mg/dL cholesterol was seen in both a PFOA-exposed community [21,24] with mean PFOA concentrations of 70–80 ng/mL and in the general population where the mean PFOA concentrations were around 4 ng/mL [23]. An increase of 16 mg/dL cholesterol was reported in an occupationally exposed group with mean serum PFOA concentrations of 22,000 ng/mL [25]. Thus the greatest increase in cholesterol concentration was seen in the general population when the increases in PFOA concentration were the smallest. In contrast to the human epidemiology data, dosing with APFO results in a decrease in cholesterol concentration in rodents [26,27] and does not impact cholesterol concentrations in monkeys [28].

The lack of a dose-response relationship in human studies between the magnitude of cholesterol increases and serum PFOA concentrations has not been explained. The magnitude of cholesterol increases is not consistently greater in occupationally exposed populations although this would be the expectation of a cause/effect relationship. There are 3 possibilities to consider: (1) that PFOA could directly increase cholesterol, (2) both PFOA and cholesterol are increased independently by some other substance or factor, or (3) high serum lipids might increase retention of PFOA in the body. In rodents, treatment with PFOA has been shown to decrease cholesterol concentrations rather than increase [29]. No other substance or other factor has been identified to date that increases both cholesterol and PFOA serum concentrations. Thus, we looked at the last possibility in an animal model by evaluating uptake and clearance of PFOA in hamsters given hyperlipidemic diets compared to hamsters fed normal diets. Hamsters were chosen for this experiment because they transport and regulate serum cholesterol in a manner that closely resembles humans [30,31]. Hamsters are different from rats and guinea pigs in that the rate of hepatic cholesterol synthesis more closely resembles those typical of humans. Both the hamster and human transport a significant proportion of plasma cholesterol in the form of low density lipoproteins (LDL). Similar mechanisms are involved in the regulation of plasma LDL-cholesterol which is responsive to both nutritional and pharmacological manipulation [32]. In addition, hamsters also are known to develop hyperlipidemia in response to high fat diets [33–35]. In this study, uptake and clearance of PFOA were evaluated by measuring serum PFOA over a 30 day dosing period in hamsters fed either a normal diet or a hyperlipidemic diet.

2. Materials and methods

2.1. Test material

The material used in this study was the linear form of ammonium perfluorooctanoate. This material was a white to slightly opaque liquid in a 20.0% solution of APFO in water. Chemical analysis showed the actual concentration in the stock solution to be 23.1%. No evidence of instability such as a change in color or physical state was observed under the conditions of this study.

2.2. Animals and husbandry

Forty-six female Golden Syrian hamsters were received at 47 days of age from the supplier (Harlan, Indianapolis, IN). Hamsters were housed in stainless-steel, wire-mesh cages in animal rooms maintained at $22 \pm 4\,^{\circ}C$, a relative humidity of $50 \pm 20\%$, and a light/dark cycle of 12/12 h. All hamsters were quarantined for 1 week during which they were fed normal diet Laboratory Rodent Diet 5001 (meal, PMI Nutritional International, St. Louis, MO), observed daily for clinically apparent signs of disease or injury and were weighed 3 times. Both water and feed were available *ad libitum*. All procedures using animals were reviewed and approved by Haskell's Institutional Animal Care and Use Committee, and the animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.3. Diet preparation

Coconut oil (Spectrums Essential, Boulder, CO) and cholesterol (J.T. Baker, Phillipsburg, NJ) were added directly to PMI 5001 ground chow and thoroughly mixed to attain homogeneous distribution of the dietary components. The blended high-fat diet had a final concentration of 10% coconut oil (w/w) and 0.05% cholesterol (w/w). Diets were prepared every other week and added to hamster feeder compartments daily with the remainder refrigerated until use. All diets were discarded within 2 weeks of preparation.

2.4. Study design

Two weeks prior to dosing, hamsters were assigned to high-fat and normal diet groups. Mean body weights between 100 and 120 g in the 2 groups were similar. Hamsters were maintained on the appropriate diet (either normal or high-fat) for 14 days prior to first administration of the test material (APFO). Groups of 5 hamsters/diet were dosed with either 0 (control), 0.1, 1, or 10 mg APFO/kg for 30 days.

2.5. Dosing methods

Dose solutions were prepared, using the nominal 20% solution (corrected for actual determined 23.1%), by serial dilution with Nanopure® water to a final concentration of 1 mg APFO/mL for the 10 mg/kg group. Additional serial dilutions were used to prepare dose solutions for the 2 lower dosing groups. All dose volumes were 10 mL/kg.

Samples of dosing solutions were analyzed for APFO concentrations near the beginning and end of the study. The vehicle control was Nanopure® water. Hamsters were dosed *via* oral gavage with care being taken not to injure the animals during treatment.

2.6. Blood collection

Approximately a 0.5 mL sample of blood was collected from the orbital sinus of each hamster under carbon dioxide anesthesia. The samples were collected 24 h after the first, 10th, 20th, and 30th dose of APFO and prior to the subsequent dose.

2.7. Analytical

Concentrations of PFOA were measured in hamster blood serum samples collected in anti-coagulant-free tubes using a modified version of the method described in Flaherty et al. [36]. Briefly, the stable isotope internal standard (dual 13C-PFOA-Perkin Elmer, Billerica, MA) was added to an aliquot of each sample to facilitate quantification. Then acetonitrile was added and the samples were centrifuged at 15,000 × *g* to precipitate protein and extract PFOA. The samples were then analyzed by reversed-phase liquid chromatography with mass spectrometric detection.

A Micromass Quattro Micro tandem mass spectrometer (MS/MS) system (Waters Associates, Milford, MA) coupled to a Hewlett Packard series 1100 liquid chromatograph (Agilent, Little Falls, DE) was used to determine perfluorooctanoate in the dosing solution and in serum. A 2.1 mm × 150 mm, 5 μm Zorbax RX-C8 column (Agilent, Little Falls, DE) was used at 35 °C. Upon injection of 20 μL of sample, a gradient consisting of Mobile Phase A (0.15% acetic acid on Nanopure® water) and Mobile Phase B (acetonitrile) was initiated at a flow rate of 0.4 mL/min starting at 5% B and moving to 80% B at 1.0 min. At 6.1 min the mobile phase was returned to 5% B until the end of the run (9 min). The electrospray interface was operated in the negative ion mode with the 413 → 369 Da transition monitored for perfluorooctanoate and the 415 → 370 Da transition monitored for the dual 13C internal standard. The limit of quantitation for PFOA was set at 0.04 ppm and the limit of detection was 0.01 ppm. Unusually high serum PFOA concentrations for 2 animals were verified by repeat analysis.

2.8. Endpoints

Clinical observations were conducted daily and body weights were determined weekly on all hamsters. Food consumption was measured weekly. Serum lipid concentrations (triglycerides, cholesterol-total, and HDL) were measured following 1, 10, 20, and 30 daily doses of APFO. These lipid parameters were determined using standard reagents on an Olympus® AU640 (Beckman Coulter) clinical chemistry analyzer (Irving, TX). Serum PFOA concentrations were analyzed at the same intervals using the analytical methods described earlier. The primary outcome tested was the serum PFOA concentration at several time points as a function of being fed either a high fat content or a normal rodent diet.

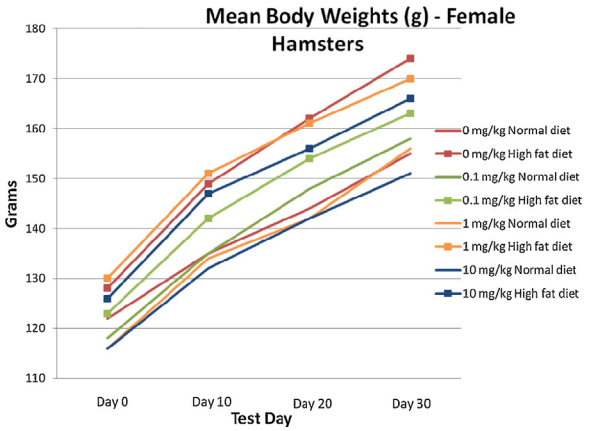

Fig. 1. Mean body weights of hamsters fed either a normal or high fat diet followed by 30-day oral dosing of APFO.

2.9. Scheduled sacrifice

After the final blood collection (30 days of dosing), hamsters were sacrificed by carbon dioxide anesthesia and exsanguinations and discarded. Extra serum was collected and saved frozen at −80 °C.

2.10. Statistical analysis

Significance was judged at $p < 0.05$. All data was first analyzed by Levene's test for homogeneity [37] and the Shapiro–Wilk test [38] for normality. If the preliminary tests were not significant, a one-way analysis of variance [39] using p-adjusted linear contrasts was performed. If the preliminary test was significant, Dunn's [40] Type 1 p-adjusted linear contrast test was employed.

3. Results

The dosing solutions contained the proper concentration of APFO during the experiment. APFO concentrations were 100.9–107.0%, 103.6–109.0%, and 89.5–111.0% of the target concentrations for the 0.1, 1.0, and 10 mg/kg dosing solutions respectively. Analysis of the dosing preparations determined that the test substance was at the targeted levels, was uniformly mixed (CV's = 0.6%, 0.0%, and 1% respectively), and was stable for 5 h at room temperature in vehicle. The vehicle dosing solution did not contain any detectable APFO.

All hamsters survived to scheduled sacrifice. No clinical observations were attributed to APFO. Hamsters in the normal and high-fat diet groups gained body weight throughout the study. Mean body weight gains were 36.0/45.7, 39.7/40.2, 40.1/40.0, and 35.1/39.8 g (normal diet/high-fat diet) for hamsters given 0, 0.1, 1, and 10 mg/kg, respectively (Fig. 1). Although not statistically significant, the 0 and 10 mg/kg animals on the high-fat diet gained 27% and 13% more weight than did their counterparts on the normal diets, respectively. The

Table 1
Serum lipid concentrations in hamsters fed either a normal or high fat diet followed by 30-day oral dosing of APFO.

	Diet Group	Normal				High Fat			
	APFO dose (mg/kg)	0	0.1	1	10	0	0.1	1	10
Parameter	Day								
Cholesterol (mg/dL)	1	104(16)	107(22)	103(16)	105(16)	215(64)	193(39)	191(46)	200(46)
	10	141(32)	149(15)	127(19)	125(17)	204(50)	208(57)	184(58)	184(50)
	20	120(25)	133(8)	117(24)	97(18)	152(13)	167(29)	156(49)	159(23)
	30	115(15)	113(7)	111(26)	97(13)	164(27)	165(33)	158(61)	151(20)
Triglycerides (mg/dL)	1	204(67)	199(76)	166(22)	189(44)	343(125)	304(59)	330(67)	354(23)
	10	259(26)	241(51)	255(88)	223(36)	386(70)	324(109)	307(36)	339(68)
	20	217(38)	242(52)	233(57)	209(42)	316(33)	273(77)	330(57)	271(68)
	30	208(22)	219(17)	223(36)	164(67)	291(59)	271(50)	366(134)	354(98)
HDL (mg/dL)	1	66(8)	71(12)	68(9)	67(9)	104(9)	115(14)	112(7)	110(14)
	10	90(17)	102(9)	86(15)	86(12)	136(23)	132(20)	119(21)	122(21)
	20	80(13)	95(2)	81(12)	76(13)	112(8)	120(9)	105(17)	121(18)
	30	83(8)	84(9)	78(17)	72(13)	115(17)	113(9)	105(22)	111(11)

Note: Numbers in parenthesis = S.D.

administration of APFO did not affect body weight gains. Hamsters sometimes slept in food compartments and food was occasionally contaminated with feces or urine thus reabsorption of the chemical was possible.

Serum lipid concentrations including total cholesterol, HDL cholesterol, and triglycerides were higher in hamsters fed the high-fat diet than were those fed the normal diet and were relatively consistent across the time points evaluated (test days 1, 10, 20, 30; Table 1). Mean serum lipid concentrations in the high-fat diet groups, as a percent of the normal diet group, ranged from 136% to 191%, 128–176%, 138–168%, and 130–234% for triglyceride, total cholesterol, and HDL cholesterol, and non-HDL respectively. Dosing with APFO did not alter serum lipid concentrations in either diet group. For all time points, serum PFOA concentrations were similar for a given dose group in both diets.

Serum PFOA concentrations were generally proportional to administered APFO (Table 2 and Fig. 2). Mean serum PFOA concentrations were approximately 0.5, 5, and 35 μg/mL for hamsters given a single dose of 0.1, 1, and 10 mg/kg APFO (normal and hyperlipidemic hamsters combined), respectively, approximating a linear dose response. After 10 doses, serum PFOA concentrations were 4–5 fold higher than after the initial dose in the 0.1 mg/kg groups and 2-fold higher in the 1 mg/kg groups. Serum PFOA concentrations in these dose groups progressively decreased at the next 2 sampling intervals, reaching concentrations of approximately 1 and 7–8 μg/mL in the 0.1 and 1 mg/kg groups respectively after 30 days of dosing. In the 10 mg/kg groups, serum PFOA concentrations progressively decreased after the first time point, and were approximately 5–6 μg/mL after 30 days of dosing. The decrease in serum PFOA concentration was more rapid in this group compared to the other dose groups.

4. Discussion

The pattern of absorption, distribution, and excretion of APFO is variable across sexes and species of laboratory animals. Both sexes of rabbits and female rats are rapid excretors while male rats and both sexes of mice are slow excretors. APFO metabolism has been studied in a single male and single female hamster following oral administration using 14C labeled material. The male hamster is a rapid excretor of PFOA and excretes more than 99% of an APFO dose within 120 h with negligible tissue retention. In contrast, the female hamster excretes PFOA more slowly with only 60% of an APFO dose excreted within 120 h of dosing. Substantial PFOA residues were present in the blood, liver, kidneys, lungs and skin at that time point [41]. Blood was identified as the tissue storage site for PFOA at 120 h post-dosing, although PFOA concentrations were not calculated. In this study, serum PFOA concentrations of female hamsters were approximately 0.5, 5, and 35 μg/mL (0.1, 1.0, and 10 mg/kg respectively) at 24 h after the first oral dose of APFO. Analyses of serum PFOA concentrations indicate that our results are similar to those of Hundley et al. [41].

Feeding of diets containing 10% coconut oil and 0.05% cholesterol did produce a hyperlipidemic state in hamsters. Serum lipid values were consistent over the duration of the study for hamsters fed either the normal or high-fat diets. The individual serum chemistry values for hamsters on the high-fat diet were above those of any animal fed the normal diet. Therefore, the high-fat diet was successful in inducing a consistently hyperlipidemic state in female hamsters.

Treating hamsters with oral doses of from 0.1 to 10 mg/kg for 30 days did not produce any clinical signs and body weight data reflected no adverse effect. Lipid measurements, such as triglycerides and cholesterol concentrations, showed no change with continuing dosing with PFOA. These lipid responses are similar to that seen in the monkey [28] and unlike the decreases seen in rodents [27].

Serum PFOA concentrations showed significant inter-individual variability as indicated by large standard deviations of means. Unusually high PFOA serum concentrations (verified by repeat chemical analysis) were observed in 2 hamsters at 1.0 mg/kg on day 10 (one from each diet group). A possible explanation for these high values and the large standard deviations is that hamsters often were observed inside their food jars, contaminating food with urine and feces and causing spillage of food. In addition, hamsters are coprophagic like several other

Table 2
Serum PFOA concentrations in hamsters fed either a normal or high fat diet followed by 30-day oral dosing of APFO.

Dose (mg/kg/day)	Diet	Test Day	1	10	20	30
0.0	Normal fat	Mean	0.000	0.000	0.000	0.000
		SD	0.000	0.000	0.000	0.000
	High fat	Mean	0.000	0.000	0.009	0.000
		SD	0.000	0.000	0.021	0.000
0.1	Normal fat	Mean	0.573	1.898	1.026	0.869
		SD	0.0043	0.557	0.357	0.276
	High fat	Mean	0.512	2.824	1.820	1.394
		SD	0.176	0.906	0.611	0.359
1.0	Normal fat	Mean	5.128	9.686	8.630	7.040
		SD	0.620	8.435	9.229	9.433
	High fat	Mean	5.440	10.352	9.028	7.818
		SD	0.986	8.852	10.711	11.539
10	Normal fat	Mean	34.280	26.242	13.556	5.694
		SD	9.964	20.531	11.544	3.652
	High fat	Mean	34.820	20.674	13.410	5.212
		SD	15.277	14.001	9.728	1.426

herbivores [42–44]. This behavior likely resulted in unreliable food consumption measurements, as well as the possibility of re-exposure to PFOA following ingestion of urine and feces-contaminated food (urine and feces are the major routes of PFOA elimination in the hamster [41]). In this experiment, food spillage and hamsters sitting in the food jars were frequent occurrences with urine and feces noted in the food jars. When the 2 high serum concentrations at 1.0 mg/kg on day 10 are excluded from the mean, the day 10 PFOA concentrations for hamsters at 0.1 and 1.0 mg/kg are consistent at days 10, 20, and 30. It is also possible that the absorption and excretion of APFO/PFOA is inherently variable in female hamsters. Single animals were used in the previous study by Hundley et al. [41] so published data concerning variability in hamster APFO kinetics is not available.

Feeding of high-fat diet did not alter serum PFOA concentrations in hamsters at any dose (0.1, 1.0, and 10 mg/kg). Although mean serum concentrations were usually slightly higher in hamsters fed a high-fat diet compared to those fed a normal diet, there was considerable overlap in the PFOA concentrations of individual animals within a given dose group and none of the differences were statistically significant. Therefore, we did not demonstrate any effect of diet on serum PFOA concentrations in hamsters dosed up to 30 days.

For both dietary groups, the serum PFOA concentrations 24 h after the first dose were approximately linear across the APFO dose range (0.1–10 mg/kg). After subsequent doses at 10 mg/kg, serum concentrations decreased progressively through day 30. At 0.1 and 1.0 mg/kg mean values were highest following the tenth dose and decreased progressively following the 20th and 30th dose. Exclusion of 2 outliers at 1.0 mg/kg resulted in generally consistent PFOA concentrations at days 10, 20, and 30; a similar pattern was observed at 0.1 mg/kg.

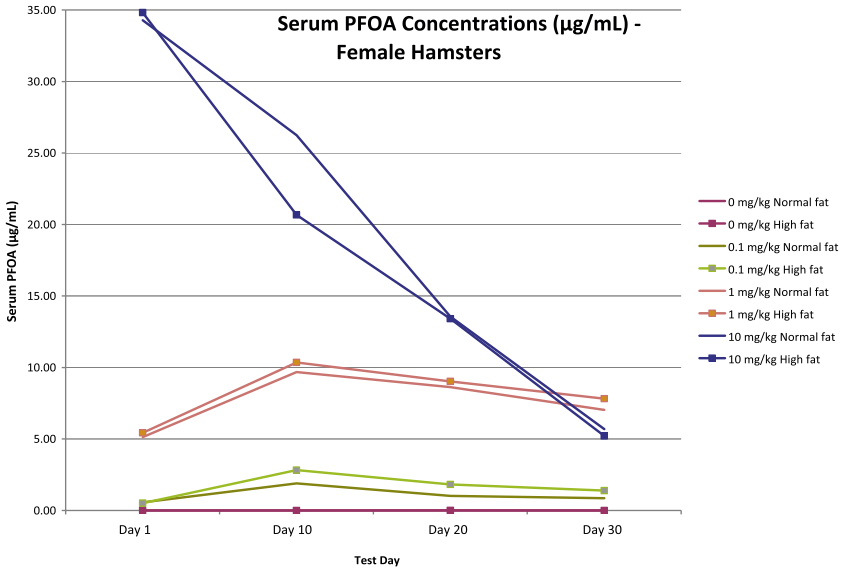

Fig. 2. Serum PFOA concentrations in hamsters fed either a normal or high fat diet followed by 30-day oral dosing of APFO.

The reason for decreasing serum PFOA concentrations in female hamsters dosed with 10 mg/kg despite continued daily dosing with APFO was not determined in this study. The pattern seen here was not seen in rats and monkeys as in those species an increase, not a decrease, in PFOA concentration with continued daily dosing (attaining a steady state concentration) was reported [28,29,45]. The observation here suggests that either absorption or excretion is being altered with repeated doses.

Enzyme induction and metabolism of APFO could result in lower concentrations with continued dosing, however, PFOA is stable in the blood and to date there is no evidence of mammalian metabolism of PFOA [46,47]. Distribution of PFOA to peripheral tissues may increase after repeated dosing, however, a depot for PFOA has not been detected in either rodents or the monkey. In rats and mice, PFOA has been shown to affect expression of renal transporters indicating that PFOA may alter its own excretion [48]. Gene array experiments have shown that PFOA decreases organic anion transporting polypeptides (Oatp) in the rat and mouse liver [49,50]. In male rats, organic anion transporters recycle PFOA from urine back to the blood resulting in a longer half-life than female rats. Rat renal transporter Oat1a1 has been shown to reabsorb PFOA from the urine [48,51,52]. It is possible that PFOA may induce a renal or biliary/intestinal transporter in hamsters that preferentially transports PFOA into urine or feces. In this study, excretion of PFOA in urine and feces was not determined

An association between serum PFOA concentrations and increased cholesterol levels has been reported in a number of cross sectional studies and in 3 studies where multiple measures were obtained (see references in introduction). A recent longitudinal assessment of lipid parameters in workers involved in demolition of perfluoroalkyl manufacturing facilities failed to show an adverse association between serum PFOA and cholesterol, non-HDL cholesterol, and HDL cholesterol [53]. Several other lines of evidence argue against causality. First, the lack of an association between serum PFOA and cholesterol concentrations in workers who are more highly exposed [1,14,18,19]. Mechanistic studies in animals which show that hypo, not hyperlipidemia occurs with increasing exposure to PFOA [5,47]. No changes in cholesterol or perhaps even a decrease in cholesterol levels in patients in a phase 1 clinical trial who have the highest PFOA concentrations ever measured in humans [54]. Finally, possible biological roles involved membrane transport, uptake and efflux, and dietary factors might be involved.

Reverse causality refers to a direction of cause-and-effect which is contrary to a common presumption [25]. It could be that the assumption that A causes B may be wrong and B might cause A. This hamster experiment was designed to test the hypothesis that higher lipid levels could lead to higher PFOA serum concentrations rather than the reverse. Other PFOA related associations have been proposed to occur due to reverse causality. For example, lower PFOA serum levels have been associated with higher birth weight (and to a lesser extent length). Physiological changes during pregnancy causing plasma volume expansion can affect pharmacokinetics and can be associated with birth outcomes. Thus the increased serum volume normally occurring during pregnancy effectively dilutes the solute (PFOA). Larger fetuses require larger volumes of serum. This will result in lowered concentrations of PFOA. Thus it is expected that larger (heavier, longer) fetuses would be associated with lower PFOA serum levels as a result of normal physiological changes, not that neonatal weight and length is affected by the PFOA serum concentration [55,56]. Similarly, longer inter-pregnancy intervals have been associated with serum PFOA concentrations. This is likely also explained by PFOA kinetics and distribution during pregnancy to the fetus and excretion of PFOA post-pregnancy in breast milk. The longer the post-pregnancy interval, the more likely it is that the PFOA concentration will recover to pre-pregnancy concentrations. Indeed, findings in nulligravid women did not support an association with longer inter-pregnancy intervals [57].

Under the conditions of this study, higher serum lipids did not affect the toxicokinetics of PFOA in hamsters as serum PFOA concentrations in hyperlipidemic hamsters were essentially the same as those fed normal diet. In this species, serum PFOA concentrations generally declined over the course of the study despite continued dosing with APFO at doses between 0.1 and 10 mg/kg. This patterns has not been seen in other species (rat: [47,58]; mouse: [28]; monkey: [45]) where serum perfluorooctanoic acid concentrations either rise or reach an apparent steady-state with repeated doses.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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