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PFOS induced lipid metabolism disturbances in BALB/c mice through inhibition of low density lipoproteins excretion

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Male BALB/c mice fed with either a regular or high fat diet were exposed to 0, 5 or 20 mg/kg perfluorooctane sulfonate (PFOS) for 14 days. Increased body weight, serum glucose, cholesterol and lipoprotein levels were observed in mice given a high fat diet. However, all PFOS-treated mice got reduced levels of serum lipid and lipoprotein. Decreasing liver glycogen content was also observed, accompanied by reduced serum glucose levels. Histological and ultrastructural examination detected more lipid droplets accumulated in hepatocytes after PFOS exposure. Moreover, transcripitonal activity of lipid metabolism related genes suggests that PFOS toxicity is probably unrelevant to PPARα's transcription. The present study demonstrates a lipid disturbance caused by PFOS and thus point to its role in inhibiting the secretion and normal function of low density lipoproteins.

Perfluorooctane sulfonate (PFOS) is a ubiquitous perfluorinated compound with a variety of uses in both industrial and consumer products, leading to its increased global exposure[1–3]. It can now be detected in the liver and blood of fish, birds and mammals, even in human serum and milk[2,4–6]. Though the PFOS concentration in general population and wildlife was at the ng/mL level, concentrations over 10 μg/mL have been detected in serum of occupational populations[7,8]. And *in vivo* studies revealed that exposure to 10 and 20 mg/kg PFOS could affect the neuroendocrine system or cause lung injury in rats[9,10]; 5, 20 and 40 mg/kg PFOS treatment induced reproductive or immune abnormalities in mice[11–13].

The liver is an important organ for detoxification and lipid metabolism. As the primary site of bioaccumulation of certain pollutants, it is likely to be a target of PFOS[14]. Previous studies reported that PFOS decreased body weight of rodents while conversely increased the liver index[9,13,15], intracellular hepatic fatty acid and cholesterol content[16]. Vacuolation and hypertrophy of hepatic cells also occurred in PFOS-treated mice and rats[15,17,18]. PFOS has been considered to disturb the expression of hepatic genes associated with fatty acid synthesis, activation, transport and oxidation pathways, as well as hormonal regulation[14,19,20]. Accordingly, PFOS-treated rodents got reduced serum cholesterol and triglyceride levels[16,18,21], decreased thyroid hormone concentrations, and elevated levels of serum corticosterone[8,22,23].

Peroxisome proliferator-activated receptor α (PPARα) is responsible for regulating the expression of genes involved in fatty acid, cholesterol metabolism, and DNA replication as well[24,25]. PFOS induces PPARα activation in both rodents and humans, this has been supposed to be a key factor in its various toxicities[26–28]. As PFOS resembles fatty acid in structure, it can bind to apolipoprotein and disturb lipid transport thus affect the physiological effects of lipids, this might also contribute to PFOS-caused toxicities[29]. However, those hypotheses are still under debate, the specific mechanism needs to be elucidated.

Feeding a high fat diet (HFD) to rodents causes increase in body weight, fat mass accumulation, and circulating concentrations of lipids, and accelerates free fatty acid metabolism[30–32]. In this study, male BALB/c mice were fed

Table 1 | Change in body weight, food consumption and organ indices after PFOS exposure

	PFOS (mg/kg)	Change of Body Weight (g)[a]	Food Consumption (g/day)	Liver Weight (g)	Liver Index	Fat Weight (g)	Fat Index
RD	0	2.46 ± 0.35	3.69 ± 0.09	1.34 ± 0.06	5.42 ± 0.19	0.47 ± 0.06	1.64 ± 0.26
	5	2.78 ± 0.29	3.62 ± 0.12	2.43 ± 0.11**	9.67 ± 0.30**	0.37 ± 0.05	1.34 ± 0.19
	20	−4.07 ± 0.46**	2.39 ± 0.15**	2.66 ± 0.20**	14.22 ± 0.96**	0.04 ± 0.03**	0.12 ± 0.09**
HFD	0	4.12 ± 0.39	3.59 ± 0.14	1.88 ± 0.05	6.77 ± 0.12	0.57 ± 0.07	2.13 ± 0.25
	5	2.81 ± 0.40*	3.46 ± 0.12	2.93 ± 0.11**	11.54 ± 0.22**	0.48 ± 0.04	1.88 ± 0.14
	20	−3.73 ± 0.41**	2.65 ± 0.22**	3.26 ± 0.13**	17.27 ± 0.50**	0.02**[b]	0.05**[b]

*$P<0.05$, **$P<0.01$ versus their respective controls, using one-way ANOVA. RD = regular diet; HFD = high fat diet. All values are means ± SE (standard error); N=16 per group.
[a]Change in body weight was calculated as [final body weight (g)- initial body weight (g)].
[b]Fat was obtained from only one individual, no fat was harvested from the remaining animals.

either a regular diet (RD) or a HFD during PFOS exposure. Considering that PFOS is structurally similar with fatty acid, a HFD here could mobilize more lipids and might relieve the competition of PFOS. Furthermore, by comparing the results of HFD-fed and RD-fed mice, we aimed to explore the molecular mechanism of PFOS-induced toxicity.

Results

Body weight and organ indices. Though consuming comparable feed, HFD-fed controls gained more weight than RD-fed ones (4.12 g compared to 2.46 g) after 14 days' exposure; the extra fat also caused increasing weight of the livers and ventral fat (Table 1). 5 mg/kg PFOS had no effect on body weight change or feed consumption of RD-fed mice, but caused body weight loss in individuals fed a HFD ($P < 0.05$). Daily feed consumption of 20 mg/kg PFOS-treated mice was reduced, which was more significant in RD-fed animals. Decreased body weight was also observed in those individuals ($P < 0.01$, Table 1).

In contrast to the body weight loss, PFOS-exposed livers were more hypertrophic, in a dose-dependent manner ($P < 0.01$, Table 1). Therefore the liver indices were significantly elevated ($P < 0.01$, Table 1). However, there was a decrease in the amount of ventral fat in PFOS-exposed mice. This was significant when treated with 20 mg/kg PFOS ($P < 0.01$), no or little ventral fat was obtained (Table 1). Accordingly, the fat indices of these mice were decreased with the increasing PFOS doses. Both RD and HFD-fed mice displayed similar changes (Table 1).

Hepatic fat and glycogen content. As enlarged livers occurred after PFOS exposure, liver fat content was determined later to assess hepatic lipid accumulation (Fig. 1). For control mice, more fat existed in the livers when fed a HFD ($P < 0.01$). Consistent with the increased liver indices, a significant increase in liver fat content occurred in PFOS-treated RD individuals ($P < 0.01$). No significant increase was observed in HFD-fed mice after PFOS exposure, but they still retained higher levels than RD-fed controls (Fig. 1).

Liver glycogen, acting as a storage form of glucose and maintaining steady blood glucose levels, was also detected. Though ingesting more fat, no obvious change of liver glycogen was observed in HFD-fed control mice. Reduced liver glycogen content occurred in PFOS-exposed RD mice (Fig. 1). HFD-fed mice got similar decrease following PFOS exposure, more obvious in 5 mg/kg dose group (2.45 mg/g liver compared to 6.17 mg/g liver, $P < 0.01$).

Histological and ultrastructural assessment of the liver. H & E stained liver sections showed hydropic degeneration and vacuolation in the hepatocytes after PFOS exposure, and more severe in 20 mg/kg dose groups (Fig. 2-B, C, E and F). HFD-fed mice demonstrated greater susceptibility to pathological changes of hepatocytes than RD individuals, with significantly more hypertrophied hepatic cells and enlarged intercellular spaces (Fig. 2-D, E and F). However, the nuclei remained unaffected after PFOS treatment (Fig. 2-B, C, E and F).

The ultrastructure of hepatocytes was observed by using a transmission electron microscopy. More lipid droplets were accumulated in hepatic cells of HFD-fed control mice (Fig. 3-A, D). Some PFOS-exposed RD mice had voids within the cytoplasm resulting from dilatations of the endoplasmic reticulum (Fig. 3-B, C), these were also observed in HFD-fed ones (Fig. 3-E, F). More lipid droplets accumulated in livers of RD and HFD-fed mice following PFOS exposure (Fig. 3-B, C, E and F), especially in 20 mg/kg dose groups (Fig. 3-C, F). This is consistent with their histological changes and increasing fat content of the livers.

Moreover, sections of kidney, heart and intestine showed no obvious pathological changesafter PFOS treatment, which verified that these organs were not the main target of PFOS.

Changes in serum biochemical parameters. Comparing with RD mice, a HFD induced higher serum glucose level in control groups (Fig. 4-A), but the liver glycogen content retained a comparable level. For both RD and HFD-fed mice, serum glucose level remained unchanged when treated with 5 mg/kg PFOS, but was decreased

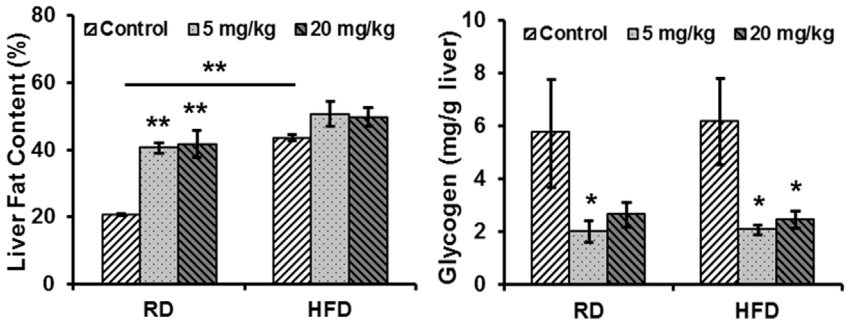

Figure 1 | Liver fat and glycogen content after 14 days of PFOS exposure. *$P < 0.05$, **$P < 0.01$ versus their respective controls or between the two groups indicated, using one-way ANOVA. RD = regular diet, HFD = high fat diet. All values are means ± SE (standard error); N = 4 per group.

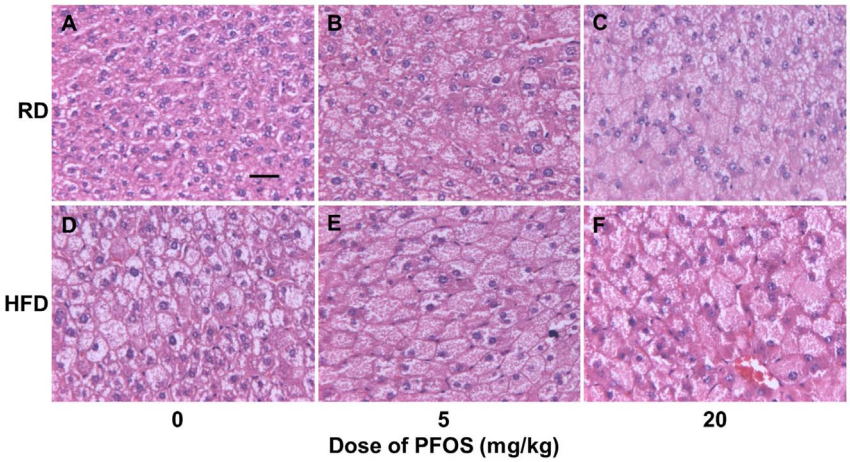

Figure 2 | H & E stained sections of livers from control and PFOS-exposed mice. Sections of livers viewed under light microscopy. (A) Control mice fed a regular diet, (B) 5 mg/kg PFOS-treated mice fed a regular diet, (C) 20 mg/kg PFOS-treated mice fed a regular diet, (D) control mice fed a high fat diet, and (E) 5 mg/kg PFOS-treated mice fed a high fat diet, (F) 20 mg/kg PFOS-treated mice fed a high fat diet. Scale bar in panel A, 200 μM.

significantly in 20 mg/kg dose groups ($P < 0.01$, Fig. 4-A). Serum albumin concentration, a reflection of liver function, was not changed by a HFD; While it increased significantly in a dose-dependent manner following PFOS exposure ($P < 0.01$ for both RD and HFD-fed mice, Fig. 4-B).

Despite the high fat in diet, serum triglyceride in HFD control mice retained similar levels with RD controls (Fig. 4-C). However, the HFD caused elevated levels of serum cholesterol ($P < 0.01$, Fig. 4-D). High-density lipoprotein cholesterol (HDL-ch) and low-density lipoprotein cholesterol (LDL-ch), according with HDL and LDL, their levels were also increased by feeding a HFD ($P < 0.01$, Fig. 4-E, F).

For RD-fed mice, serum triglyceride level was slightly elevated after 5 mg/kg PFOS exposure, while decreased significantly in 20 mg/kg dose group ($P < 0.01$, Fig. 4-C). HFD-fed mice displayed similar changes in serum triglyceride concentration following PFOS exposure (Fig. 4-C). PFOS-treated RD mice had lower levels of serum cholesterol, HDL-ch and LDL-ch, which was more significant in 20 mg/kg dose group (Fig. 4-D, E and F). A dose-dependent decrease in serum cholesterol occurred in HFD-fed mice with PFOS exposure (Fig. 4-D), so as the serum HDL-ch (Fig. 4-E). Though not so significant, slight decrease in LDL-ch concentrations was also detected in PFOS-treated HFD individuals, this tended to be more obvious in 20 mg/kg dose group (Fig. 4-F).

Alterations in serum hormone levels. Comparing the median, HFD controls had lower testosterone level (4.54 ng/mL) than RD controls (6.06 ng/mL). PFOS exposure also reduced the average level of testosterone in both RD and HFD-fed mice. However, due to the great fluctuation in the parallel individuals, serum testosterone concentrations did not differ between those PFOS-treated and control mice (Fig. 5). A HFD caused slightly increase in serum estradiol level, 53 pg/mL compared to 34 pg/mL of RD controls (Fig. 5). PFOS exposure had no significant effect on serum

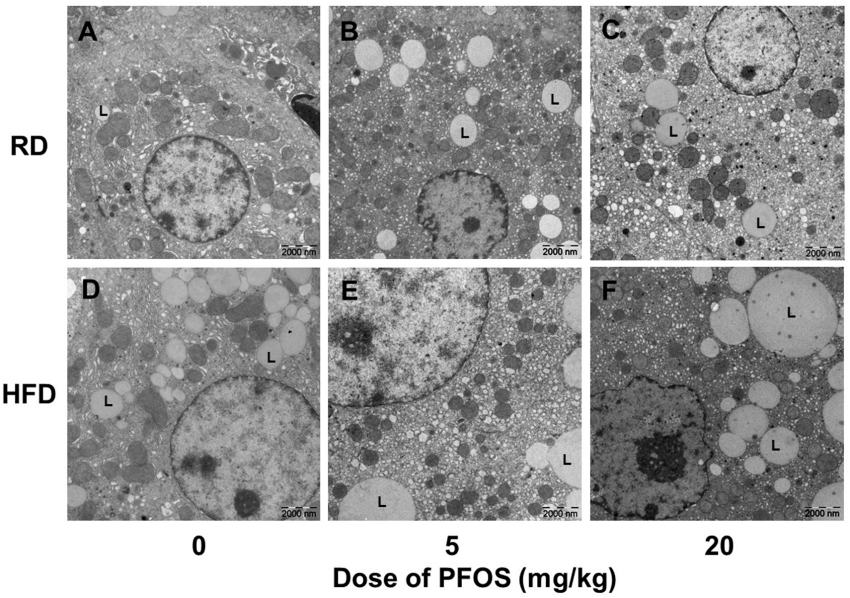

Figure 3 | Hepatocellular ultrastructure in control and PFOS-exposed mice. Transmission electron microscopy images of representative liver samples. (A) Control mice fed a regular diet, (B) 5 mg/kg PFOS-treated mice fed a regular diet, (C) 20 mg/kg PFOS-treated mice fed a regular diet, (D) control mice fed a high fat diet, and (E) 5 mg/kg PFOS-treated mice fed a high fat diet, (F) 20 mg/kg PFOS-treated mice fed a high fat diet. L = Lipid droplet. Scale bar is shown in each panel.

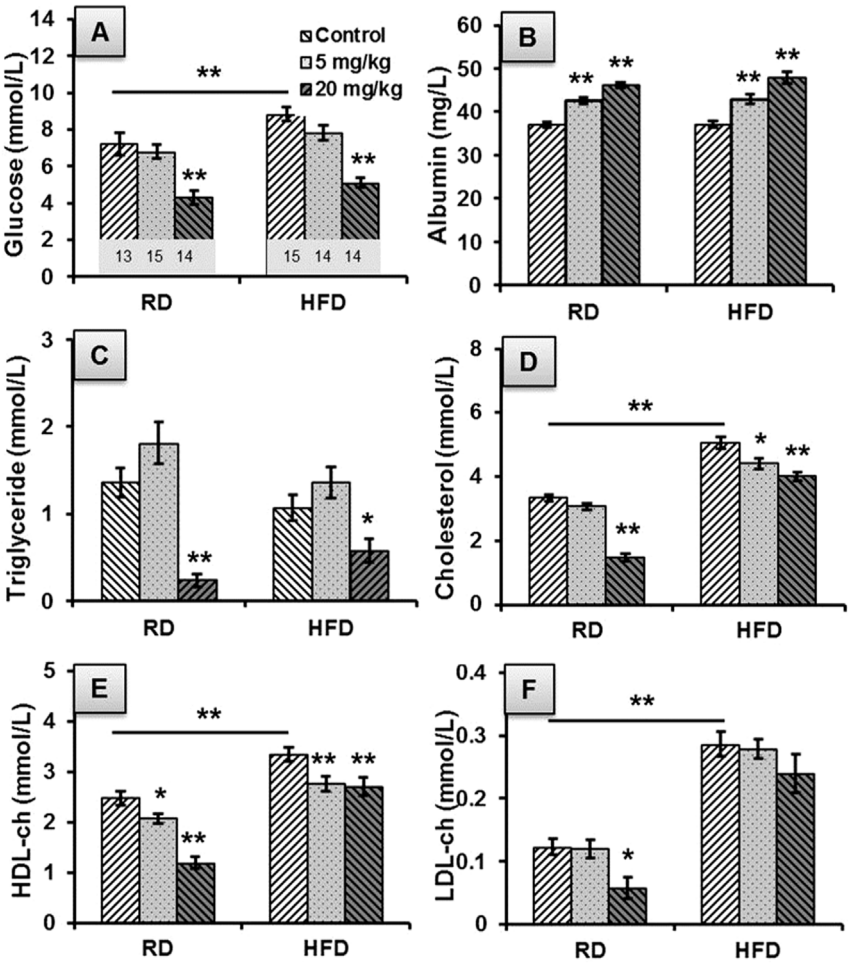

Figure 4 | Serum biochemical parameters after 14 days of PFOS exposure. The legend and numbers of mice per group are detailed in panel A. $*P < 0.05$, $**P < 0.01$ versus their respective controls or between the two groups indicated, using one-way ANOVA. RD = regular diet, HFD = high fat diet. All values are means ± SE (standard error).

estradiol levels of RD-fed mice, but a moderate decrease in HFD ones (Fig. 5).

Expression of PPARα and lipid metabolism related genes in liver. PPARα controls the transcriptional expression of key enzymes that are involved in FA uptake and β-oxidation. Feeding extra fat resulted in greater PPARα gene expression in control mice, but not so significant (Fig. 6). Expression of PPARα was not up-regulated by 5 mg/kg or 20 mg/kg PFOS in livers of RD-fed mice. However, this expression was inhibited in HFD-fed individuals, in a dose-dependent manner, turned out to be very significant in 20 mg/kg dose group ($P < 0.01$, Fig. 6).

Carnitinepalmitoyl transferase 1A (CPT1A), responsible for transportation of fatty acids into the mitochondria and its catabolism; cholesterol 7α-hydroxylase (CYP7A1), key gene in the transformation of cholesterol into bile acids, their expressions were further investigated. Adding more fat in diet caused activation in CPT1A and CYP7A1 expression ($P < 0.01$, Fig. 7). When feeding a RD, CPT1A expression was up-regulated by 5 and 20 mg/kg PFOS ($P < 0.01$); 5 mg/kg PFOS exposure also caused slight increase in CYP7A1 expression, while no change occurred after 20 mg/kg PFOS treatment. Nonetheless, this trend was reversed by a HFD following PFOS exposure (Fig. 7). Expression of CPT1A gene was inhibited in HFD-fed mice, displaying a dose-dependent effect ($P < 0.01$, Fig. 7).

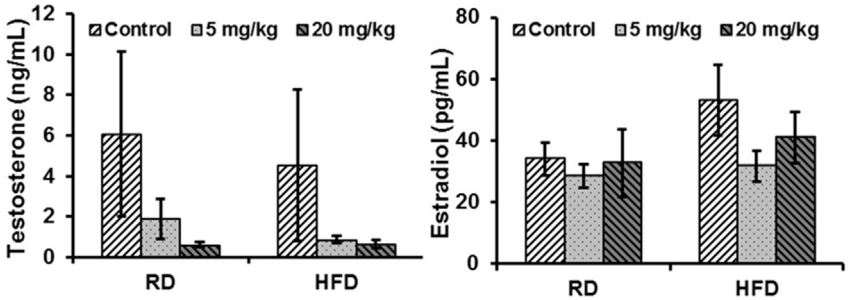

Figure 5 | Serum sex hormone levels after 14 days of PFOS exposure. $*P < 0.05$, $**P < 0.01$ versus their respective controls or between the two groups indicated, using one-way ANOVA. RD = regular diet, HFD = high fat diet. All values are means ± SE (standard error); N = 6 per group.

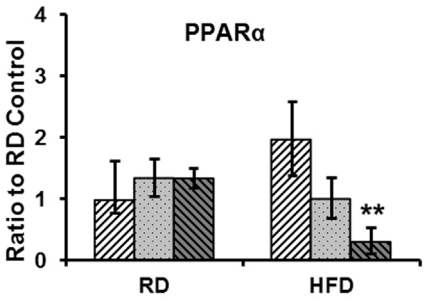

Figure 6 | Expression of PPARα after 14 days of PFOS exposure. *$P < 0.05$, **$P < 0.01$ versus their respective controls or between the two groups indicated, using one-way ANOVA. RD = regular diet, HFD = high fat diet. All values are means ± SE (standard error); N = 4 per group.

CYP7A1 gene expression was inhibited significantly in those mice, $P < 0.01$ and $P < 0.05$ for 5 and 20 mg/kg dose group respectively (Fig. 7).

Discussion

PFOS has been considered to disturb lipid metabolism in rodents and human because of its similar structure to fatty acid[33-35]. 5 mg/kg PFOS here did not cause significant change in body weight or food consumption, but more lipids were accumulated in the livers. These mice also had lower serum cholesterol level, as well as the HDL and LDL. However, serum triglyceride level was elevated, indicating the ventral fat store might be released. Increasing serum albumin levels might also contribute to the released triglyceride and fatty acid. We also detected declined liver glycogen storage, which suggested sustaining the serum glucose levels. With increasing dose of PFOS, severe body weight loss was observed. The decreasing liver glycogen storage was not sufficient to release glucose, leading to the reduction in serum glucose levels. Serum triglyceride and cholesterol concentrations were also decreased after 20 mg/kg PFOS exposure, accompanied by much lower levels of HDL and LDL. Additionally, more lipids accumulated in the livers following 20 mg/kg PFOS treatment while the ventral fat index reduced sharply. The accumulation of lipid droplets within hepatic cells was further proved by pathological and ultrastructural assessments. We also examined spleen and thymus cells but found no lipid accumulation[36]. These results suggest that PFOS exposure disturbed homeostasis of lipid metabolism in BALB/c mice, and caused declined liver glycogen storage as well as serum glucose levels.

Besides a RD feeding, here extra fat were added to the feed during PFOS exposure, which is different from former PFOS-related studies. It was reported previously that increasing body weight, hyperglycemia and steatohepatitis occurred in HFD-fed rodents[31,32]. Similarly, the liver fat content of HFD-fed mice here was significantly elevated, as were serum glucose, cholesterol, HDL and LDL concentrations. Expression of key genes related to lipid metabolism such as CPT1A and CYP7A1 were also up-regulated in the livers. But unlike the control mice, the PFOS-treated HFD mice showed lipid accumulated livers along with decreased serum glucose and lipid levels. Those results were also consistent with performance of RD-fed mice. Therefore, supplement of dietary fat could not moderate the disturbances of PFOS on lipid metabolism.

Molecular mechanism of PFOS-caused toxicities remains unclear. Major opinions focused on the activation of PPARα[37,38]. Some researchers have demonstrated that PFOS elicited its hepatotoxicity by causing increased expression of PPARα-regulated genes[39]. However, one study done by Abbott showed that PPARα knockout mice also got developmental toxicity and enlarged livers following PFOS exposure, which occurred in wild type mice[40,41]. In our study, a HFD indeed induced a slight increase in PPARα expression of control mice. But unlike previous finding[39], PFOS caused no stimulation of PPARα gene expression in RD-fed mice while inhibited its expression in HFD-fed individuals. Nevertheless, both RD and HFD-fed mice displayed similar differences in body weight, liver index and lipid accumulation with PFOS exposure; Those mice also got similar fluctuating trend in the serum lipid and lipoprotein levels. Moreover, PFOS altered the expression of CPT1A and CYP7A1 gene,in a different pattern from PPARα. CPT1A and CYP7A1 have been reported to have PPRE response elements in their promoter regions, indicating that they are possibly regulated by PPARα[42]. We thus consider activation of PPARα might not be the main cause of PFOS-induced changes in lipid metabolism. Due to their key roles in regulating the metabolism, the alteration of CPT1A and CYP7A1 expression might indicate that transportation of fatty acids into the mitochondria and transformation of cholesterol into bile acids was blocked by PFOS. Therefore, the rate of lipid catabolism would be decreased. This may directly contribute to the lipid accumulation in PFOS-exposed livers.

Increased serum albumin level was observed here in PFOS-treated mice, which ascended with the exposure doses. Serum albumin was reported to play an important role in maintaining colloid osmotic blood pressure, and transport, distribution of various molecules including fatty acids[43,44]. The increasing fatty acids released from the ventral fat might be the cause of elevated albumin level here. Since albumin is originally synthesized in the liver, its normal function indicated that the liver remained undamaged. We thus infer that accumulation of lipid in livers of PFOS-treated mice might not be caused by hepatic injury, the disturbance in transport and metabolism of lipids should account for it.

Particularly, 20 mg/kg PFOS-exposed mice got reduced serum glucose level in contrast to their lipid-accumulated livers. This decline might cause an acute increase in energy demand by selective oxidation of liver glycogen, following the decreased liver glycogen content. As it went on, the gluconeogenesis pathway would be activated, including gluconeogenesis from glycerol[43]. Additionally, less

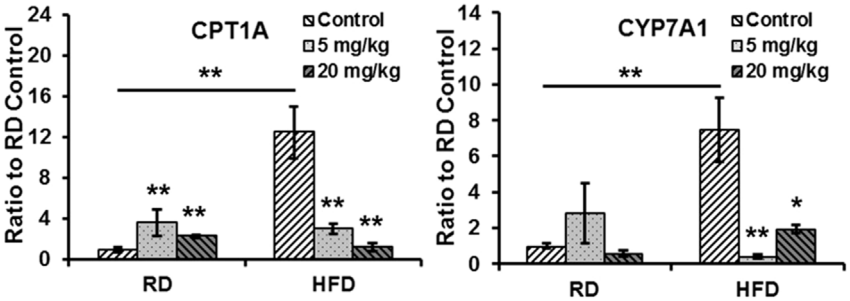

Figure 7 | Expression of key genes involved in lipid metabolism after 14 days of PFOS exposure. *$P < 0.05$, **$P < 0.01$ versus their respective controls or between the two groups indicated, using one-way ANOVA. RD = regular diet, HFD = high fat diet. All values are means ± SE (standard error); N = 4 per group.

Table 2 | Primer sequences of target genes for RT-PCR analysis

Target gene	Gene bank accession No.	5′ → 3′ Primer sequences
β-actin	AK167825	Forward: TCGTGCGTGACATCAAAG Reverse: GAAGGAAGGCTGGAAAAG
PPARα	NM_001113418	Forward: TGAACAAAGACGGGATGC Reverse: GCCACAAACAGGGAAATG
CPT1A	NM_013495	Forward: TGTCCAAGTATCTGGCAGTC Reverse: TGTCCAAGTATCTGGCAGTC
CYP7A1	BC021642	Forward: CTCCGTACTTTGAGCAGC Reverse: TCACTCGTGGACATCCC

exogenous fat might be ingested, and then more glycerol should be mobilized from ventral fat to supply glucose, accompanied by release of fatty acids[43]. As a result, more fatty acids might be transported by albumin to the liver, while the ventral fat atrophied. Similar phenomenon was observed in our research on PFOA, mice treated with increasing doses of PFOA got reduced serum glucose levels while lipid-accumulated livers[43]. However, mechanism of this phenomenon as well as its relationship with the disturbed lipid homeostasis needs to be explored.

Plasma lipoproteins mediate the transport and delivery of triglyceride, cholesterol and certain lipid-soluble vitamins to body tissues[46,47]. HDL participates in reverse cholesterol transport from peripheral tissues to the liver; LDL, revolves from very low density lipoproteins (VLDL), carries endogenous lipids (including triglyceride and cholesterol) from the liver to peripheral tissues for their subsequent processing, storage or secretion[46,47]. A HFD here significantly elevated serum HDL and LDL levels, aiming to transport the extra fat. Nevertheless, both RD and HFD-fed mice got reduced HDL and LDL levels after PFOS exposure. We previously reported similar results with PFOA-exposed BALB/c mice, but lipid droplets occurred in the hepatocyte nuclei of those mice[45]. However, considering their structural resemblance, PFOS may act like PFOA in disturbing VLDL synthesis and excretion. As a result, the secretion and normal function of LDL would be also blocked, this further might lead to reduced serum LDL levels. According to this hypothesis, as more lipids were released from ventral fat following PFOS exposure while lipids exportation from livers was blocked, then lipid accumulated-livers and atrophic ventral fat would occur.

In conclusion, a diet high in fat cannot relieve the lipid metabolism disturbance caused by PFOS exposure. We consider the PFOS-caused toxicities were probably irrelevant to PPARα gene expression. As triglyceride in ventral fat were mobilized for gluconeogenesis following decreased serum glucose levels, more free fatty acids might be transported to the liver by albumin; By inhibiting the secretion and normal function of LDL, PFOS could possibly block the exportation of lipids from the liver to peripheral tissues. As a result, more lipids accumulated in the liver but ventral fat store was decreased, serum triglyceride and cholesterol levels were also remarkably reduced. Nevertheless, further researches are required to demonstrate the hypothesis of those mechanisms.

Methods

Animals and Diet. Male BALB/c mice aged 4–5 weeks old were purchased from the Hubei Laboratory Animal Research Center (Hubei, China). Mice at this age gain body weight continuously and lipid metabolism is the most vigorous. The mice were housed in a climate-controlled facility with a temperature of 24 ± 2°C, relative humidity of 60–70%, and a 12:12 hr light: dark cycle. Following acclimatization for one week, they were randomly divided into six groups (A, B, C, D, E and F), 8 individuals for each group and housed 4 per cage. During PFOS exposure, mice in groups A, B and C were fed the RD, whereas mice in groups D, E and F were fed a HFD containing 10% more lard and 3% more cholesterol than the RD. The feed was purchased from Beijing HFK Bio-Technology Co., LTD.

Experimental protocol. This experiment was repeated twice for its reliability. The animal treatment was approved by the Ethics Committee of Jianghan University (Wuhan, China). The methods were carried out in accordance with the approved guidelines. PFOS (CAS number 2795-39-3, purity >98%, Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.5% Tween 20 (Sigma-Aldrich), and given via daily oral gavage to mice for 14 days at doses of 5 mg/kg (groups B and E) and 20 mg/kg (groups C and F). Control mice (groups A and D) received 0.5% Tween 20 without PFOS. The two doses were chosen by referring previous research on PFOS-exposed mice[11–13], and comparable PFOS concentrations (more than 10 μg/mL) have also been detected in occupational populations[7,8]. During the exposure, body weight and feed consumption were measured every 2 days, and the dose of PFOS was adjusted to the changing body weight every 4 days.

Blood samples were collected by retro-orbital bleeding into sterile polystyrene tubes, and the mice were then sacrificed by cervical dislocation. The livers and ventral fat pad were quickly harvested and weighed. Organ indices were calculated as [organ weight (g)/body weight (g)] × 100. Organ samples were fixed immediately either in 4% paraformaldehyde or 2.5% glutaraldehyde for later histopathological or ultrastructural assessment. Additional liver samples were stored at −80°C for following detections.

Detection of serum biochemical parameters and hormone levels. The blood samples were centrifuged at 3000 rpm for 10 min to separate serum for biochemical analysis. Serum albumin, glucose, cholesterol, triglyceride, HDL-ch and LDL-ch were measured using an Abbott Aeroset automated instrument analyzer (Abbott Diagnostics, Abbott Park, Illinois, USA). LDL-ch and HDL-ch were detected as cholesterol content in serum lipoprotein molecules and are regarded here as representative of serum LDL and HDL levels. Serum concentrations of estradiol and testosterone were also determined using an Abbott AXSYM System automated immunoassay analyzer (Abbott Diagnostics, Abbott Park, Illinois, USA).

Determination of liver fat content. Liver fat content was determined as follows: (1) Fresh tissue wrapped in aluminum foil was freeze-dried in an Alpha1-2LD plus freeze dryer (Christ, Osterode am Harz, Germany) for two days. (2) The dried tissue was ground, weighed, and denoted as M1. Samples were then mixed with 15 ganhydrous sodium sulfate and extraction was performed using an ASE300 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) set to the following parameters: solvent dichloromethane/n-hexane (1 : 1 v/v), pressure 15 M Pa, temperature 150°C. (3) The extractant solution was then transferred into a pre-weighed dry flask (flask weight = W1) and dried using a Laborota 4002 rotary evaporator (Heidolph, Schwabach, Germany). (4) The extract was further dried under a gentle stream of nitrogen, and the flask was reweighed (weight of flask + extract = W2). The final dry weight of the fat (M2) was calculated as the difference between W2 and W1. Liver fat content was calculated as M2/M1 × 100%.

Measurement of hepatic glycogen content. Weighed liver samples were homogenized in normal saline. Hepatic glycogen levels were then measured using a hepatic/muscle glycogen detecting assay kit from the Nanking Jiancheng bioengineering research institute (Nanking, China).

Histopathological observation. Following overnight fixation in 4% paraformaldehyde, the livers were dehydrated by stepwise transfer into increasing concentrations of ethanol (70%, 80%, 85%, 90%, 95% and 100%) and embedded in paraffin. Embedded tissues were sliced into 4 μm sections using a Leica automatic microtome (Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin (H & E) for histological assessment under light microscopy (Olympus BX41, Tokyo, Japan).

Transmission electron microscopy examination. Liver samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 hr. Samples were then washed twice with phosphate buffer (0.1 M, pH 7.4) and post-fixed for 20 min with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). After dehydration, samples were embedded in Epon-812 epoxy resin and ultrathin sections were made using a LKB-V ultramicrotome (LKB, Bromma, Sweden). Sections were mounted on copper grids and stained with 2% uranyl acetate in a 1% solution of lead citrate for 30 min. The ultrastructure of the liver samples was visualized using a FEI Tecnai G^2 (FEI, Hillsboro, OR, USA) transmission electron microscopy operating at 200 kV. Sections were photographed with a Gatan 832 CCD camera (Gatan, Pleasanton, CA, USA).

Expression of hepatic lipid metabolism genes. Total RNA was isolated from liver samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was verified by confirming that the optical density 260/280 ratio was between 1.8 and 2.0, and by electrophoresis on a 1% agarose formaldehyde gel. Reverse-transcribed cDNA was obtained using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed on a PTC-200 thermal cycler (MJ Research, Watertown, CA, USA) using a SYBR Green Master Mix Reagent Kit (Toyobo, Tokyo, Japan). Threshold cycle (CT) values of PPARα, CPT1A and CYP7A1 were analyzed with Opticon Monitor 3 analysis software (Opticon, Luton, UK). Gene expression levels were normalized to the housekeeping gene β-actin. Relative changes in gene expression were assessed using the $2^{-\Delta\Delta C}{}_{T}$ method[48]. Primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, USA) and listed in Table 2.

Statistical analysis. All data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Results were expressed as the mean value ± standard error from at least 4 test experiments. One-way ANOVA was used to determine between-group differences followed by Fisher's least significant difference (LSD) test. $P < 0.05$ was considered significant, $P < 0.01$ was considered very significant.

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Author contributions

Y.L. conceived the idea and designed the research with L.W. L.W., Y.W., Y.L. and J.L. carried out the experiment. L.W. analyzed the data and wrote the manuscript. Y.C.L. and J.Z. supplied theoretical background for the explanation. All the authors contributed to discussion of the results. J.J.F. and A.Q.Z. revised the manuscript. G.B.J. supervised the project.

Additional information

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