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The effects of perfluorooctanoic acid (PFOA) on fetal and adult rat testis

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a widely dispersed synthetic chemical, which accumulates in living organisms and has been connected with male reproductive disorders. To monitor the effects of PFOA, fetal rat testes or seminiferous tubule segments (stage VII-VIII) of adult rats were cultured in 0–100 μg/ml PFOA for 24 h. Afterwards, cAMP, progesterone, testosterone and StAR protein levels were measured from the fetal testes culture. Measurements were combined with immunohistochemistry, immunofluorescence, TUNEL and flow cytometric analysis to monitor cell death in somatic and germ cells. This study shows that the levels of cAMP, progesterone, testosterone and expression of StAR decreased significantly in PFOA 50 and 100 μg/ml. PFOA affected cell populations significantly by decreasing the amount of diploid, proliferating, meiotic I and G2/M-phase cells in adult rat testis. However, PFOA did not affect fetal, proliferating or adult rat Sertoli cells but an increased tendency of apoptosis in fetal Leydig cells was observed.

1. Introduction

During the last decades an increasing prevalence of male reproductive disorders, such as hypospadias, cryptorchidism, testicular germ cell cancer and decline in sperm counts, has been reported in several countries [1–4]. The underlying cause of these disorders is often of fetal origin [5]. There is accumulating evidence that developmental and modern lifestyle exposure to endocrine disrupting chemicals (EDC) can cause adverse reproductive effects [6].

Perfluorooctanoic acid (PFOA) is a synthetic fluorosurfactant consisting of a perfluorinated alkyl chain of 7 carbons and a terminal carboxylate group. A strong carbon-fluorine bond makes PFOA stable and non-degradable. Thus, it is a persistent and dispersed chemical in the environment and it has an elimination half-life of approximately 3.5 years in humans [7,8]. PFOA has been produced since the 1940s. Due to the stain, fat and water resistant features of the perfluorinated tail of the chemical, PFOA is widely used as a coating in industrial products like in outdoor clothes, footwear, nonstick cookware, food packages, carpets and firefighting foams [9–11].

PFOA has been detected in the blood of humans and animals worldwide and there is evidence that PFOA can cross the placenta [7,12,13]. It has also been found in umbilical cord blood, human and rodent fetuses and fetal organs reflecting *in utero* exposure of the fetus [12–14]. In addition to prenatal exposure, PFOA has also been found in newborns and in breast milk contributing to the postnatal exposure [12,14]. Regarding potential effects on male fertility in humans, PFOA has been found in seminal plasma [15,16], and some studies have shown that PFOA disturbs the function of blood testis barrier *in vivo* and *in vitro* [17–19]. Furthermore, exposure to PFOA has been shown to cause weight loss, liver enlargement, changes in lipid metabolism and increased incidence of liver, pancreas, and testicular tumors in rodent studies [7]. *In utero* exposure to PFOA is associated with small birth

Abbreviations: Ab, Antibody; AGD, Anogenital distance; AR, Androgen receptor; BSA, Bovine serum albumin; cAMP, Cyclic adenosine monophosphate; CV, Coefficients of variation; DAB, Diamino benzidine; DMSO, Dimetylsulfoxide; ED, Embryonic day; EDC, Endocrine disrupting chemicals; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; hCG, Human chorionic gonadotropin; Ki67, Protein, associated with cell proliferation; LH, Luteinizing hormone; NC, Negative control; PBS, Phosphate buffered saline; PC, Positive control; PFOA, Perfluorooctanoic acid; P4, Progesterone; RIA, Radioimmunoassay; RT, Room temperature; SEM, Standard error of the mean; SOX-9, SRY (Sex-Determining Region Y)-Box 9; StAR, Steroidogenic acute regulatory protein; T, Testosterone; Tunel, Terminal deoxynucleotidyl transferase dUTP nick end labeling; 3β-HSD, 3β-Hydroxysteroid dehydrogenase; 17β-HSD3, 17β-Hydroxysteroid dehydrogenase

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weight and size, delayed physical development and neonatal mortality [7,20]. In adult male rats PFOA may reduce testosterone levels by inhibiting HSD3-17-B3 and 3B-HSD activity and increase estradiol levels through the induction of hepatic aromatase [7,21]. In humans, *in utero* exposure to PFOA is suspected to affect reproductive hormone levels and semen quality in adult men later in life [9,22].

Due to several toxicological findings, PFOA has been classified as an endocrine disrupting chemical [10]. Concerns of PFOA's potential health effects combined with its broad distribution and persistence have led to phasing out the production of PFOA by many manufacturers. While the industrial production of PFOA has decreased in Europe and USA, the latest statistics show that in some areas in Asia the production has increased [23].

Although the potential reproductive and developmental toxicity of PFOA has been shown in many studies [7,9,22,24–28], the mechanisms behind are not yet well established. The present study was conducted to analyze the direct effects of PFOA on embryonic day (ED) 17.5 rat testis and adult rat seminiferous tubule. Our focus was to analyze the effects on steroidogenesis and testicular cell development and survival. The ED 17.5 and 24 h exposure time in tissue culture was selected because that targets the sensitive developmental time window for programming development of male reproductive organs [29].

2. Materials and methods

2.1. Chemicals

Perfluorooctanoic acid (PFOA), 96% purity, CAS No. 335–67-1 and dimethylsulfoxide (DMSO), > 997% purity, CAS No. 67-68-5 were purchased from Sigma-Aldrich (Saint Louis, USA).

2.2. Experimental animals and housing

Young adult Sprague-Dawley female rats (2–3 months old) and male rats (>2 months old) were obtained from Animal Center of the Turku University, (Turku, Finland). All animals were housed in a 12 h light/12 h dark cycle in a climate-controlled room at 21 ± 3 °C with relative humidity of 55% ±15%. Water and standard food (Rat and Mouse breeder and Grower, SDS diets/LBS Biotech, UK) were distributed *ad libitum*. Female rats were paired overnight with males and the following morning the presence of the mating plug was checked. This morning was considered as embryonic day (ED) 0.5. All animal experiments were carried out according to the institutional and ethical policies of the University of Turku. The local Ethics Committee on Animal Experimentation approved the animal experiments.

2.3. Sample collection of fetal rat testes

Pregnant rats were sacrificed on ED 17.5 by CO_2 asphyxiation and cervical dislocation. The uteri were removed, placed on ice and the number of fetuses, body weight and sex were recorded. Fetuses were sacrificed by decapitation. Fetal rat testes were collected under a stereo microscope and transferred into four-well culture dishes for tissue culture and PFOA exposure.

2.4. In vitro fetal testis culture

Two types of fetal testis culture were used in this study. In the first condition [30,31], testes were placed in a 4-well-plate containing in each well 1 ml of Dulbecco's modified Eagle's medium (DMEM high glucose, pyruvate and GlutMAX; Invitrogen Co., Paisley, UK) supplemented with 0.1% bovine serum albumin (BSA) and 0.1 g/l of gentamycin (Biological Industries, BethaEmek, Israel). The control culture contained the vehicle, 1 µl of dimethylsulfoxide (DMSO; Sigma chemical Co., St. Louis, MO, USA) and PFOA (Sigma chemical Co., St. Louis, MO, USA) concentrations were 10, 50 or 100 µg/ml in the culture. The PFOA concentrations for the present study were selected based on the report that the highest serum PFOA levels were 0.135–2.44 mg/l (approx. 250 µM) in occupational studies [32]. The highest exposure concentration in our study is 241.5 µM (100 µg/ml of PFOA).

Fetal testes were cultured for 24 h at 37 °C in a humidified atmosphere containing 95% air and 5% of CO_2. Testes were stimulated by 7 IU/ml of human chorionic gonadotropin (hCG; Pregnyl®, Shering-Plough, N.V. Organon, Oss Holland) or forskolin 50 µM (LC Laboratories, USA) 3 h before ending the culture. One hour before the hCG and forskolin stimulation, 0.1 mM of 3-isobutyl-1-methyl xanthine (IBMIX; Sigma-Aldrich Finland Oy, Helsinki, Finland) was added as described previously [33]. At the end of the exposure, the culture medium was collected, warmed up to 60 °C for 5 min and frozen in −20 °C and testes were stored in −80 °C. The cultured fetal testes samples were used for the Western blot analysis and the cyclic AMP (cAMP), progesterone and testosterone measurements were done from the culture medium, respectively.

In the first fetal testis culture setup, five replicates for each condition were used (n = 5). The fetal testes for each replicate were derived from different litters. Thereby each replicate is representing n = one.

Another set of experiments was carried out on Millicell filters (pore size 0.4 µm; Millipore, Billerica, MA, USA) as described previously [34,35]. Filters were placed in a 24-well-plate containing 320 µl of medium containing PFOA as described above and incubated for 24 h without hCG and forskolin stimulation. After the culture, testes were collected for morphological analysis, they were fixed in 4% paraformaldehyde and embedded in paraffin and the culture medium was stored at −20 °C. Four dams were sacrificed and the collected fetal testes (n = 4) for the immunofluorescence and TUNEL assay were from different litters.

2.5. cAMP, progesterone and testosterone assays in fetal testis culture

The cAMP measurements were carried out from the medium using a radioimmunoassay which detected femtomole amounts of cAMP by acetylating the cyclic nucleotides at the 2′0 position with acetic anhydride as described previously [36]. Secreted levels of progesterone (P4) and testosterone (T) were measured from the medium by time-resolved fluoroimmunoassays (DELFIA; Perkin-Elmer Life and Analytical Sciences, Turku, Finland) as described earlier [37]. The sensitivities of the assay were 100 pg/ml and 250 pg/ml for progesterone and testosterone, respectively. The intra- and inter-assay coefficients of variation (CV) were below 6% and 12%, respectively.

2.6. Immunofluorescence and TUNEL assay of cultured fetal testes

Four fetal testes per experimental condition (n = 4) at ED 17.5 after 24 -h culture were fixed in 4% paraformaldehyde and embedded in paraffin. Four-µm-thick sections were cut and every tenth sections were attached to glass slides for staining. Double indirect immunofluorescence assessment of proliferation and apoptosis of somatic cells was performed. Sertoli cells were detected by using goat polyclonal anti-SOX-9 primary antibody (ab5535, Merck Millipore, Frankfurt, Germany, final concentration 2.1 µg/ml) coupled to Alexa 594 donkey anti-rabbit (A-21207, Thermo Fisher, Waltham, USA; final concentration 0.4 µg/ml). Rat Monoclonal anti-Ki67 primary antibody (14-5698-82, Thermo Fisher, Waltham, USA; final concentration 1 µg/ml) coupled to Alexa 488 donkey anti-rat (A-21208, Thermo Fisher, Waltham, USA; final concentration 1.7 µg/ml) was used for the detection of proliferative cells. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was done as described previously [38]. Briefly, deparaffinized and rehydrated sections were permeabilized in a pressure cooker in 0,1 M citrate buffer, pH 6. Quenching of autofluorescence was performed by submerging the slides in 100 mM NH_4Cl. Then sections were incubated with the TUNEL reaction mixture for 60 min at 37 °C

and reaction was stopped by using 300 mM NaCl, 30 mM NaCitrate. To detect the TUNEL positives cells, an incubation with streptavidin Alexa 488 (S32354, Thermo Fisher, Waltham, USA; final concentration 0.4 μg/ml) was performed. The positive control was performed by adding 1U/μl DNAse for 30 min at 37 °C and for the negative control, terminal deoxynucleotidyl transferase (TdT) was omitted. All (100–300) Sertoli cells in proliferation (Ki67-positive, SOX-9-positive) and undergoing apoptosis (TUNEL-positive, SOX-9-positive) were quantified in 1–3 non-consecutive sections from four fetal testes (n = 4). All (300–400) Leydig cells (interstitial TUNEL-positive) were quantified in 1–3 non-consecutive sections from four fetal testes (n = 4). Graphs are shown as percentage of single or double positive cells. All immunofluorescence samples were imaged using Pannoramic MIDI FL slidescanner with the 40x Plan Apochromatic objective (Zeiss) and all images were processed with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.7. Western blot of cultured fetal testis

A pool of 5–6 fetal testis (ED 17.5) were homogenized in 35–40 μl of ice-cold lysis buffer (0.2% Nonidet P-40, in phosphate buffered saline (PBS) and protease inhibitor tablet; Complete mini, Roche Diagnostics, Mannheim, Germany) and the homogenate was centrifuged at 13,000 rpm for 15 min at 4 °C. Total protein concentration of each supernatant was measured *via* the bicinchoninic acid detection method (BCA protein assay kit, Pierce, Rockford USA). For each sample, 20 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresisand transferred onto nitrocellulose membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, UK). To block the unspecific antibody binding, the membrane was incubated for 1 h with PBS supplemented with 5% fat free milk and 0.2% of Tween 20.

The protein immunodetection was performed by incubating the membrane overnight at room temperature with a polyclonal rabbit StAR antibody (Abcam, Cambridge, UK) diluted into 1:2000 PBS with 1% fat free milk and 0.2% of Tween 20. Monoclonal mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (HyTest, Turku, Finland) diluted at 1:5000 was used as a loading control. The antibody binding was detected using corresponding horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies (Amersham Life Science, Buckinghamshire, UK) diluted in 1:10,000 for 1 h at room temperature (RT) and revealed by ECL chemiluminescence (Western Lighting® ECL Pro, Perkin Elmer, Inc., USA) on Fujifilm LAS 4000. ImageJ 1.46 r (Wayne Rasband, National Institutes of Health, USA) was used to analyze the Western blots. The StAR was normalized with the GAPDH and the results are represented as relative protein abundance. At least 4 separate samples were analyzed in each experiment that were repeated four times (n = 4).

2.8. Microdissection of adult rat seminiferous tubules

Adult male rats were killed by CO_2 asphyxiation and cervical dislocation. For the seminiferous tubule experiments, one testis of adult male rat was freshly collected direct after the death. Litter effect was eliminated by using the experimental testes always from a different litter. Adult rat testes were carefully decapsulated on a petri dish containing PBS (phosphate buffered saline). After decapsulation, the seminiferous tubules were placed on another petri dish with PBS. The stages of the seminiferous epithelial cycle were identified visually under a transilluminating microscope on the basis of specific light absorption patterns, and two-mm long segments at the defined stages of the seminiferous tubules (VII-VIII) were carefully cut with McPherson-Vannas scissors as described previously [39]. Tubule segments were individually transferred in 20 μl of PBS to 96-well-plates and 80 μl of culture medium (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine and 15 mM HEPES, without sodium bicarbonate, powder) supplemented with 0,1% bovine serum albumin (BSA) and 005 g/l of gentamycin was added. The microdissection time was limited to less than 1 h.

2.9. In vitro exposure to PFOA in adult seminiferous tubule culture

For the squash sample preparation, 16 seminiferous tubule segments (2 mm) of stage VIII of each adult rat testis (n = 7) were dissected for culture with 0, 10, 50, and 100 μg/ml PFOA in 96-well plates with one piece of tubule per well. Thus, four parallel tubule samples per concentration were analyzed. For flow cytometry, 40 tubule segments (20 + 20) of stage VII-VIII were collected from 4 adult rat testes (n = 4) and cultured at 0 and 100 μg/ml PFOA for 24 h at 34 °C in a humidified atmosphere containing 5% CO_2 in the air. All the adult rat testes used here were from the different litters.

2.10. Preparation of squashed cultured adult seminiferous tubules

After 24 h of cell culture the tubule segments were transferred in 20 μl of culture medium and pipetted on to a microscope slide. Each tubule segment were gently squashed between a coverslip and a microscope slide until a cloud like cell monolayer spread on the microscope slide [39]. This step was monitored under Leica DMR light microscope (Leica Microsystems, Wetzlar, Germany) and the desired spermatogenic stage VIII was verified by inspecting the polarization of the nucleus and the shape of the acrosome in step 8 spermatids. After squashing, the samples were dropped into liquid nitrogen for freezing. The coverslip was flipped off with a scalpel and the sample was first dipped in ice-cold 96% EtOH and then fixed in 4% PFA for 10 min. After fixation the samples were stored in PBS at 4 °C until the next day.

2.11. Immunohistochemistry for cultured and squashed adult seminiferous tubules

The squashed tubule segments were treated with 1% H_2O_2 in PBS for 15 min, then washed twice for 5 min in PBS. Afterwards, they were treated for 15 min in 0.1% Triton-X in PBS at RT and blocked with 0.5% BSA in 0.1% Triton-X PBS for 30 min in a humid chamber. Cleaved caspase-3 was used as an apoptosis marker. The first antibody (ab) (rabbit ab; #9661S, Cell Signalling, Leiden, Netherlands) was added in 1:100 dilution with 0.5% BSA and 0.1% Triton-X for 1 h at RT in a humid chamber, and washed three times in PBS. The second antibody, EnVision + System-HRP -labelled polymer anti-rabbit ab (K4003, Dako North America, California, USA) was used as 1:1 for 30 min and washed three times in PBS. The samples were stained with diamino benzidine (DAB) (Dako North America, California, USA) for 2 min, and counterstained slightly with hematoxylin and eosin. They were mounted with aquamount and scanned with Pannoramic 250 slide scanner and analyzed with Pannoramic viewer software. The cleaved caspase-3 positive cells were quantified and the length of tubule segments was measured. The apoptotic cells are reported per 1 mm of seminiferous tubule.

2.12. Analysis of adult testicular cell populations by flow cytometry

The analysis of cell composition in seminiferous tubules was done by following a protocol previously described [40]. Briefly, for each treatment, 20 individual seminiferous tubules (2 mm) of each testes (n = 4) were pooled and placed in digestion medium. The digestion medium contained 1 mg/ml collagenase/dispase (10269638,001; Roche, Basel, Switzerland), 1 mg/ml hyaluronidase (H3506; Sigma–Aldrich, Saint Louis, MO, USA) and 1 mg/ml DNAse1 (DN-25; Sigma–Aldrich, Saint Louis, MO, USA). Tubules were disintegrated using McPherson-Vannas scissors for 1 min. The samples were incubated at 37 °C for 15 min, and they were mechanically disrupted at 10 and 5 min interval times. The obtained cell suspensions were filtered through 40 μm pore size strainer (FlowMI, H13680-0040, New Jersey, USA) and washed with PBS by centrifugation. Single cells were fixed

with 4% paraformaldehyde followed by a permeabilization with 90% methanol. The samples were stored frozen in −20 °C prior to analysis.

To evaluate the amount of somatic and germ cells after PFOA exposure, Vimentin antibody detection and DNA staining was performed. Monoclonal rabbit anti-vimentin antibody conjugated to Alexa 488 (9854; Cell Signaling Technologies, final concentration 4 μg/ml) to detect Sertoli cells. FxCycle Far-Red DNA stain (F-10348; Life Technologies Ltd, Paisley, UK, final concentration 200 nmol/ml per 1×10^6 cells) was used to assess the germ cells on the basis of their DNA contents. A defined volume of suspension was analyzed by the flow cytometer (BD LSRII, Becton Dickinson, Franklin Lakes, NJ, USA) and the number of cells were normalized to cells/mm of seminiferous tubule. Analyses were performed with the noncommercial Flowing Software 2.5 (Mr. Perttu Terho; Turku Centre for Biotechnology, Finland; www.flowingsoftware.com) as previously described in detail [40].

2.13. Statistical analysis

All data from hormone assays, Western blots and squash samples were analyzed using JMP Pro 12 for checking if the data were normally distributed. Data from immunofluorescence and flow cytometry were analyzed using Systat software (Systat software Inc SigmaPlot 13.0.1 for windows) for checking whether the data were normally distributed. Afterwards Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) software was used either for *t*-test or one-way-ANOVA (depending on the number of experimental groups), followed by Dunnett's Multiple Comparison test. Significance level was at $p = 0.05$.

3. Results

3.1. PFOA exposure decreased cAMP, progesterone and testosterone levels and the expression of StAR in fetal rat testis culture

Levels of cAMP decreased significantly at all PFOA concentrations (10, 50 and 100 μg/ml) of fetal testis culture in basal and hCG-stimulated conditions (Fig. 1). In the forskolin-stimulated culture there was a significant decrease of cAMP levels at PFOA 50 and 100 μg/ml as compared to the control (Fig. 1).

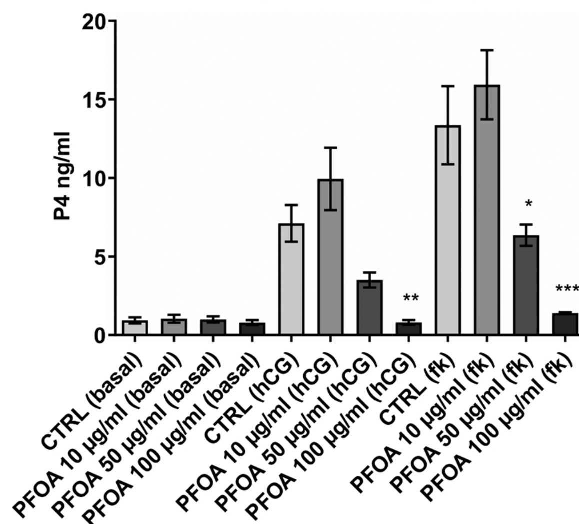


Fig. 2. PFOA decreased progesterone secreted from hCG- and forskolin-stimulated fetal rat testes *in vitro*. Progesterone levels measured from the fetal testis (ED 17.5) culture medium after 24-h incubation in control and PFOA concentrations of 10, 50 and 100 μg/ml. Basal (no stimulation), hCG- (7 IU/ml), forskolin (50 μM) -stimulated cultures. Bars represent means +/-SEM from 5 litters, n = 5. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

Progesterone levels were significantly lower in PFOA 100 μg/ml than in control after hCG stimulation, but the hormone levels did not differ in basal condition (Fig. 2). Similarly, in the forskolin-stimulated culture, the levels of progesterone were significantly lower in PFOA 50 and 100 μg/ml than in control (Fig. 2). Furthermore, testosterone levels were significantly lower in hCG-stimulated fetal testes cultures in PFOA 50 and 100 μg/ml and in forskolin-stimulated cultures in PFOA 100 μg/ml than in controls (Fig. 3).

In order to assess whether early steroidogenesis was influenced by the action of PFOA, testicular expression of StAR was analyzed. The

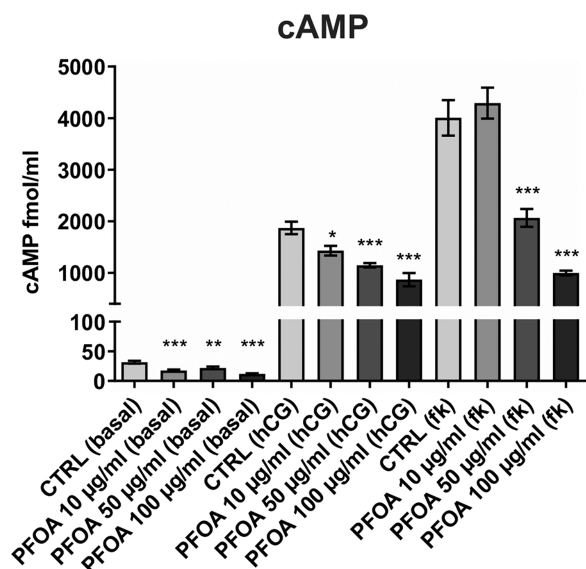


Fig. 1. PFOA decreased cAMP levels significantly in all exposure conditions of fetal testis cultures. Cyclic AMP levels measured from the fetal testis (ED 17.5) culture medium after 24-h incubation in control and PFOA concentrations of 10, 50 and 100 μg/ml. Basal (no stimulation), hCG- (7 IU/ml), forskolin (50 μM) -stimulated cultures. Bars represent means +/-SEM from 5 litters, n = 5. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

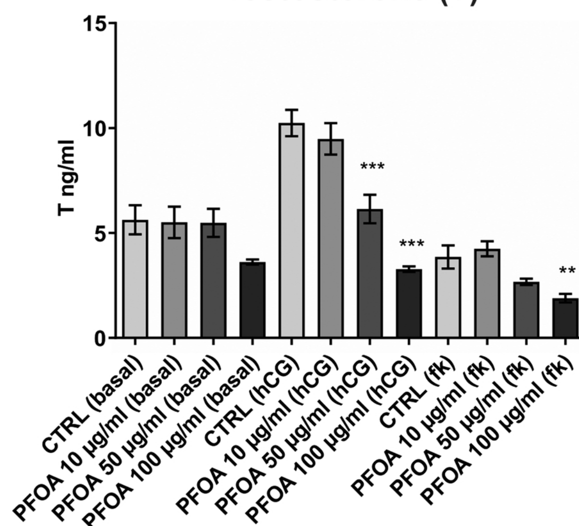
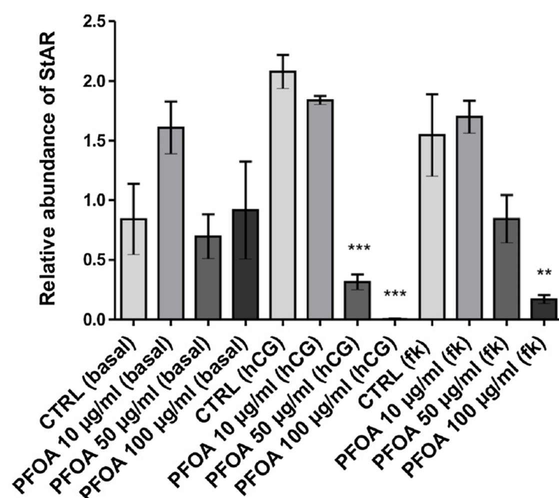


Fig. 3. PFOA decreased testosterone secreted from hCG- and forskolin-stimulated fetal rat testes *in vitro*. Testosterone levels measured from the fetal testis (ED 17.5) culture medium after 24-h incubation in control and PFOA concentrations of 10, 50 and 100 μg/ml. Basal (no stimulation), hCG- (7 IU/ml), forskolin (50 μM) -stimulated cultures. Bars represent means +/-SEM from 5 litters, n = 5. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

A



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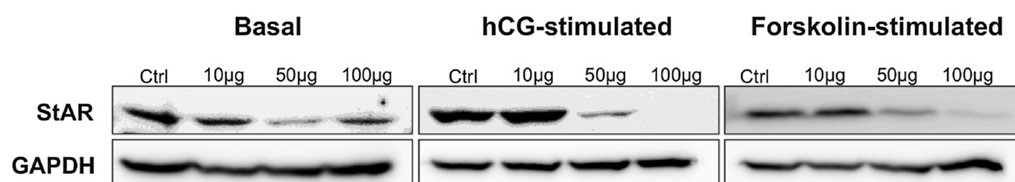


Fig. 4. PFOA decreased significantly the expression of StAR. Statistical graph (A) and the representative pictures (B) of Western blot analysis of StAR (30 kDa) protein expression levels in ED 17.5 rat testis after 24 -h exposure to 0, 10, 50 or 100 µg/ml of PFOA. In both hCG- and forskolin-stimulated testis culture, the StAR level was decreased significantly after 100 µg/ml exposure to PFOA as compared to the respective control. In hCG-stimulated testis culture the expression level of StAR protein also decreased significantly after 50 µg/ml exposure to PFOA. GAPDH (36 kDa) was used as a loading control. Bars represent means +/- SEM of four separate samples normalized to GADPH, n = 4 Statistical differences were determined using one-way ANOVA, followed by Dunnetts multiple comparison test. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

Western Blot results (Fig. 4) show that in the basal condition there was no clear difference in the levels of StAR between the PFOA-exposed and control samples. However, a significant decrease in StAR protein expression at 50 and 100 µg/ml PFOA in hCG-stimulated testis was observed (Fig. 4). In the forskolin-stimulated fetal rat testis culture the levels of StAR protein decreased significantly at PFOA 100 µg/ml (Fig. 4).

3.2. PFOA did not have an effect on proliferating fetal Sertoli cells, but PFOA affected fetal Leydig cell survival

In order to assess whether PFOA affected proliferation of fetal Sertoli cells in cultured fetal rat testes (ED 17.5), immunofluorescence staining with SOX-9 and Ki67 was analyzed (Fig. 5 A). There was no significant difference in the amount of proliferating Sertoli cells (Fig. 5 B).

The immunofluorescence analysis of fetal cultured testes (ED 17.5) with SOX-9 and TUNEL staining did not show any TUNEL and SOX-9 double positive Sertoli cells indicting that there were no detectable apoptotic fetal Sertoli cells after 24 -h culture and exposure to PFOA (Fig. 6 A). However, TUNEL positive fetal Leydig cells were morphologically recognized and quantified (Fig. 6 A) and an increased tendency was observed, albeit the difference was not significant (Fig. 6 B).

3.3. PFOA induced apoptosis and decreased the number of diploid, proliferating and 4C cells in adult seminiferous tubules

The flow cytometric analysis of the cell composition of the adult seminiferous tubules after exposure to 100 µg/ml PFOA for 24 h resulted in loss of all cell types except for haploid spermatids as compared to the controls (Fig. 7). Immunohistochemistry staining (Fig. 8 A) showed that PFOA 100 µg/ml induced apoptosis in the seminiferous tubule segments at stage VIII (Fig. 8 B).

4. Discussion

PFOA has been suspected to be toxic for male reproductive health but the underlying mechanisms are still partly unknown. The present study shows that 24 h PFOA exposure *in vitro* inhibits fetal rat (ED 17.5) testicular steroidogenesis. In the presence of PFOA, production of cAMP was significantly reduced, and at PFOA concentrations of 50 µg/ml and 100 µg/ml, hCG- and forskolin-stimulated progesterone and testosterone production was significantly decreased.

Welsh et al. have earlier identified the window ranging from ED 15.5 and 18.5 in rats as a sensitive masculinization programming window, where the androgens ensure the correct development of the male reproductive tract [29]. Fetal rat testis start to produce testosterone during ED 15.5 and the whole masculinization window includes the timepoint within ED 15.5–21.5 [29,41]. This window range can be divided in three parts: early window ED 15.5–17.5, middle window ED 17.5–19.5 and late window ED 19.5-21.5. When studying the toxicity of potential endocrine disrupting chemicals it is important to cover the critical time window. For fetal rats this means early and/or middle masculinization window range where the potential harm effect for the androgen sensitive cells and tissues may occur [5,29]. Disruptions of hormonal levels at this sensitive time range may lead to cryptorchidism, hypospadias, short anogenital distance (AGD), micropenis and decreased sperm count [5,29]. We chose this exposure period (ED 17.5) and 24 h exposure time because it targets the critical window for antiandrogen-induced reproductive toxicity and secondly because the

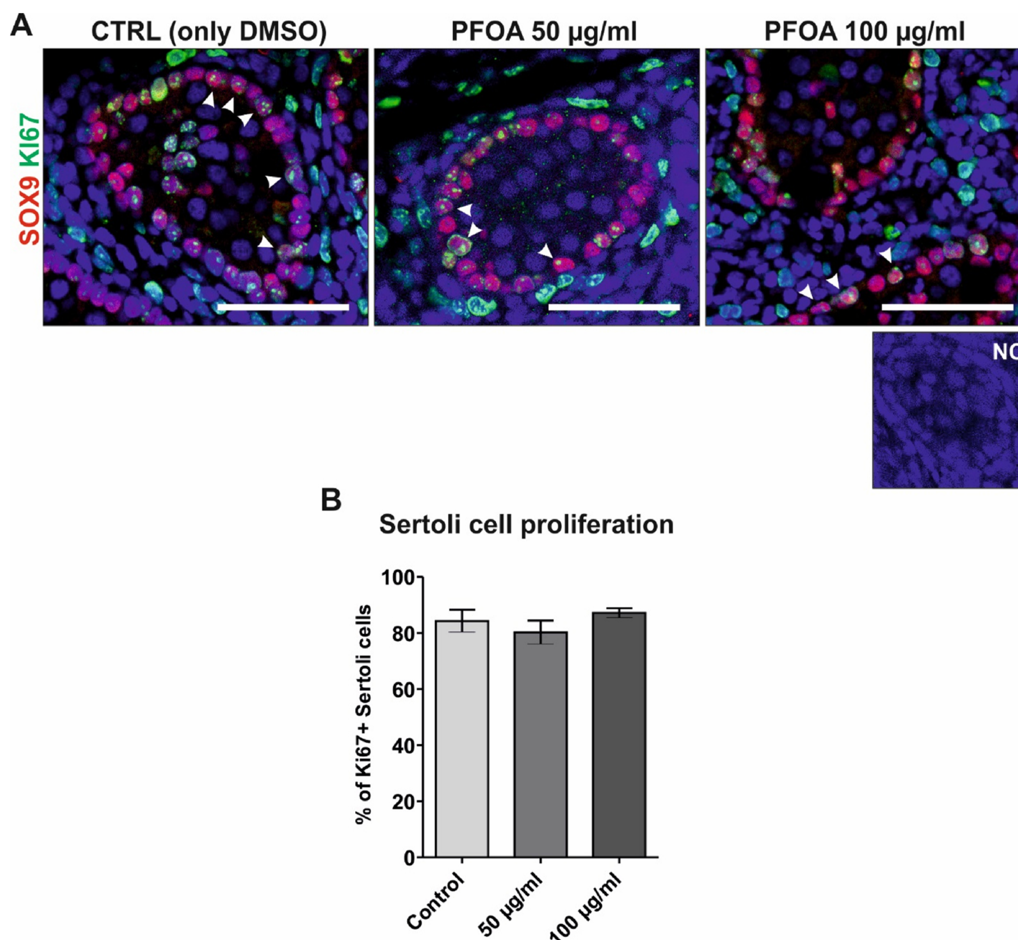


Fig. 5. PFOA did not have a significant effect on the amount of proliferating fetal Sertoli cells. Quantification of immunofluorescence images of testicular sections immunostained with Ki67 and SOX-9 (A) did not show significant difference in appearance of proliferating Sertoli cells per 100 Sertoli cells (B). Bars represent means +/- SEM, n = 4. NC: negative control, Scale bar 50 µm.

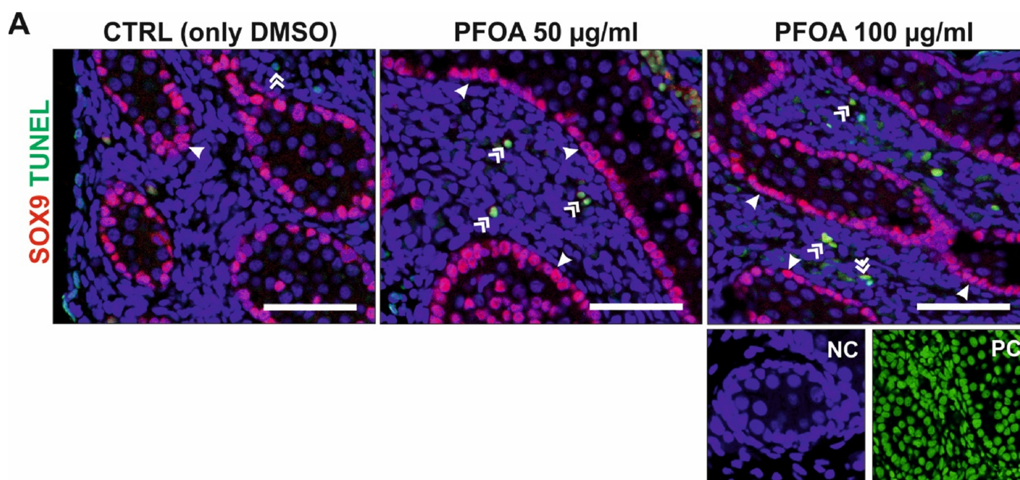
fetal testes are reliably identified and dissected from the mesonephros.

Since the decline in cAMP, progesterone and testosterone levels by the action of PFOA was observed we wanted to analyze if an early event in steroidogenesis is also impaired by the action of PFOA. Thus, we measured testicular expression of StAR, steroidogenic acute regulatory protein. Western blot results showed that the levels of StAR in the cultured fetal testis were significantly lower after hCG and forskolin stimulation in PFOA-exposed testes cultures than in controls. This was also reflected by lower progesterone and testosterone levels in PFOA-exposed cultures than in control. StAR protein is involved in early phase of steroidogenesis and it is synthesized in response to LH [42]. These findings are in line with previous studies [7,9].

Rosenmai et al. and Vinggaard et al. have previosly shown that PFOA does not function as an antagonist or agonist for androgen receptor [43,44]. By contrast, Nisio et al. have recently published experimental evidence which show that PFOA can act as an antagonist to AR [45]. Moreover, their clinical evidence showed that increased levels of PFOA in plasma and seminal fluid positively correlate with higher testosterone and LH levels in serum and reduction of semen quality, testicular volume, penile length, and AGD [45]. In our study, 24 -h PFOA exposure inhibited the increase of cAMP both in the hCG- and forskolin-stimulated cultures. In the light of the present results we can conclude that PFOA affects steroidogenesis on gonadotropin signaling level, but also other mechanisms are possible, like disturbance on the receptor level. Our results are in line with other studies, in which PFOA has been shown to decrease testosterone levels on isolated rat Leydig cells *in vitro* [21]. However, our results may be interpreted as not agreeing with recent clinical results from Di Nisio et al. where PFOA exposed men had increased testosterone and LH levels in serum [45]. When PFOA acts as an AR antagonist, the hypothalamus-pituitary axis is missing the negative feedback of testosterone resulting in increased LH and testosterone levels. This physiological feedback effect can not be seen in *in vitro* studies.

The present results showed also an increased tendency of apoptosis in fetal Leydig cells. Biegel et al. showed that ammonium perfluorooctanoate, which is an ammonium salt of PFOA, decreased testosterone production and increased estradiol levels in adult rats *in vivo*. In the same study the increase of Leydig cell adenomas/hyperplasia was observed after *in vivo* PFOA exposure in rats which may partly be explained by disturbed steroidogenesis [26]. Recently it was shown that concentrations of 17.7 and 53.2 µM of PFOA (corresponding concentrations 7.3 µg/ml and 22.0 µg/ml of PFOA) inhibited also 3β-HSD and 17β-HSD3, which are key enzymes involved in steroidogenesis, in adult rat Leydig cells [21]. The present results are in line with these findings.

By contrast, the current study shows that PFOA does not affect either apoptosis or proliferation of fetal Sertoli cells after 24 -h exposure in tissue culture. In adult rat testes we tested the effect of PFOA in 24 -h



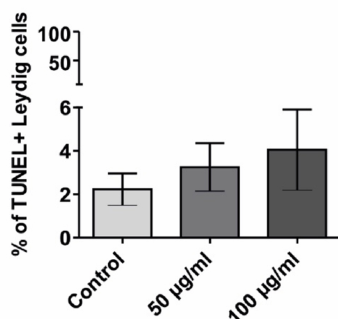


Fig. 6. PFOA affected fetal Leydig cell survival *in vitro* but did not induce apoptosis in fetal Sertoli cells. Immunofluorescence images of cultured fetal rat testes (ED 17.5) immunostained with SOX-9 and Tunel show that Tunel positive Sertoli cells were not found after 24 h exposure to 0, 50 and 100 μg/ml of PFOA (A). Morphologically identified and quantified fetal Tunel positive Leydig cells (A) showed a tendency of increase, but the difference was not significant (B). Bars represent means +/- SEM, n = 4. NC: negative control, PC: positive control, Scale bar 50 μm.

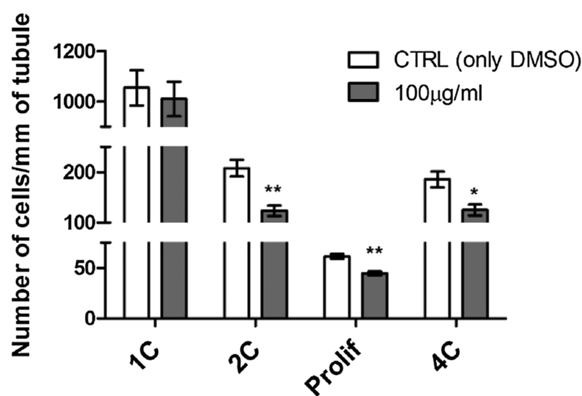


Fig. 7. PFOA decreased significantly the amount of diploid, proliferating, meiotic I and G2/M –phase cells. Cell population distribution of seminiferous tubule segments (stage VII-VIII) after 24 h incubation in DMSO 1 μl/ml and to 100 μg/ml of PFOA. Graph shows the amounts of different testicular cells/mm of tubule. 1C = haploid cells, 2C = diploid cells, Prolif. = proliferating cells and 4C = germ cells in meiosis I and cells in G2/M-phase. Bars represent means +/-SEM, n = 4. ****p* < 0.001, ***p* < 0.01 and **p* < 0.05.

exposure on seminiferous tubule segments at stage VII-VIII. The flow cytometric analysis showed that PFOA decreased significantly the amounts of cells in 2C (diploid cells), proliferating cells and in 4C (cells in meiosis and in G2/M-phase of cell cycle, including primary spermatocytes) populations in adult rat testis. PFOA did not have a significant effect on 1C (haploid germ cells) population of rat testicular cells. Apoptosis of germ cells was also quantified by immunohistochemistry corroborating flow cytometric findings.

Previously it has been shown that the most vulnerable testicular cells to undergo apoptosis in adult rat testis include spermatogonia, early and meiotically dividing spermatocytes [46–48]. Secondary spermatocytes, mid to late spermatocytes and spermatids seldom undergo apoptosis [46–48]. This is in agreement with present flow cytometry and immunohistochemistry data. Due to this finding we can consider that the affected adult rat testicular cells during the PFOA exposure in seminiferous tubule culture were mostly spermatogonia in 2C population and primary spermatocytes in 4C cells. Furthermore, with longer time the decrease of 4C cell population after PFOA exposure should be seen in 1C and 2C testicular cell populations as well. In the present *in vitro* study the 24 h exposure time was too short to show the secondary effect of the loss of 2C, proliferating and 4C cells.

Epidemiological studies have reported adverse effects of PFOA on male reproductive function in human [9,22,24,27] and several animal studies support this finding [7,25,26,28,49]. *In vitro* and *in vivo* studies have previously shown concrete and direct toxicological effects of PFOA on male reproductive functions. Bach and Vested et al. have recently reviewed this and there is some evidence that PFOA exposure may affect sperm motility, number of morphologically normal sperm, DNA fragmentation index, rates of sperm aneuploidy and PFOA may also have an effect on reproductive hormone levels of men, but there were some inconsistent results between different studies [9]. The effects of PFOA on fetal rat testis has not been well studied to date and the *in vitro* effects of PFOA on seminiferous tubules has not been previously analyzed.

As a conclusion, the present study shows that 24 -h PFOA exposure *in vitro* inhibits fetal rat steroidogenesis by decreasing cAMP, progesterone and testosterone levels. Furthermore, decreased StAR protein expression levels were observed. There was no significant difference in the amount of apoptotic Leydig cells, but an apparent trend for dose

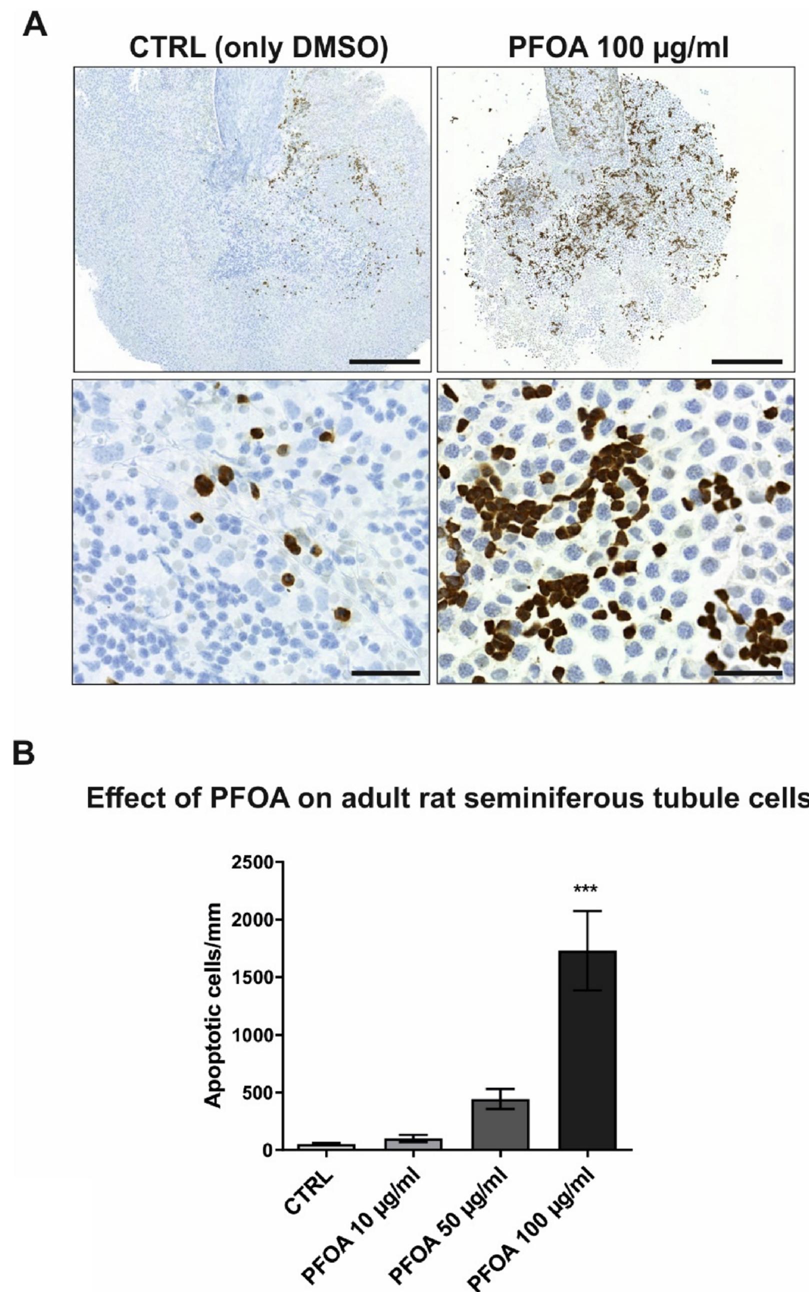


Fig. 8. PFOA induced apoptosis in rat seminiferous tubules. Cultured 2 mm long tubule segments of stage VIII were squashed and apoptotic cells were detected in the cell monolayer by immunohistochemistry with cleaved caspase-3 as the first antibody. The apoptotic cells are the deep brown cells in the pictures (A) of squashed tubule segments after 24 h exposure to DMSO 1 µl/ml or to 100 µg/ml of PFOA. Upper scale bar 500 µm, lower scale bar 50 µm. Quantification of the apoptotic cells per 1 mm shows that PFOA 100 µg/ml induced significantly apoptosis (B). Bars represent means +/-SEM, n = 7. ***$p <$ 0001.

response relationship was observed. In adult rat seminiferous tubules PFOA induces germ cell apoptosis. Taken together, our results suggest that PFOA has adverse effects on both fetal and adult rat testis.

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References

[1] H.E. Virtanen, N. Jorgensen, J. Toppari, Semen quality in the 21(st) century, Nat. Rev. Urol. 14 (2017) 120–130.

[2] C. Le Cornet, J. Lortet-Tieulent, D. Forman, R. Beranger, A. Flechon, B. Fervers, et al., Testicular cancer incidence to rise by 25% by 2025 in Europe? Model-based predictions in 40 countries using population-based registry data, Eur. J. Cancer 50 (2014) 831–839.

[3] N. Jorgensen, M. Vierula, R. Jacobsen, E. Pukkala, A. Perheentupa, H.E. Virtanen, et al., Recent adverse trends in semen quality and testis cancer incidence among Finnish men, Int. J. Androl. 34 (2011) e37–48.

[4] J. Toppari, H.E. Virtanen, K.M. Main, N.E. Skakkebaek, Cryptorchidism and hypospadias as a sign of testicular dysgenesis syndrome (TDS): environmental connection, Birth Defects Res A Clin Mol Teratol 88 (2010) 910–919.

[5] A. Juul, K. Almstrup, A.M. Andersson, T.K. Jensen, N. Jorgensen, K.M. Main, et al., Possible fetal determinants of male infertility, Nat. Rev. Endocrinol. 10 (2014) 553–562.
[6] N.E. Skakkebaek, A brief review of the link between environment and male reproductive health: lessons from studies of testicular germ cell Cancer, Horm. Res. Paediatr. (2016).
[7] C. Hodes, C. Lau, K. Anitole, D. Lai, A. Pfahles-Hutchens, J. Seed, Perfluoroalkyl acids: a review of monitoring and toxicological findings, Toxicol. Sci. 99 (2007) 366–394.
[8] G.W. Olsen, J.M. Burris, D.J. Ehresman, J.W. Froehlich, A.M. Seacat, J.L. Butenhoff, et al., Half-life of serum elimination of perfluorooctanesulfonate,perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers, Environ. Health Perspect. 115 (2007) 1298–1305.
[9] C.C. Bach, A. Vested, K.T. Jorgensen, J.P. Bonde, T.B. Henriksen, G. Toft, Perfluoroalkyl and polyfluoroalkyl substances and measures of human fertility: a systematic review, Crit. Rev. Toxicol. (2016) 1–21.
[10] A. Bergman, G. Becher, B. Blumberg, P. Bjerregaard, R. Bornman, I. Brandt, et al., Manufacturing doubt about endocrine disrupter science–A rebuttal of industry-sponsored critical comments on the UNEP/WHO report "State of the Science of Endocrine disrupting Chemicals 2012″, Regul. Toxicol. Pharmacol. 73 (2015) 1007–1017.
[11] A.B. Lindstrom, M.J. Strynar, E.L. Libelo, Polyfluorinated compounds: past, present, and future, Environ. Sci. Technol. 45 (2011) 7954–7961.
[12] P.M. Hinderliter, E. Mylchreest, S.A. Gannon, J.L. Butenhoff, G.L. Kennedy Jr., Perfluorooctanoate: placental and lactational transport pharmacokinetics in rats, Toxicology 211 (2005) 139–148.
[13] L.S. Mamsen, R.D. Bjorvang, D. Mucs, M.T. Vinnars, N. Papadogiannakis, C.H. Lindh, et al., Concentrations of perfluoroalkyl substances (PFASs) in human embryonic and fetal organs from first, second, and third trimester pregnancies, Environ. Int. 124 (2019) 482–492.
[14] R. Cariou, B. Veyrand, A. Yamada, A. Berrebi, D. Zalko, S. Durand, et al., Perfluoroalkyl acid (PFAA) levels and profiles in breast milk, maternal and cord serum of French women and their newborns, Environ. Int. 84 (2015) 71–81.
[15] C. Guerranti, C. La Rocca, S. Tait, L. Busani, F. Ciardo, B. Bergamasco, et al., Exposure to endocrine disruptors and nuclear receptors gene expression in infertile and fertile men from italian areas with different environmental features, Int. J. Environ. Res. Public Health 12 (2015) 12426–12445.
[16] K.S. Guruge, S. Taniyasu, N. Yamashita, S. Wijeratna, K.M. Mohotti, H.R. Seneviratne, et al., Perfluorinated organic compounds in human blood serum and seminal plasma: a study of urban and rural tea worker populations in Sri Lanka, J. Environ. Monit. 7 (2005) 371–377.
[17] Y. Lu, B. Luo, J. Li, J. Dai, Perfluorooctanoic acid disrupts the blood-testis barrier and activates the TNFalpha/p38 MAPK signaling pathway in vivo and in vitro, Arch. Toxicol. 90 (2016) 971–983.
[18] A.C. Dankers, M.J. Roelofs, A.H. Piersma, F.C. Sweep, F.G. Russel, M. van den Berg, et al., Endocrine disruptors differentially target ATP-binding cassette transporters in the blood-testis barrier and affect Leydig cell testosterone secretion in vitro, Toxicol. Sci. 136 (2013) 382–391.
[19] B. Lindeman, C. Maass, N. Duale, K.B. Gutzkow, G. Brunborg, A. Andreassen, Effects of per- and polyfluorinated compounds on adult rat testicular cells following in vitro exposure, Reprod. Toxicol. 33 (2012) 531–537.
[20] C. Lau, J.L. Butenhoff, J.M. Rogers, The developmental toxicity of perfluoroalkyl acids and their derivatives, Toxicol. Appl. Pharmacol. 198 (2004) 231–241.
[21] B. Zhao, G.X. Hu, Y. Chu, X. Jin, S. Gong, B.T. Akingbemi, et al., Inhibition of human and rat 3beta-hydroxysteroid dehydrogenase and 17beta-hydroxysteroid dehydrogenase 3 activities by perfluoroalkylated substances, Chem. Biol. Interact. 188 (2010) 38–43.
[22] A. Vested, C.H. Ramlau-Hansen, S.F. Olsen, J.P. Bonde, S.L. Kristensen, T.I. Halldorsson, et al., Associations of in utero exposure to perfluorinated alkyl acids with human semen quality and reproductive hormones in adult men, Environ. Health Perspect. 121 (2013) 453–458.
[23] E.T. Chang, H.O. Adami, P. Boffetta, H.J. Wedner, J.S. Mandel, A critical review of perfluorooctanoate and perfluorooctanesulfonate exposure and immunological health conditions in humans, Crit. Rev. Toxicol. 46 (2016) 279–331.
[24] U.N. Joensen, R. Bossi, H. Leffers, A.A. Jensen, N.E. Skakkebaek, N. Jorgensen, Do perfluoroalkyl compounds impair human semen quality? Environ. Health Perspect. 117 (2009) 923–927.
[25] A.D. Benninghoff, W.H. Bisson, D.C. Koch, D.J. Ehresman, S.K. Kolluri, D.E. Williams, Estrogen-like activity of perfluoroalkyl acids in vivo and interaction with human and rainbow trout estrogen receptors in vitro, Toxicol. Sci. 120 (2011) 42–58.
[26] L.B. Biegel, R.C. Liu, M.E. Hurtt, J.C. Cook, Effects of ammonium perfluorooctanoate on Leydig cell function: in vitro, in vivo, and ex vivo studies, Toxicol. Appl. Pharmacol. 134 (1995) 18–25.
[27] U.N. Joensen, B. Veyrand, J.P. Antignac, M. Blomberg Jensen, J.H. Petersen, P. Marchand, et al., PFOS (perfluorooctanesulfonate) in serum is negatively associated with testosterone levels, but not with semen quality, in healthy men, Hum. Reprod. 28 (2013) 599–608.
[28] H.T. Wan, Y.G. Zhao, M.H. Wong, K.F. Lee, W.S. Yeung, J.P. Giesy, et al., Testicular signaling is the potential target of perfluorooctanesulfonate-mediated subfertility in male mice, Biol. Reprod. 84 (2011) 1016–1023.
[29] M. Welsh, P.T. Saunders, M. Fisken, H.M. Scott, G.R. Hutchison, L.B. Smith, et al., Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism, J. Clin. Invest. 118 (2008) 1479–1490.
[30] L.J. Brokken, A. Adamsson, J. Paranko, J. Toppari, Antiandrogen exposure in utero disrupts expression of desert hedgehog and insulin-like factor 3 in the developing fetal rat testis, Endocrinology 150 (2009) 445–451.
[31] N.A. Adamsson, L.J. Brokken, J. Paranko, J. Toppari, In vivo and in vitro effects of flutamide and diethylstilbestrol on fetal testicular steroidogenesis in the rat, Reprod. Toxicol. 25 (2008) 76–83.
[32] F.M. Hekster, R.W. Laane, P. de Voogt, Environmental and toxicity effects of perfluoroalkylated substances, Rev. Environ. Contam. Toxicol. 179 (2003) 99–121.
[33] H. Nikula, T. Talonpoika, M. Kaleva, J. Toppari, Inhibition of hCG-stimulated steroidogenesis in cultured mouse Leydig tumor cells by bisphenol A and octylphenols, Toxicol. Appl. Pharmacol. 157 (1999) 166–173.
[34] G. Livera, G. Delbes, C. Pairault, V. Rouiller-Fabre, R. Habert, Organotypic culture, a powerful model for studying rat and mouse fetal testis development, Cell Tissue Res. 324 (2006) 507–521.
[35] R. Habert, I. Devif, M.N. Gangnerau, L. Lecerf, Ontogenesis of the in vitro response of rat testis to gonadotropin-releasing hormone, Mol. Cell. Endocrinol. 82 (1991) 199–206.
[36] J.F. Harper, G. Brooker, Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′0 acetylation by acetic anhydride in aqueous solution, J. Cyclic Nucleotide Res. 1 (1975) 207–218.
[37] T. Haavisto, K. Nurmela, R. Pohjanvirta, H. Huuskonen, F. El-Gehani, J. Paranko, Prenatal testosterone and luteinizing hormone levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol, Mol. Cell. Endocrinol. 178 (2001) 169–179.
[38] E. Rotgers, A. Rivero-Muller, M. Nurmio, M. Parvinen, F. Guillou, I. Huhtaniemi, et al., Retinoblastoma protein (RB) interacts with E2F3 to control terminal differentiation of Sertoli cells, Cell Death Dis. 5 (2014) e1274.
[39] J. Toppari, M. Parvinen, In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle morphologic and immunolocalization analysis, J. Androl. 6 (1985) 334–343.
[40] E. Rotgers, S. Cisneros-Montalvo, K. Jahnukainen, J. Sandholm, J. Toppari, M. Nurmio, A detailed protocol for a rapid analysis of testicular cell populations using flow cytometry, Andrology 3 (2015) 947–955.
[41] R. Habert, R. Picon, Testosterone, dihydrotestosterone and estradiol-17 beta levels in maternal and fetal plasma and in fetal testes in the rat, J. Steroid Biochem. 21 (1984) 193–198.
[42] D.M. Stocco, StAR protein and the regulation of steroid hormone biosynthesis, Annu. Rev. Physiol. 63 (2001) 193–213.
[43] A.K. Rosenmai, F.K. Nielsen, M. Pedersen, N. Hadrup, X. Trier, J.H. Christensen, et al., Fluorochemicals used in food packaging inhibit male sex hormone synthesis, Toxicol. Appl. Pharmacol. 266 (2013) 132–142.
[44] A.M. Vinggaard, J. Niemela, E.B. Wedebye, G.E. Jensen, Screening of 397 chemicals and development of a quantitative structure–activity relationship model for androgen receptor antagonism, Chem. Res. Toxicol. 21 (2008) 813–823.
[45] A. Di Nisio, I. Sabovic, U. Valente, S. Tescari, M.S. Rocca, D. Guidolin, et al., Endocrine disruption of androgenic activity by perfluoroalkyl substances: clinical and experimental evidence, J. Clin. Endocrinol. Metab. 104 (2019) 1259–1271.
[46] K. Henriksen, H. Hakovirta, M. Parvinen, In-situ quantification of stage-specific apoptosis in the rat seminiferous epithelium: effects of short-term experimental cryptorchidism, Int. J. Androl. 18 (1995) 256–262.
[47] T. Sjoblom, A. West, J. Lahdetie, Apoptotic response of spermatogenic cells to the germ cell mutagens etoposide, adriamycin, and diepoxybutane, Environ. Mol. Mutagen. 31 (1998) 133–148.
[48] W. Yan, J. Suominen, M. Samson, B. Jegou, J. Toppari, Involvement of Bcl-2 family proteins in germ cell apoptosis during testicular development in the rat and pro-survival effect of stem cell factor on germ cells in vitro, Mol. Cell. Endocrinol. 165 (2000) 115–129.
[49] Y. Wei, J. Dai, M. Liu, J. Wang, M. Xu, J. Zha, et al., Estrogen-like properties of perfluorooctanoic acid as revealed by expressing hepatic estrogen-responsive genes in rare minnows (Gobiocypris rarus), Environ. Toxicol. Chem. 26 (2007) 2440–2447.