Chronic Exposure to Perfluorohexane Sulfonate Leads to a Reproduction Deficit by Suppressing Hypothalamic Kisspeptin Expression in Mice

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Research

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Abstract

Background

Perfluorohexane sulfonate (PFHxS) is a six-carbon perfluoroalkyl sulfonic acid found as an environmental contaminant. This study aims to investigate the effects of PFHxS exposure on female reproduction and the underlying mechanism in mice.

Methods

Eight-week-old ICR mice were divided randomly into four groups: corn oil (vehicle) and PFHxS at doses of 0.5, 5, and 50 mg/kg/day for 42 days by intragastric administration. Body weight, ovarian weight, estrous cycle, follicle counts, and serum sex hormone levels were evaluated. Expression of kisspeptin and gonadotropin releasing hormone (GnRH) in the hypothalamus was also detected.

Results

Compared to vehicle exposure, 5 mg/kg/day PFHxS prolonged the estrous cycle, especially the duration of diestrus, after 42 days of treatment. The numbers of antral follicles and corpus lutea were significantly reduced in PFHxS-treated mice. Moreover, compared with the control group, PFHxS-treated mice showed decreases in serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estrogen (E2), and reduced GnRH mRNA levels, along with the lack of an LH surge. Furthermore, PFHxS-treated mice had lower levels of kisspeptin immunoreactivity and kiss-1 mRNA in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV). After intraventricular administration of kisspeptin-10, the numbers of antral follicles and corpus lutea recovered, along with the levels of GnRH mRNA, FSH, and LH in mice treated with 5 mg/kg/day PFHxS.

Conclusion

These results indicate that chronic exposure of mice to 5 mg/kg/day PFHxS affects reproductive functions by inhibiting kisspeptin expression in the ARC and AVPV regions, leading to the deficit of follicular development and ovulation.

Background

Perfluorohexane sulfonate (PFHxS) belongs to the family of perfluoroalkyl compounds (PFASs) that are widely used in the production of a variety of industrial and chemical products, including paper, upholstery, food packaging, and fire-fighting foam [1]. Due to their global distribution, high persistence, bioaccumulation potential, and strong toxicity, the European Chemicals Agency recommends them to be listed as persistent organic pollutants in accordance with the Stockholm Convention [2]. Abundant researches from labs have shown that adverse impacts, such as tumor-inducing possibilities, fetal growth interruption, toxic for nerves, and disruption of endocrine, are related to the contact with PFASs [3]. PFHxS has been detected in soil, drinking water, several consumer products, and animals [4]. Human exposure to
PFHxS is mainly through ingestion of contaminated water and food. It can also be passed through the placenta to the embryo, and through breast milk to infants [5]. PFHxS is persistent and poorly metabolized with estimated half-lives of 1 month, 4 months, and 7.3 years in mice, monkeys, and humans, respectively [6,7].

The contents of PFASs in circulating blood are similar to those in ovarian follicular fluid. This indicates that serum samples can be used as an appropriate substitute index for the ovarian exposure level [8]. The level of PFHxS was negatively correlated with the concentration of triiodothyronine (T3) and thyroxine (T4) in 202 serum samples from Chinese individuals [9]. A previous study with human breast cancer MCF-7 cells has shown that PFHxS is a weak agonist of estrogen receptor α (ERα) and a potential weak antagonist of the androgen receptor (AR) [10]. Another study showed mild to moderate liver and thyroid hypertrophy with PFHxS exposure at two different doses (3 and 10 mg/kg) [11]. Although several studies have evaluated the effects of PFHxS on reproduction, there are very few reports exploring the mechanism of the imparted toxicity.

The hypothalamic-pituitary-gonadal (HPG) axis regulates the ovary activity along with menstruation period. Gonadotropin releasing hormone (GnRH) regulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and these sex hormones, in turn, regulate FSH, LH and GnRH. Kisspeptin, also known as metastin, serves as a gatekeeper of puberty and is mainly expressed in the hypothalamus of rodents, especially the arcuate nucleus (ARC) and the anterioventral periventricular nucleus (AVPV) areas [12], in addition to the paraventricular nucleus and amygdala [13]. Kisspeptin is a 145 amino acid neuropeptide encoded by kiss-1 gene, and is cleaved into short peptides of different lengths to function. About 90% of GnRH neurons express the kisspeptin receptor, also known as G protein-coupled receptor 54 (GPR54) [14], which can be strongly activated by kisspeptin neurons in the AVPV [15]. GPR54 is mainly expressed in rodent brains [16], especially GnRH neurons [17], along with liver and pancreas [18]. Kisspeptin is also involved in the feedback regulation of GnRH/LH by estrogen (E2) [19]. Kisspeptin can regulate the secretion of GnRH through the activation of GPR54 in the hypothalamus and then participates in reproductive endocrine functions [20]. Because PFHxS disrupts reproductive functions, we hypothesized that it may exert these effects by affecting the expression of kisspeptin.

Among several PFASs currently in use, only perfluorooctane sulfonic acid (PFOS) is regulated under the Stockholm Convention. There are very few reports related to the uptake and elimination of PFHxS, and its effects on reproductive and endocrine functions remain elusive [21]. In the present study, we established a mouse model of chronic exposure to PFHxS and then chose the best dose of PFHxS for this study. The effects of PFHxS on the general state of mice, follicular development, normal ovulation, HPG axis regulation, and its possible molecular mechanism were explored.

**Methods**

**Animals and treatment**
We complied with the approval of Nanjing Medical University and Institutional Animal Care and Use Committee (IACUC) as to the utilization of animals. Our research used female ICR mice which were eight weeks old (Oriental Bio Service, Nanjing, China). To minimize additional exposure to endocrine disrupting chemicals, we adopted cages of stainless steel and wooden plates to raise mice. There was a temperature of 23 ± 2 °C and humidity at 55 ± 5% along with a light/dark period of 12:12 hours. Mice could eat and drink at their will without interruption. Fed as dry pellets to limit spillage, diets, which were packed in vacuum, were kept in a room at 8 °C before use. Mouse body weights were measured daily. Mice were allowed to acclimatize for one week before the start of the study. We purchased PFHxS with a purity higher than 98% from Sigma-Aldrich (CAS-No: 3871-99-6, SigmaAldrich, Copenhagen, Denmark). After dissolved in dimethyl sulfoxide (DMSO), PFHxS was diluted using corn oil (eventual concentration of 0.5 percent of DMSO). Casually, we divide mice into four groups: one control group along with three groups with PFHxS exposure. Control mice were given corn oil containing 0.5% DMSO, and the three groups which were treated with PFHxS were given corn oil with 0.5, 5, and 50 mg/kg/d PFHxS respectively for 42 days. All treatments were through intragastric administration. In the last week of exposure, a related pharmacological experiment was carried out whereby kisspeptin-10 (Kp-10, 1nmol/3 μl) or 0.9 % saline was injected into the lateral ventricle of mice. On the final day of chemical administration, mice were sacrificed under anesthesia by intraperitoneal injection of ketamine. The serum, brain, and ovaries were collected for subsequent analyses.

**Estrous cycle**

The estrous cycle was monitored every day at 0800–0900 h and assessed by vaginal cytology according to a previously reviewed procedure [22]. In line with morphological standards, we observed cells from vaginas by light microscopy. Proestrus was featured by a large number of round nucleated epithelial cells and a small number of cornified cells, estrus was featured by extensive cornified squamous epithelial cells, metestrus was featured by both epithelial cells and leukocytes, and diestrus was featured by leukocytes. Each cycle length was determined as time between two consecutive occurrences of estrus.

**Ovarian morphology**

Observing by normal histology protocols, we fixed ovaries in paraformaldehyde at a density of 4% for 24 hours. Paraffin-embedded ovaries were sliced by a thickness of 5 μm. Dyeing the slices with HE (hematoxylin & eosin), we put them on slides. Scored as antral follicles morphologically, the number of follicles was calculated throughout the whole ovary five by five: 1-2 antral spaces in early antral follicles, and oocytes and surrounding follicle cells are squeezed to one side of the follicle to form a cumulus. The number of corpora lutea was counted randomly by one slice from one ovary of each mouse.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

Before the storage at a temperature of -80 °C until observation, there was a collection of brain sections in frozen state at a thickness of 200 μm, which included the POA region (0.76 mm in front of bregma as well as 0.50 mm behind the bregma) along with the anteroventral periventricular nucleus (AVPV) region (0.50
mm in front of bregma as well as 0.02 mm behind the bregma) at proestrus, and the arcuate nucleus (ARC) region (-1.46 mm in front of bregma as well as -1.70 mm behind the bregma) at diestrus. By Trizol reactant (Invitrogen, Carlsbad, CA, USA), we isolated the overall RNA from areas of the POA, AVPV, along with ARC. In the light of the guidance of NovoScript® 1st Strand cDNA Synthesis SuperMix (gDNA Purge) (Novoprotein Scientific, Shanghai, China), we carried out the reversal process of transcription. The synthesized cDNA was kept at a temperature of -20 °C before the process of qPCR. We adopted the primers as follows: GnRH F-5′-GGGAAAGAGAAACACTGAACAC-3′, R-5′-GGACAGTACATTGGAAGTCTG-3′; kiss-1 F-5′-GAATGATCTCAATGGCTTCTTGG-3′, R-5′-TTTCCCAGGCATTAACGAGTT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F-5′-ACCACAGTCCATGCCATCAC-3′, R-5′-TCCACCACCCTGTGCTTGTA-3′. As to every gene and GAPDH (as the internal control), there was triplicate for each specimen. By the $2^{-\Delta\Delta CT}$ approach and normalized expression of GAPDH, we confirmed gene expression relatively.

**Measurements of serum hormones**

Blood was collected on the day of diestrus, kept at normal temperature for 30 minutes, centrifuged (1500 g) at a temperature of 4 °C for 10 minutes, and used for analysis. In order to explore the LH-surge, orbital blood (100 μL each time) was acquired at 1600, 1700, and 1800 h of proestrus. Serum concentrations of FSH, LH, and E2 were surveyed by merchant enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science, Houston, TX, USA) according to the manufacturer's protocols. Intra-assay as well as inter-assay variabilities were both under 15%. The sensitivities were 2.0 pg/mL regarding E2, 0.2 ng/mL regarding LH, and 0.4 ng/mL regarding FSH.

**Immunohistochemistry of kisspeptin neurons**

Mice were narcotized and perfused with pre-cooled phosphate-buffered saline (4 °C) through the left ventricle, and fixed with 4% paraformaldehyde. The entire brain was removed and fixation continued for 24 h. Then, the tissue was dehydrated in 15% and 30% sucrose. After sinking to the bottom, coronal sections were sliced continuously with a cryostat. The brain slices were completely immersed in the antigen retrieval solution and incubated in a constant temperature water bath at 80 °C for 20 minute. Subsequently the slices were preincubated in 1% standard foetal goat serum for 1 hour, afterwards with a rabbit anti-kisspeptin polyclonal antibody (1:1000, Millipore, Billerica, MA, USA) at a temperature of 4 °C for 24 hours. The next day, the slices were incubated by using biotin-conjugated goat anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA, USA) at room temperature for two hours, then treated with Ni-3, 3′-diaminobenzidine (DAB). Finally, the color-developed section was attached to a glass slide, dried, and mounted with a neutral resin, and the kisspeptin-positive (kisspeptin+) neurons were observed under a fluorescence microscope.

**Statistical analyses**

By SPSS (IBM, Chicago, IL, USA), a 20.0 version, we analyzed the whole data displayed as averages ± SEM (standard error of the mean). Significant diversities within groups were determined by the
application of a multiple-comparison one-way or two-way analysis of variance (ANOVA), with Bonferroni corrections. Using Student's t-test, we made comparisons between the two groups. A value of $P < 0.05$ was considered to be statistically significant. At the fewest, there were three repetitious for each test.

**Results**

**Effect of PFHxS exposure on the general state of mice**

Figure 1 shows the general state, body weights, and ovary weights of the control and PFHxS-treated mice. PFHxS exposure at a dose of 50 mg/kg/d changed the general state of mice, such as their overall size and hair condition (Fig. 1A). In addition, the mean body and ovary weights were significantly decreased in the 50 mg/kg/d group (body: $P = 0.011$; ovary: $P = 0.023$; Fig 1B-D). Mean body weights of 0.5 and 5 mg/kg/d PFHxS-treated mice were not different from the control mice (0.5 mg: $P = 0.811$; 5 mg: $P = 0.362$), nor were the mean ovary weights (0.5 mg: $P = 0.759$; 5 mg: $P = 0.676$; Fig 1B-D). To avoid influences of the general state on reproductive and endocrine functions, only mice treated with 0.5 and 5 mg/kg/d PFHxS were analyzed for subsequent experiments.

**Effect of PFHxS exposure on the estrous cycle**

By vagina cell observation, we surveyed the period of estrous. “Regular cyclers”, which were animals having regular periods lasting four to five days, included one day at proestrus, one day at estrus, two to three days at diestrus (one day at metestrus) (Fig. 2A). In comparison with the regular estrous cycle observed in control mice, PFHxS-treated mice at a dose of 5 mg/kg/d displayed a prolonged estrous cycle ($P = 0.002$; Fig. 2B), especially an increased duration of diestrus ($P = 0.003$; Fig. 2C). No significant changes of the estrous cycle were evident in mice treated with 0.5 mg/kg/d PFHxS ($P = 0.139$; Fig. 2B).

**Effect of PFHxS exposure on follicle development and ovulation**

By morphology standards, numbers of the corpora lutea along with antral follicles in diestrus were calculated so as to explore the impacts of long-term contact of PFHxS as to follicle growth as well as ripeness. Samples within the control and 0.5 mg/kg/d PFHxS mice displayed a typical diestrus period (Fig. 3A). We observed extensive corpora lutea in these two groups, proving that there was obvious ovulation recently. Mice treated with 5 mg/kg/d PFHxS displayed a remarkable decrease in the number of antral follicles ($P = 0.025$; Fig. 3B) and corpora lutea ($P = 0.022$; Fig. 3C). Because there was no obvious adverse effect on follicular development and maturation in the 0.5 mg/kg/d group, mice treated with 5 mg/kg/d PFHxS were applied for tests afterwards.

**Effect of PFHxS exposure on the HPG axis**

To explore the underlying mechanism of PFHxS exposure on follicle development and ovulation, we measured serum hormone levels to investigate effects of PFHxS on the HPG axis. As shown in Figure 4, the GnRH mRNA level in the hypothalamus decreased significantly in PFHxS mice ($P = 0.013$; Fig. 4A). In comparison with the control group at diestrus, the serum levels of FSH ($P = 0.011$; Fig. 4B), LH ($P = 0.038$;
Fig. 4C), and E2 (P = 0.025; Fig. 4D) were significantly reduced in PFHxS mice. Moreover, a surge in LH release (LH surge) in proestrus was observed between 1600 and 1800 in control but not PFHxS mice (P = 0.020; Fig. 5A). These results indicated that chronic exposure to PFHxS could inhibit the function of the HPG axis.

**Effect of PFHxS exposure on hypothalamic kisspeptin expression**

To identify the possible mechanism of the effect of PFHxS on the HPG axis, we measured kisspeptin expression in the AVPV and ARC. The number of AVPV-kisspeptin+ cells (Fig. 5B) and the level of AVPV-kiss-1 mRNA (P = 0.015; Fig. 5C) were significantly reduced at proestrus after PFHxS exposure. Similarly, the number of ARC kisspeptin+ cells (Fig. 5D) and the level of ARC-kiss-1 mRNA (P = 0.021; Fig. 5E) were also significantly decreased at diestrus in PFHxS mice. These results indicated that PFHxS suppressed the activation of kisspeptin neurons and reduced kiss-1 mRNA expression.

**Kp-10 restored follicle development and the function of the HPG axis**

As shown in Figure 6A and B, the numbers of antral follicles (P = 0.034) and corpora lutea (P = 0.032) significantly increased after treatment with Kp-10 in mice given 5 mg/kg/d PFHxS. Furthermore, the GnRH mRNA level (P = 0.031; Fig. 6C), and serum levels of FSH (P = 0.033; Fig. 6D) and LH (P = 0.034; Fig. 6E) were also higher in PFHxS+Kp-10 mice than in PFHxS+saline mice. These results indicated that Kp-10 could partially restore follicle development and function of the HPG axis that were suppressed by PFHxS exposure.

**Discussion**

The present study provides in vivo evidence that chronic exposure to PFHxS affects HPG axis functions by inhibiting the expression of kisspeptin in the ARC and AVPV regions. This disrupts follicular development and ovulation in mice. The toxicity of PFHxS can be overcome by subsequent administration of Kp-10.

The reproductive endocrine system in women is regulated by the HPG axis, which is affected by many factors including genetic, iatrogenic, environmental, psychological, and nutritional factors [23,24]. Endocrine disruptors (EDCs) are exogenous chemical substances that can mimic natural hormones thereby exerting agonistic or antagonistic effects and interfering with their actions [25]. PFASs are a kind of EDCs that resist degradation, and persist in environmental and biological samples [26,27]. In our previous study, we analyzed the levels of 10 common PFASs in the plasma of 120 premature ovarian insufficiency (POI) patients and 120 matched controls. We found that the plasma levels of perfluorooctanate (PFOA), PFOS, and PFHxS in POI patients were significantly higher than the controls, and high exposure to PFOA, PFOS, and PFHxS was associated with an increased risk of POI [28]. Another epidemiological study in Shanghai showed that increased exposure to PFOA, PFOS, and PFHxS was related to menstrual cycle disorders [29]. The underlying molecular mechanisms of PFOA and PFOS toxicity on reproduction have been analyzed in many in vivo and in vitro studies in recent years [30,31],
while the effect and possible mechanism of PFHxS on reproduction remains elusive and needs to be further explored.

In humans, PFHxS is mainly distributed in plasma proteins, liver, and kidney [32]. The elimination of PFHxS mainly depends primarily on reabsorption and filtration by the kidney proximal tubules [33]. A previous study found that menstruation was another pathway for removal of PFOA and PFOS, and that this process may also be involved in the clearance of PFHxS [34]. Current epidemiological studies on PFHxS have mainly focused on its effects on thyroid, liver, and immune functions [35-37], whereas very few have analyzed its effect and mechanism on reproductive functions. To investigate the possible mechanism underlying the adverse effect of PFHxS on reproduction, we established a mouse model of chronic PFHxS exposure. In the present study, PFHxS exposure at a dose of 50 mg/kg/d changed the general state of mice and reduced the mean body and ovary weights significantly. To avoid the influence of these general effects on reproductive functions, only mice treated with 0.5 and 5 mg/kg/d PFHxS were analyzed for the subsequent experiment.

In comparison with the regular estrous cycle observed in control mice, mice treated with 5 mg/kg/d PFHxS had a prolonged estrous cycle, especially an increased duration of diestrus. Moreover, mice given 5 mg/kg/d PFHxS showed a significant decrease in the numbers of antral follicles and corpora lutea, suggesting a deficit of follicle development and maturation. As no obvious adverse effect on reproduction was observed in the 0.5 mg/kg/d group, a dose of 5 mg/kg/d was considered appropriate for this mouse model of chronic exposure to PFHxS. The HPG axis regulates reproductive endocrine functions. We found that the serum levels of FSH, LH, and E2 were obviously reduced in PFHxS mice, along with the GnRH mRNA level in the hypothalamus. These results suggest that PFHxS affects the synthesis or release of HPG hormones, and this effect may occur at the hypothalamic level.

Generation of the LH peak before ovulation is an important factor in ovulation and a normal estrus cycle [38]. Changes in the pattern (time and amplitude) of the LH peak may directly disrupt ovarian function [39]. After chronic PFHxS exposure, the LH pulse disappeared, which may at least partly explain the follicular development disorder. The kiss-1 gene expresses kisspeptin and may participate in regulating the HPG axis. An abundance of evidence indicates that AVPV-kisspeptin neurons regulate the generation of GnRH and the LH surge, and ARC-kisspeptin neurons are involved in the rhythm of the GnRH pulse [40]. Smith et al. analyzed kiss-1 mRNA expression in the AVPV and ARC in response to E2 and found that E2 could stimulate AVPV kisspeptin neurons and inhibit ARC kisspeptin neurons [41,42]. Another study found that central or peripheral administration of kisspeptin had a strong stimulating effect on gonadal hormones [43]. Low dose intraventricular injection of kisspeptin can significantly increase the secretion of LH and FSH [44], which is expressed by activating GnRH neurons. To confirm whether the toxic effect of PFHxS on reproduction was associated with the activity and synthesis of kisspeptin, we detected its expression levels in the AVPV and ARC areas of the hypothalamus. We found that the numbers of kisspeptin-positive neurons in the AVPV and ARC areas decreased simultaneously, along with the mRNA level of kiss-1 in the hypothalamus.
To verify the role of kisspeptin in PFHxS-mediated toxicity, we injected Kp-10 into the lateral ventricle of mice and found that the reproductive toxicity caused by PFHxS improved to a certain extent. Specifically, a significant increase in the numbers of antral follicles and corpus lutea were observed, along with an increase in gonadal hormone levels. This result further supports our hypothesis that PFHxS may affect the HPG axis by reducing the expression of kisspeptin in the hypothalamus, thereby affecting follicular development and ovulation in mice.

There are few studies that reported mild to no effect of PFHxS on reproductive toxicity in mice. One such study by Chang et al. found that daily PFHxS treatment did not affect the weight, food consumption, hematology, serum thyroid stimulating hormone (TSH) level, and reproductive function in mice, including the estrous cycle, fertility, conception, pregnancy, and birth [45]. However, that study used a maximum PFHxS dose of 3 mg/kg/d, whereas we studied the effect of 5 mg/kg/d PFHxS. This dose difference may be a factor in the differing results we obtained.

To date, several epidemiological studies have identified associations between PFHxS and altered liver, thyroid, energy and lipid metabolism, protein biosynthesis, and immune function, which may be indirectly involved with the reproductive toxicity caused by PFHxS [46-49]. In addition, AVPV- and ARC- kisspeptin neurons express prolactin receptors, suggesting the possible participation of prolactin in the toxicity of PFHxS [50-51]. It is still unclear whether PFHxS can suppress the biosynthesis of E2 directly, like PFOS, through reducing histone acetylation of the steroidogenic acute regulatory protein (StAR) [52]. All of the above are problems waiting to be solved and we would like to address these in our future studies.

Conclusions

Although the mechanism underlying PFHxS-induced suppression of kisspeptin expression remains elusive, our data in the present study indicate that chronic exposure of mice to PFHxS at a dose of 5 mg/kg/day affects reproductive function by inhibiting kisspeptin expression in the ARC and AVPV regions, leading to the deficit of follicular development and ovulation. These findings may be helpful for understanding the effect of PFHxS exposure on reproductive endocrine functions and reproductive health in humans.

Abbreviations

PFHxS: perfluorohexane sulfonate; GnRH: gonadotropin releasing hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estrogen; ARC: arcuate nucleus; AVPV: anteroventral periventricular nucleus; Kp-10: kisspeptin-10; PFASs: perfluoroalkyl compounds; EDCs: endocrine-disrupting chemicals; DMSO: dimethyl sulfoxide; GPR54: G proteincoupled receptor 54; DAB: diaminobenzidine; DNA: deoxyribonucleic acid; ECL: enhanced chemiluminescence; ELISA: enzyme-linked immuno sorbent assay; H&E: hematoxylin and eosin;

Declarations
Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC No.2000528).

Consent for publication

Not applicable.

Availability of data and materials

All the data is contained in the manuscript.

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

XRY and TTD performed the experiments and drafted the article. XYC and ZNL helped analyzing the data. JYX and SYZ are the correspondence authors of this manuscript, who designed the study and approved the final version.

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Figure 1

The general state of mice after chronic exposure to PFHxS. (A) Overall size and hair condition. (B) Body weights (g) of mice. (C) Shapes of the ovaries. (D) Ovary weights (mg). *P < 0.05 vs. control mice (n = 6, one-way ANOVA).
Figure 2

Changes of the estrous cycle after chronic exposure to PFHxS. (A) Typical periods of estrous anterior to and posterior to PFHxS contact lasting 42 days. Spots connected represent the diestrus time (0), proestrus (1), or estrus (2) separately. * persistent diestrus; ↓ proestrus loss. (B) The average length (day) of a single estrous cycle. (C) The average time (day) of proestrus (P), estrus (E), and diestrus (D) separately, per estrous cycle. **P < 0.01 vs. control mice (n = 6, one-way ANOVA).
Figure 3

Follicle development and ovulation after chronic exposure to PFHxS. (A) Representative images of ovaries stained with hematoxylin and eosin within control as well as PFHxS-treated mice. * for corpora lutea; # for antral follicles; ↑ for atretic follicles. Scale bars = 200 µm. (B) Average number of antral follicles at diestrus. (C) Mean number of corpora lutea at diestrus. *P < 0.05 vs. control mice (n = 6, one-way ANOVA).
Figure 4

Effect of PFHxS exposure on the HPG axis. Bar graphs show levels of GnRH mRNA (A), serum FSH (B), LH (C), and E2 (D) respectively. *P < 0.05 vs. control mice (n = 6, Student's t-test).
Figure 5

Effect of PFHxS exposure on the LH surge and the expression of kisspeptin. (A) Average serum LH level standardized by 1600 h. *P < 0.05 vs. control mice (n = 6, two-way ANOVA). (B) Representative image of kisspeptin immunostaining in AVPV. Arrows indicate AVPV-kisspeptin+ cells. Scale bar = 100 µm. (C) Relative levels of AVPV-kiss1 mRNA. (D) Representative image of kisspeptin immunostaining in ARC. Arrows indicate ARC-kisspeptin+ cells. Scale bar = 100 µm. (E) Relative levels of the mRNA of ARC-kiss1. *P < 0.05 vs. control mice (n = 6, Student's t-test).
Figure 6

Effect of Kp-10 on follicle development and the function of HPG axis. (A) Characteristic illustrations with regard to ovaries in PFHxS+saline and PFHxS+Kp-10 treated mice. * for corpora lutea; # for antral follicles; † for atretic follicles. Scale bar = 200 µm. (B) Average number regarding corpora lutea along with antral follicles. (C-E) Relative levels of GnRH mRNA, serum FSH, along with LH levels within PFHxS+saline and PFHxS + Kp-10 mice. *P < 0.05 vs. PFHxS+saline mice (n = 6, Student’s t-test).

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