

Testicular Signaling Is the Potential Target of Perfluorooctanesulfonate-Mediated Subfertility in Male Mice¹

H.T. Wan,³ Y.G. Zhao,³ M.H. Wong,³ K.F. Lee,⁴ W.S.B. Yeung,⁴ J.P. Giesy,^{5,6} and C.K.C. Wong^{2,3}

Croucher Institute of Environmental Sciences,³ Department of Biology, Hong Kong Baptist University, Hong Kong, China
Department of Obstetrics and Gynecology,⁴ The University of Hong Kong, Hong Kong, China
Department of Veterinary Biomedical Sciences & Toxicological Center,⁵ University of Saskatchewan, Saskatoon, Saskatchewan, Canada
Department of Biology and Chemistry,⁶ City University of Hong Kong, Hong Kong, China

ABSTRACT

Perfluorooctanesulfonate (PFOS) was produced and used by various industries and in consumer products. Because of its persistence, it is ubiquitous in air, water, soil, wildlife, and humans. Although the adverse effects of PFOS on male fertility have been reported, the underlying mechanisms have not yet been elucidated. Here, for the first time, the effects of PFOS on testicular signaling, such as gonadotropin, growth hormone, insulin-like growth factor, and inhibins/activins were shown to be directly related to male subfertility. Sexually mature 8-wk-old CD1 male mice were administered by gavages in corn oil daily with 0, 1, 5, or 10 mg/kg PFOS for 7, 14, or 21 days. Serum concentrations of testosterone and epididymal sperm counts were significantly lower in the mice after 21 days of the exposure to the highest dose compared with the controls. The expression levels of testicular receptors for gonadotropin, growth hormone, and insulin-like growth factor 1 were considerably reduced on Day 21 in mice exposed daily to 10 or 5 mg/kg PFOS. The transcript levels of the subunits of the testicular factors (i.e., inhibins and activins), *Inha*, *Inhba*, and *Inhbb*, were significantly lower on Day 21 of daily exposure to 10, 5, or 1 mg/kg PFOS. The mRNA expression levels of steroidogenic enzymes (i.e., StAR, CYP11A1, CYP17A1, 3 β HSD, and 17 β HSD) were notably reduced. Therefore, PFOS-elicited subfertility in male mice is manifested as progressive deterioration of testicular signaling.

environment, gene regulation, male sexual function, sperm

INTRODUCTION

Perfluorinated compounds (PFCs) are synthetic compounds with unique chemical properties that can be degraded to several persistent products. They have been widely applied in various industries and consumer products, especially as surfactants.

¹Supported by the Super Faculty Research Grant, Hong Kong Baptist University and Collaborative Research Fund (HKBU 1/CRF/08), University Grants Committee, to C.K.C.W. J.P.G. was supported by the Canada Research Chair program and an at-large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong.

²Correspondence: Chris K.C. Wong, Croucher Institute of Environmental Sciences, Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China. FAX: 852 3411 5995; e-mail: ckcwong@hkbu.edu.hk

Received: 19 October 2010.

First decision: 14 November 2010.

Accepted: 28 December 2010.

© 2011 by the Society for the Study of Reproduction, Inc.

eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

They are ubiquitous in the environment [1]. Among various PFCs, perfluorooctanesulfonate (PFOS) is the most well-known and abundant PFC in the environment [2]. Concentrations of PFOS of 2 pg m⁻³ in air in Germany [3], 245 ng/L in rivers of the United States [3, 4], and 5 ppm in indoor house dust [5] have been reported. The half-life of PFOS in humans is approximately 5.4 yr; considerable levels of it can be accumulated and detected in human serum samples [5, 6]. An average concentration of 50 ng/ml PFOS in the blood serum of Americans has been reported. However, occupational exposure resulted in a concentration of 1.5 μ g/ml PFOS in blood serum [7]. In China, concentrations of PFOS in human breast milk ranged from 45 to 360 ng/L [8]. Because of the potential adverse health effects of PFOS, industrial production of PFOS was phased out in most countries in 2000 [5], but it is still manufactured and used in China.

Perfluorooctanesulfonate is one of the nine new persistent organic pollutants (POPs) listed under Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009. Perfluorooctanesulfonate has been reported to have an effect on animal hepatic and reproductive functions [5, 9]. Adverse effects of PFOS on reproduction include lower serum testosterone levels, reduced fertility, and increased mortality in newborns [6, 10–13]. Most studies have investigated the effects of PFOS on changes in the concentrations of steroid hormones in blood serum. However, the underlying mechanisms of these effects have not yet been elucidated. In this study, effects of PFOS on the hypothalamic-pituitary-gonadal (HPG) axis of male mice were investigated. With the benefit of hindsight, serum concentrations of approximately 179 μ g/ml PFOS were detected in the mice exposed to 10 mg/kg PFOS per day for 18 days [6]. Based on human safety factors [14] and reported concentrations of PFOS in human blood serum, daily dosages of 1, 5, or 10 mg/kg PFOS were used in this study.

MATERIALS AND METHODS

Experimental Animals and Chemicals

All experimental animals were housed and handled in accordance with the Guidelines and Regulations for Hong Kong Baptist University. Male CD-1 mice (6–8 wk old) were purchased from the Laboratory Animal Service Centre of the Chinese University of Hong Kong (Hong Kong, China). The entire study was conducted in triplicate with mice that were received in three separate batches. The animals were acclimatized for 1 wk before experiments. The mice were randomly divided into four groups (four individuals per group). Each group was housed in polypropylene cages with sterilized bedding and was maintained under controlled temperature (23.5°C) and 12L:12D cycles (0600–1800 h). The mice were weighed by an electronic balance (Shimadzu, Tokyo, Japan) and orally administered 1, 5, or 10 mg/kg PFOS by gavage in corn oil for 7, 14, or 21 days in the morning. Perfluorooctanesulfonate (98% purity; Sigma-Aldrich) was dissolved in dimethyl sulfoxide before mixing with corn

TABLE 1. DNA sequences of primers used in the present study.

Gene	Forward	Reverse
Kisspeptin (<i>Kiss1</i>)	GAATGATCTCAATGGCTTCTTGG	TTTCCCAGGCATTAACGAGTT
Kisspeptin receptor (<i>Gpr54</i>)	GCTCACTGCATGTCCCTACAG	GCCTGTCTGAAGTGTGAACC
Gonadotropin-releasing hormone (<i>Gnrh</i>)	GGGAAAGAGAAACACTGAACAC	GGACAGTACATTCGAAGTGTG
Gonadotropin-releasing hormone receptor (<i>Gnrhr</i>)	CTCTATGTATGCCCCAGCTTTCA	GCAAAGACAATGCTGAGAATCCA
Luteinizing hormone (<i>Lh</i>)	CCTAGCATGGTCCGAGTACT	GCTACAGGAAAGGAGACTATGG
Follicle-stimulating hormone (<i>Fsh</i>)	GCTGCTCAACTCCTCTGAAG	GGCAATACCTTGGGAAATTCTG
LH receptor (<i>Lhr</i>)	GCACCTCCAGAGTTGTCTG	AGGGAGATAGGTGAGAGATAGTC
FSH receptor (<i>Fshr</i>)	TCTGCATGGCCCAATTTTA	GGTAGAACAGAACTAGGAGGATC
Steroidogenic acute regulatory protein (<i>Star</i>)	GGAACCCAAATGTCAAGGAGATCA	GCACGCTCACGAAGTCTCGA
Cytochrome P450 α (<i>Cyp11a1</i>)	AGCTGGGCAACATGGAGTCA	CCTCTGGTAATACTGGTGATAGGC
Cytochrome P450 17A1 (<i>Cyp17a11</i>)	GATCTAAGAAGCTCAGGCA	GGGCACTGCATCACGATAAA
3 β -hydroxysteroid dehydrogenase (<i>Hsd3b</i>)	GTGACAGTGTGGAAGGAGACA	GACATCAATGACAGCAGCAGTG
17 β -hydroxysteroid dehydrogenase (<i>Hsd17b</i>)	CTGGTGAAGTGCATGAGGTTCT	TCAAATGAATAGGCTTTCCCGATG
5 α -reductase (<i>Srd5a</i>)	GCTGGTGGTGACGTATGTCTT	AAAATGGGAGGAAACGCTTTCC
Insulin-like growth factor-1 (<i>Igf1</i>)	GCCCCACTGAAGCCTACAAAA	GTACTTCTTTCTTCTCTCTTTG
Insulin-like growth factor-1 receptor (<i>Igf1r</i>)	CAGACACTACTACAAAGCGT	GATAACGAGCATCCGAGTCA
Growth hormone receptor (<i>Ghr</i>)	ACTGTCCAGTGTACTCATTGAGAA	CATGCTTCCAATATGTTCTGCTGA
<i>Actin</i>	TCTACGAGGGCTATGCTCTCC	TCTTTGATGTCACGCAGATTTCC
<i>Inha</i>	ATGCACAGGACCTCTGAACC	GGATGGCCGGAATACATAAG
<i>Inhba</i>	GGAGAACGGGTATGTGGAGA	TGGTCTGGTTCTGTTAGCC
<i>Inhbb</i>	CCTGAGTGAATGCACACCAC	CGAGTCCAGTTTCGCCTAGT
<i>Inhbc</i>	GACTCCAACCACAGTAGTGAAAC	CACTGGCCGACTGAGTATGG
Follistatin (<i>Fst</i>)	AAAACCTACCGCAACGAATG	TTCAGAAGAGGAGGGCTCTG

oil. The control group was given corn oil. The mice were fed standard food (Rodent Diet 5001; LabDiet) and water (in glass bottles) ad libitum.

The mice were killed by cervical dislocation in the morning on designated dates. Blood was collected by cardiocentesis, and serum was prepared by centrifugation at 3000 \times g. The sera were stored at -20°C until further analysis. The testes of each animal were collected and weighed. Histological evaluation was conducted on the testes fixed in 4% paraformaldehyde (PFA; Sigma). The rest were immediately frozen in liquid nitrogen and stored at -80°C until biochemical analysis.

Real-Time PCR

Total RNA with $A_{260}:A_{280}$ ratios between 1.8 and 1.9 was used for real-time PCR analyses. Complementary DNA was synthesized from 150 ng of total cellular RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). Gene-specific primers were designed from published sequences (Table 1). Real-time PCR was conducted with a program that consisted of 3 min at 95°C followed by 40 cycles of 95°C for 15 sec, 56°C for 20 sec, and 72°C for 30 sec. Standards and cDNAs from samples were quantified using the StepOne Real-Time PCR system using SYBR Green Master mix (Applied Biosystems). Copy numbers for each sample were calculated, and all data were normalized with the transcript levels of mouse actin. Occurrences of primer-dimers and secondary products were evaluated using melting curve analysis. Control amplifications were done either without RT or without RNA. All glassware and plasticware were treated with diethyl pyrocarbonate and autoclaved.

ELISA for Testosterone

Concentrations of testosterone in blood serum were determined using ELISA kits (MP Biomedicals). A total of 100 μl of working hormone-HRP conjugate reagent, 50 μl of rabbit anti-hormone reagent, and 25 μl of samples or standard were added to each well and incubated at 37°C for 90 min. The wells were rinsed five times with distilled water, followed by an addition of 100 μl of 3,3',5,5'-tetramethylbenzidine and incubation at room temperature. The reaction was then stopped by 1 N HCl solution. Absorbance was measured at 450 nm.

Sperm Quantification

The epididymis was isolated, chopped into small pieces, dispersed in PBS with 3% bovine serum albumin, and incubated for 1 h at 37°C . Epididymal spermatozoa were then stained with eosin-nigrosin (0.67% eosin Y, 10% nigrosin, and 0.9% sodium chloride) to identify altered membranes at the heads of spermatozoa. At least 200 epididymal spermatozoa (magnification $\times 400$) were examined under a light microscope.

Statistical Analysis

Statistical evaluations were conducted by use of SPSS16. All data were tested to be normally distributed and independent by using the Normal Plots in SPSS and Shapiro-Wilk significance of 0.05. Differences between treatment groups and corresponding control groups were tested for statistical significance by ANOVA followed by Duncan multiple-range test (significance at $P < 0.05$) SPSS16. Results are presented as the mean \pm SEM.

RESULTS

Survival, Growth, and Male Fertility

The exposure of the animals to 1 or 5 mg/kg PFOS for 3 wk did not cause any significant changes in body weight compared with the control groups (Fig. 1A). However, animals treated daily with 10 mg/kg PFOS were found to have significant lower body weight by the end of the exposure. Throughout the experiments, no death among the mice was observed. The liver weights were significantly higher in mice exposed to 5 or 10 mg/kg PFOS daily for 14 and 21 days (Fig. 1B).

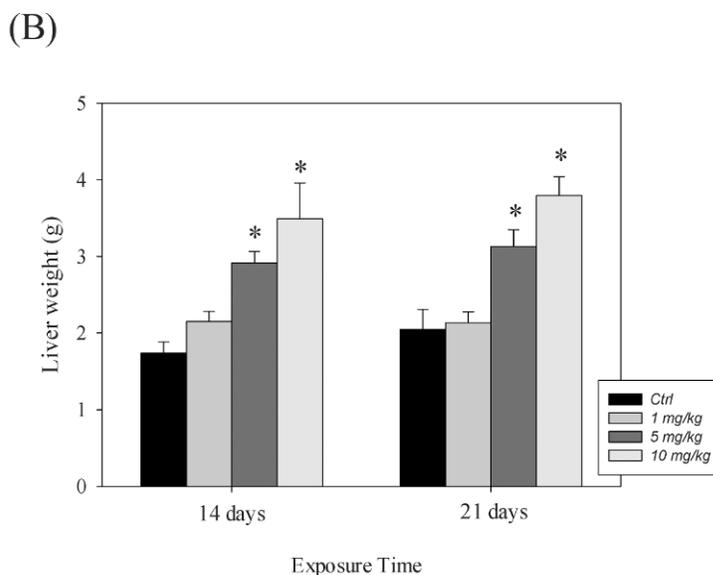
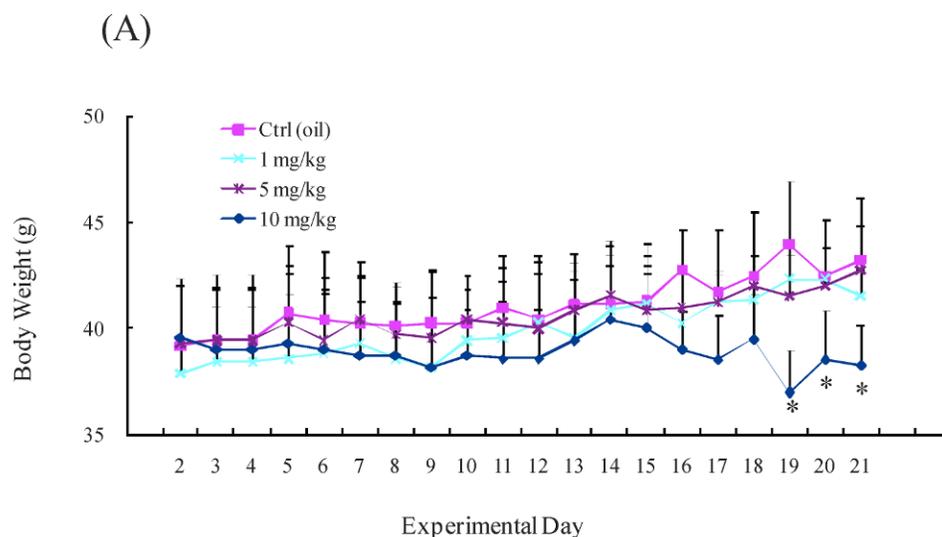
Serum testosterone levels and epididymal sperm counts of PFOS-exposed male mice are shown in Figure 2. Exposure to 10 mg/kg PFOS daily for 21 days resulted in significantly lower serum testosterone levels (Fig. 2A) and epididymal sperm counts (Fig. 2B). In mice exposed to lower doses of PFOS, no significant changes in serum testosterone levels and epididymal sperm counts were noted. There were no statistically significant differences in weights (data not shown) or gross histology of testes between the control and the PFOS-exposed mice (Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org).

Expression of Hormones and Receptors of the HPG Axis

There were no statistically significant differences in transcript levels of *Kiss1*, *Gpr54*, or *Gnrh* in the hypothalami (Fig. 3A) or *Gnrhr*, *Fsh*, or *Lh* in the pituitaries (Fig. 3B) among the control and the PFOS-exposed mice.

Expression of genes related to steroidogenesis and spermatogenesis was significantly lower in the testes of the mice exposed to PFOS for 21 days. The transcript levels of *Ghr*, *Igf1r*, and *Igf1* (Fig. 4A), *Fshr* and *Lhr* (Fig. 4B), and the

FIG. 1. Effects of 1, 5, or 10 mg/kg PFOS exposure by gavage daily for 14 or 21 days on body and liver weights of mice. **A**) Body weight records of the animals during PFOS exposure periods. * $P < 0.05$ compared with the control group (Ctrl). **B**) Weights of livers of mice exposed to PFOS for 14 and 21 days. * $P < 0.001$ compared with the control group according to the results of one-way ANOVA followed by Duncan multiple-range test.



steroidogenic enzymes (i.e., StAR, CYP11A1, CYP17A1, 3 β -HSD, and 17 β -HSD; Fig. 4C) were significantly reduced in groups receiving 10 or 5 mg/kg daily. The transcript levels of *Hsd17b* were significantly reduced in the mice exposed to 1 mg/kg PFOS daily. No statistically significant differences between male mice exposed to any of the doses of PFOS after 14 days of treatment were observed (Supplemental Fig. S2).

Testicular Inhibins, Activin, and Follistatin

Perfluorooctanesulfonate exposure reduced the expression levels of some inhibin subunits in the testes. On Day 14, the transcript levels of *Inhbc* were significantly lower in all PFOS-exposed mice (Fig. 5A). On Day 21, the mRNA levels of *Inhba* were reduced in all of the treatment groups (Fig. 5B). The inhibin β subunits, such as *Inhba* and *Inhbb*, were significantly lower in the male mice exposed to 10 or 5 mg/kg PFOS daily.

No statistically significant differences in the transcript levels of *Fst* were observed among the treatments or between the treatments and the controls.

DISCUSSION

Human epidemiological data revealed PFC exposure decreased male fertility. The decrease was associated with reduced serum testosterone levels and sperm counts [15]. Reductions in serum testosterone levels have also been reported in rodents upon PFOS exposure [11, 15, 16]. However, the mechanistic actions of PFOS on the male reproductive system are not clear. In this study, PFOS-exposed mice (10 mg/kg daily) showed substantial decreases in serum testosterone levels and epididymal sperm counts after 3 wk of exposure. The reductions were found to be the consequences of a significant decrease in the expression levels of testicular

receptors for follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH), and insulin-like growth factor 1 (IGF1), and different inhibin subunits, and impairment of testicular steroidogenesis.

The decreased body weights and the increased liver weights in the male mice exposed to PFOS observed in this study were similar to those in another report in which mice were exposed to 7 mg/kg PFOS for 28 days [17]. The lower body weights caused by PFOS was associated with neurological and behavioral changes, which lead to loss of appetite and less food intake [11]. The higher liver weights may be attributed to lipid accumulation, a common manifestation of metabolic disorders that results in fatty livers [6, 11, 13, 17–19].

Production of testosterone and sperm are regulated by feedback mechanisms along the HPG axis. The hypothalamic KISS1/GPR53 system is essential for puberty maturation and gonadal function [20]. The activation of KISS1/GPR54 stimulates the release of gonadotropin-releasing hormone, which controls the synthesis of gonadotropin LH and FSH by the pituitary. Luteinizing hormone and FSH act on testicular Leydig and Sertoli cells to regulate the processes of steroidogenesis and spermatogenesis, respectively [21, 22]. The major testicular hormonal factors, testosterone, inhibins, and activins then fine-tune the secretions by the hypothalamus and pituitary to maintain homeostasis. Therefore, understanding the effects of PFOS on the hypothalamic circuitry onto the reproductive axis is necessary. In the present study, PFOS exposure for 21 days showed no noticeable effects on the gene expression levels of the reproduction-related hormones and receptors in the hypothalamus and pituitaries. This observation reveals that short-term exposure to PFOS may not affect regulatory circuits at the hypothalamic and pituitary levels. Because the concentrations of PFOS were inversely proportional to the fertility of the male mice, the effects of PFOS on the expressions of *Lhr* and *Fshr* mRNA in the testes were investigated. Intriguingly, the transcript levels of *Lhr* and *Fshr* were significantly reduced in the testes of mice exposed to 10 or 5 mg/kg PFOS daily. Luteinizing hormone stimulates Leydig cells to produce testosterone, which acts with FSH to facilitate Sertoli-Leydig cell communication and to permit optimal spermatogenesis [23, 24]. The decrease of testicular receptor levels for gonadotropin can inhibit the process of steroidogenesis and reduce the efficacy of spermatogenesis [25, 26]. The effects on LHR and FSHR observed in this study are consistent with the lesser concentration of testosterone in the blood serum and epididymal sperm counts in the PFOS-exposed mice.

Development of male reproductive function is not controlled exclusively by gonadotropin. Other endocrine and autocrine/paracrine hormones, such as GH, IGF1, inhibins, and activins, are also involved [27–29]. Growth hormone acts on hepatic growth hormone receptors (GHRs) to stimulate synthesis and release of IGF1, which can bind to the testicular IGF1 receptor to stimulate testosterone synthesis [30] and maintain normal fertility [29]. Because GHR is also expressed in testes, the local synthesis and autocrine/paracrine actions of IGF1 are important for testicular functions [31–34]. In this study, transcription of *Ghr* and *Igf1* mRNA in livers was significantly reduced by daily exposure to 10 mg/kg PFOS for 21 days (unpublished data). The expression levels of *Ghr* and *Igf1r* mRNA in the testes were also significantly reduced in the mice exposed to 10 mg/kg PFOS daily than in the control. The lesser expressions may lead to the impairment of both GH and IGF1 signaling, and thus the production of testosterone in the testes [31]. Previously, effects of PFOS on the GH-IGF axis have not been reported. However, effects on the GH-IGF axis by the synthetic

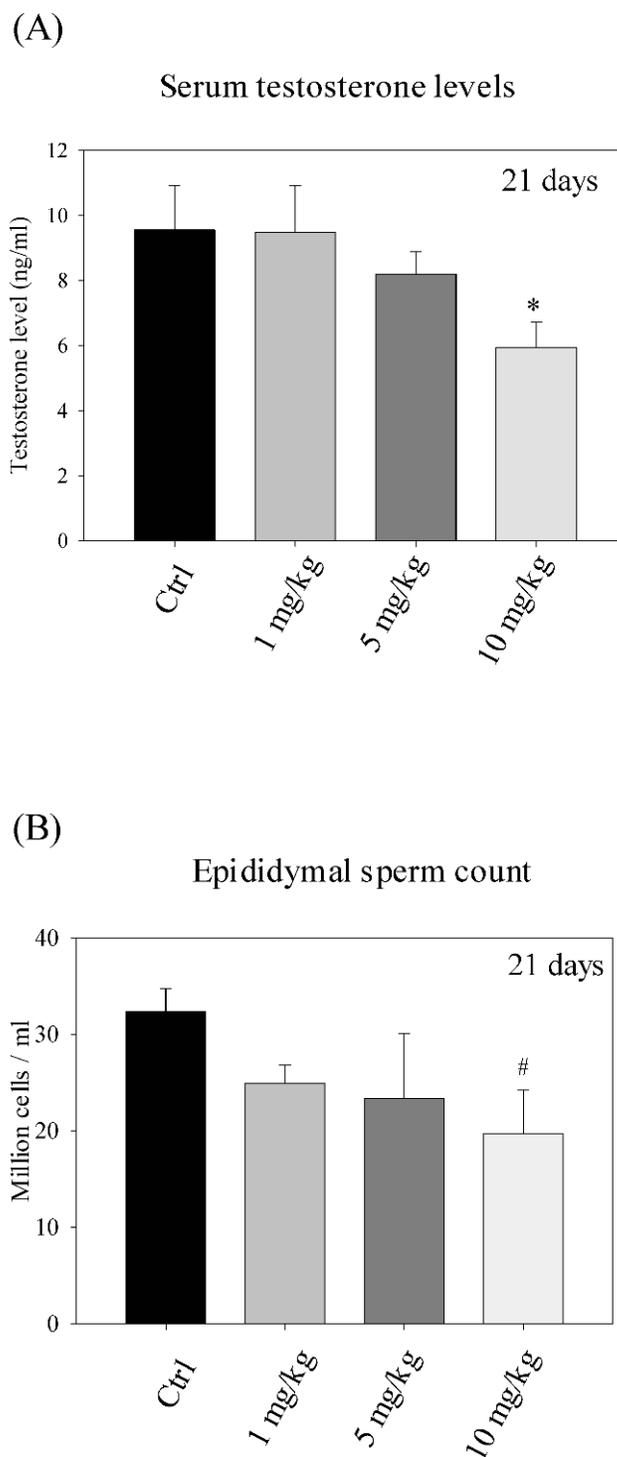
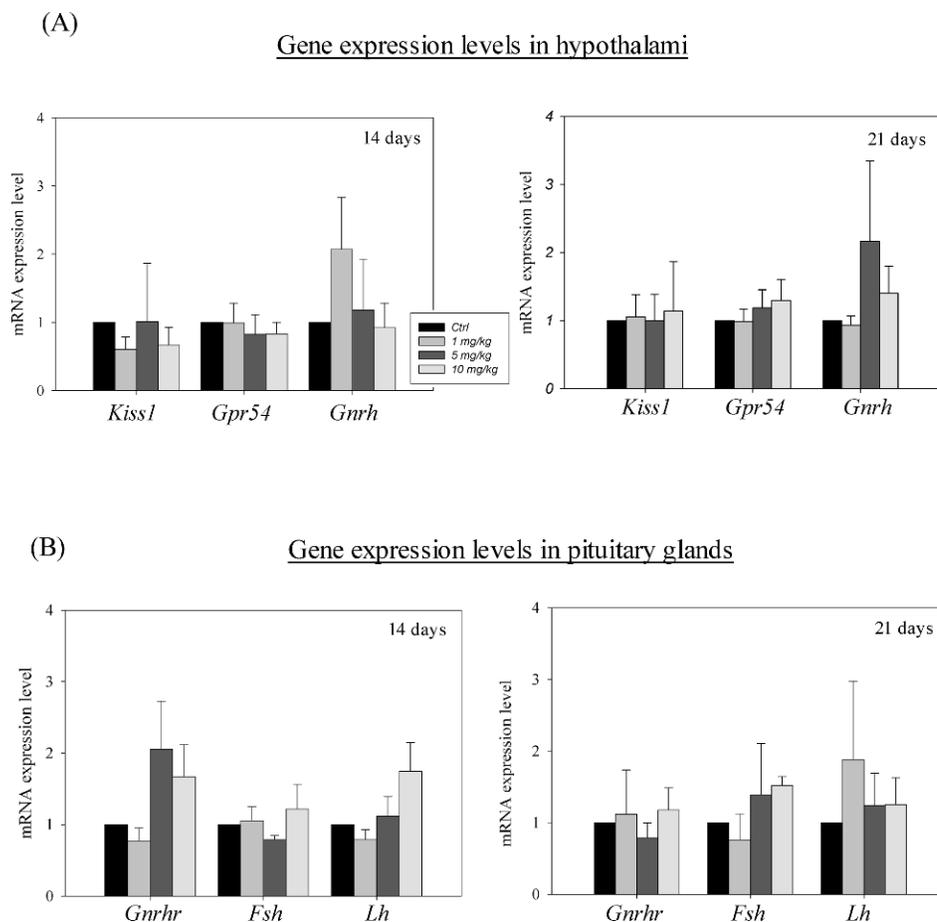


FIG. 2. Effects of 1, 5, or 10 mg/kg daily PFOS on serum concentrations of testosterone and the number of epididymal sperm in mice. Significant reductions in (A) serum testosterone levels and (B) sperm counts were observed in mice exposed daily to 10 mg/kg PFOS for 21 days. * $P < 0.005$, # $P < 0.01$ compared with the control according to the results of one-way ANOVA followed by Duncan multiple-range test.

estrogen and PCBs have been demonstrated [35–37]. In addition to GH and IGF1, the local factors inhibins and activins are known to regulate the secretion of FSH, and to mediate Sertoli cell proliferation [27, 38–40]. Inhibins A and B are heterodimers of an α subunit, encoded by *Inha*, and a β subunit (β_A or β_B), encoded by *Inhba* or *Inhbb*, that can

FIG. 3. Effects of PFOS on the expression levels of hormones and receptors in the hypothalami and pituitaries of mice after 14 and 21 days of exposure. Gene expression levels of (A) hypothalamic hormones/receptors and (B) pituitary hormones/receptors of the mice are shown. No significant changes in the gene expression levels were detected in the hypothalami and pituitaries of the control (Ctrl) and the PFOS-exposed mice. GnRH, gonadotropin-releasing hormone.



stimulate testosterone production [27, 41]. Activin is a homodimer or heterodimer of β subunits ($\beta_A\beta_A$, $\beta_B\beta_B$, $\beta_A\beta_B$) that acts as a local factor to stimulate testicular development [38]. The β_C subunit, encoded by *Inhbc*, can act as a dominant negative regulator of activin A [42]. In the present study, the mRNA expression levels of β_C were significantly reduced in the testes of the PFOS-exposed mice after 14 days of the treatment, but they went back to normal after 21 days of the treatment. Moreover α , β_A , and β_B subunits of inhibins were significantly decreased in the testes of mice exposed daily to 5 or 10 mg/kg PFOS for 21 days. Studies have shown the reciprocal pattern of β_A and β_C expression [27, 43]. Although the role of the β_C subunit in testicular function has remained elusive, the reduction in its expression levels may indicate a compensatory mechanism occurring at early PFOS exposure to increase activin A signaling for the stimulation of Sertoli cell proliferation and FSH secretion. At the later stage of PFOS exposure, the downregulation of α , β_A , and β_B subunit expressions reduced the levels of inhibins ($\alpha\beta_A$, $\alpha\beta_B$) and activins ($\beta_A\beta_A$, $\beta_B\beta_B$, $\beta_A\beta_B$), and was manifested by the reduction of serum testosterone and epididymal sperm counts. Therefore, in addition to the reduced expression levels of testicular gonadotropin receptors, our data reveal for the first time that the decreased expression levels of GHR, IGF1R, inhibins, and activins may contribute to the negative influence of PFOS on male fertility.

In this study most of the measured hormonal factors are involved in the regulation of testicular steroidogenesis [27, 44, 45]. The significant reductions of their respective signaling cascades could decrease steroidogenesis. Consistently significant reductions in the expressions of mRNA encoding for

steroidogenic enzymes, including StAR, CYP11A1, CYP17A1, 3 β -HSD, and 17 β -HSD, were observed in the testes of mice exposed daily to 5 or 10 mg/kg PFOS. These effects could lead to lesser concentrations of testosterone in the blood serum. Other studies have suggested that the lower serum testosterone levels in rodents exposed to PFOS could be due to a decrease in serum cholesterol concentration [11, 16, 46]. However, in the present study, it was found that PFOS can inhibit steroidogenic enzymes in the testes. This result is similar to that observed in the male rats exposed to perfluorododecanoic acids (0.5 mg/kg daily) for 110 days [30]. A noteworthy study has proposed that steroidogenesis is a major target for endocrine-disrupting chemicals [47]. Zhao et al. [48] demonstrated that PFOS inhibited the activities of rat HSD3B and human HSD17B using testicular microsomal preparations. The data support the notion that the inhibition of steroidogenic enzyme activity is a contributing factor to the inhibitory effects of PFOS on testicular steroid production.

Collectively, the results of this study demonstrated the negative effects of PFOS on the fertility of male mice. The underlying mechanisms leading to this consequence were the disruptions of normal functioning on testicular signaling. Although both 5 and 10 mg/kg daily PFOS exerted similar inhibitory effects on the expression levels of the testicular steroidogenic enzymes and LHR, the significant reductions in serum testosterone levels and epididymal sperm counts were only revealed in the highest-dose group. This difference in the biological outcomes is probably attributed to the significantly lower expression levels of testicular GHR, IGF1R, FSHR, and INHBA in the mice treated daily with 10 mg/kg PFOS. Thus, the correlative observations of the significant reductions in the

Gene expression levels in testes

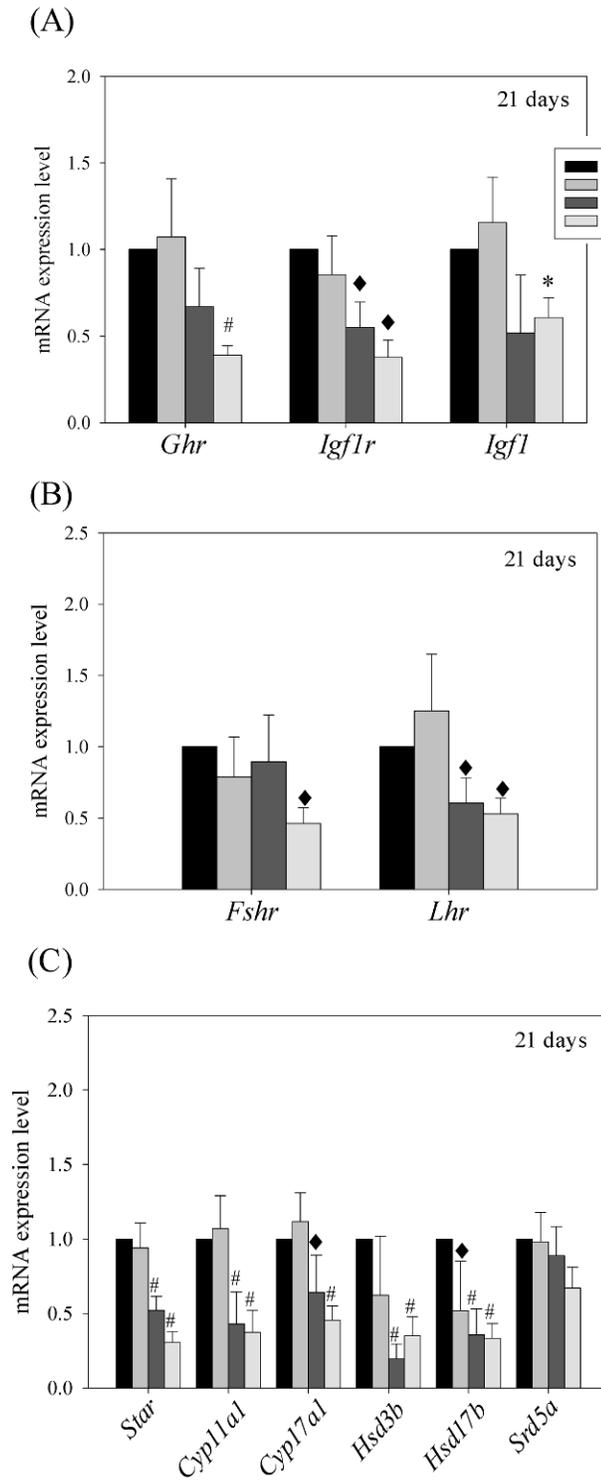


FIG. 4. Effects of PFOS on the expression levels of hormones, receptors, and steroidogenic enzymes in the testes of mice after 21 days of the exposure. Expressions of mRNA for (A) growth hormone receptor (*Ghr*); insulin-like growth factor 1 (*Igf1*) and its receptor (*Igf1r*); (B) follicle-stimulating hormone (*Fshr*) and luteinizing hormone (*Lhr*) receptors; and (C) steroidogenic enzymes of testes are shown. * $P < 0.001$, # $P < 0.01$, ♦ $P < 0.05$ compared with the respective control according to the results of one-way ANOVA followed by Duncan multiple-range test.

Gene expression levels in testes

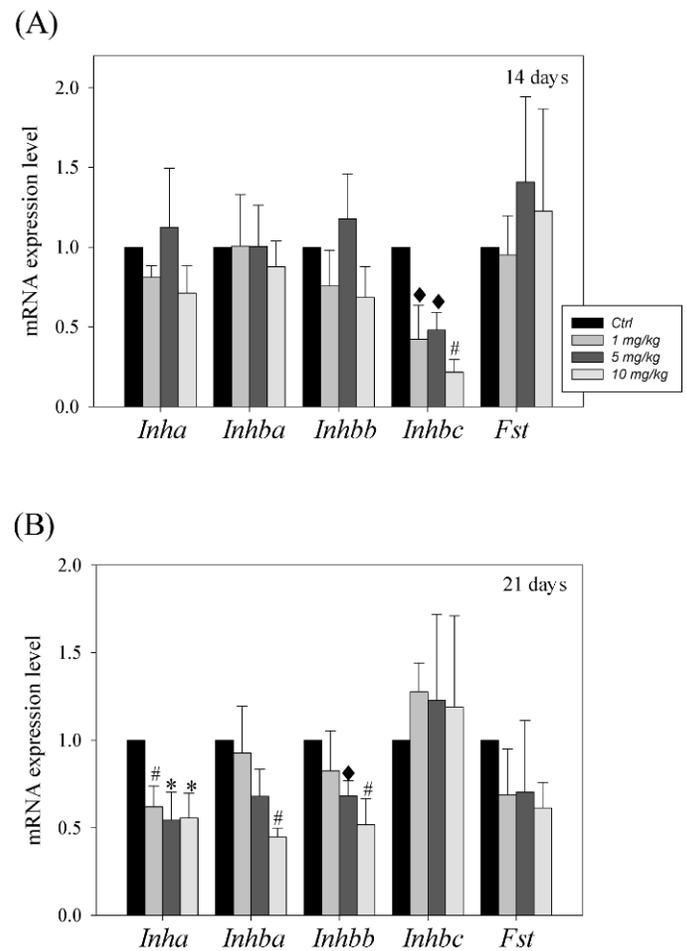


FIG. 5. Effects of PFOS exposure on the mRNA expression levels of inhibin subunits and follistatin in the testes of the mice after (A) 14 and (B) 21 days of exposure. * $P < 0.001$, # $P < 0.01$, ♦ $P < 0.05$ compared with the respective control according to the results of one-way ANOVA followed by Duncan multiple-range test.

expression levels of receptors for gonadotropin, GH, IGF1, and the local factors, inhibins and activins, in the testes of the PFOS-exposed mice may lead to an impairment of testicular steroidogenesis and spermatogenesis. These effects then resulted in less testosterone in the blood serum and a lower number of sperm in the epididymis. Although significant effects of PFOS on male subfertility are identified, the doses used in this study are higher than the tolerable daily intake dosage for the general public. In considering the factor of the body surface area normalization between human and mouse [49], the highest dose (10 mg/kg daily) of PFOS used in this study would be around 10-fold and 300-fold higher, respectively, than the reported occupational and general public exposure levels. At the lowest dose (1 mg/kg daily) of PFOS exposure, the dose is about 30-fold higher than the human equivalent dose of exposure for general public. Although at the lowest dosage we did not observe a noticeable reduction in serum testosterone levels and epididymal sperm counts, significant reductions in the expression levels of the testicular inhibin subunits, INHA and INHBC, and the steroidogenic enzyme 17 β -HSD were observed. Taking into account the

synergistic and/or additive effects of human exposure to multiple endocrine disruptors [50], the data found in this study are still relevant for human exposure. The elucidation of these mechanisms is useful in predicting effects of mixtures of PFCs and in selecting potential biomarkers of exposure to PFCs.

REFERENCES

- Giesy JP, Kannan K. Global distribution of perfluorooctane sulfonate in wildlife. *Environ Sci Technol* 2001; 35:1339–1342.
- Giesy JP, Kannan K. Perfluorochemical surfactants in the environment. *Environ Sci Technol* 2002; 36:146A–152A.
- Dreyer A, Weinberg I, Temme C, Ebinghaus R. Polyfluorinated compounds in the atmosphere of the Atlantic and Southern Oceans: evidence for a global distribution. *Environ Sci Technol* 2009; 43:6507–6514.
- Nakayama SF, Strynar MJ, Reiner JL, Delinsky AD, Lindstrom AB. Determination of perfluorinated compounds in the upper Mississippi river basin. *Environ Sci Technol* 2010; 44:4103–4109.
- Jensen AA, Leffers H. Emerging endocrine disruptors: perfluoroalkylated substances. *Int J Androl* 2008; 31:161–169.
- Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME, Butenhoff JL, Stevenson LA. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. *Toxicol Sci* 2003; 74:382–392.
- Olsen GW, Church TR, Miller JP, Burriss JM, Hansen KJ, Lundberg JK, Armitage JB, Herron RM, Medhizadehkashi Z, Nobiletta JB, O'Neill EM, Mandel JH, et al. Perfluorooctanesulfonate and other fluorochemicals in the serum of American Red Cross adult blood donors. *Environ Health Perspect* 2003; 111:1892–1901.
- So MK, Yamashita N, Taniyasu S, Jiang Q, Giesy JP, Chen K, Lam PK. Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. *Environ Sci Technol* 2006; 40:2924–2929.
- Beach SA, Newsted JL, Coody K, Giesy JP. Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS). *Rev Environ Contam Toxicol* 2006; 186:133–174.
- Johansson N, Eriksson P, Viberg H. Neonatal exposure to PFOS and PFOA in mice results in changes in proteins which are important for neuronal growth and synaptogenesis in the developing brain. *Toxicol Sci* 2009; 108:412–418.
- Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicol Sci* 2007; 99:366–394.
- Rosen MB, Schmid JE, Das KP, Wood CR, Zehr RD, Lau C. Gene expression profiling in the liver and lung of perfluorooctane sulfonate-exposed mouse fetuses: comparison to changes induced by exposure to perfluorooctanoic acid. *Reprod Toxicol* 2009; 27:278–288.
- Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Barbee BD, Richards JH, Butenhoff JL, Stevenson LA, Lau C. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations. *Toxicol Sci* 2003; 74:369–381.
- Hasegawa R, Koizumi M, Hirose A. Principles of risk assessment for determining the safety of chemicals: recent assessment of residual solvents in drugs and di(2-ethylhexyl) phthalate. *Congenit Anom (Kyoto)* 2004; 44: 51–59.
- Joensen UN, Bossi R, Leffers H, Jensen AA, Skakkebaek NE, Jorgensen N. Do perfluoroalkyl compounds impair human semen quality? *Environ Health Perspect* 2009; 117:923–927.
- Martin MT, Brennan RJ, Hu W, Ayanoglu E, Lau C, Ren H, Wood CR, Corton JC, Kavlock RJ, Dix DJ. Toxicogenomic study of triazole fungicides and perfluoroalkyl acids in rat livers predicts toxicity and categorizes chemicals based on mechanisms of toxicity. *Toxicol Sci* 2007; 97:595–613.
- Qazi MR, Nelson BD, Depierre JW, Abedi-Valugerdi M. 28-Day dietary exposure of mice to a low total dose (7 mg/kg) of perfluorooctanesulfonate (PFOS) alters neither the cellular compositions of the thymus and spleen nor humoral immune responses: does the route of administration play a pivotal role in PFOS-induced immunotoxicity? *Toxicology* 2010; 267: 132–139.
- Bjork JA, Lau C, Chang SC, Butenhoff JL, Wallace KB. Perfluorooctane sulfonate-induced changes in fetal rat liver gene expression. *Toxicology* 2008; 251:8–20.
- Wang F, Liu W, Jin Y, Dai J, Yu W, Liu X, Liu L. Transcriptional effects of prenatal and neonatal exposure to PFOS in developing rat brain. *Environ Sci Technol* 2010; 44:1847–1853.
- Castellano JM, Roa J, Luque RM, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M. KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. *Peptides* 2009; 30:139–145.
- Pineda R, Aguilar E, Pinilla L, Tena-Sempere M. Physiological roles of the kisspeptin/GPR54 system in the neuroendocrine control of reproduction. *Prog Brain Res* 2010; 181:55–77.
- Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. *Hum Reprod Update* 2009; 15:203–212.
- Sairam MR, Krishnamurthy H. The role of follicle-stimulating hormone in spermatogenesis: lessons from knockout animal models. *Arch Med Res* 2001; 32:601–608.
- O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH. Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. *Mol Cell Endocrinol* 2009; 306:2–8.
- Heckert LL, Griswold MD. The expression of the follicle-stimulating hormone receptor in spermatogenesis. *Recent Prog Horm Res* 2002; 57: 129–148.
- Misrahi M, Beau I, Meduri G, Bouvattier C, Atger M, Loosfelt H, Ghinea N, Hai MV, Bougneres PF, Milgrom E. Gonadotropin receptors and the control of gonadal steroidogenesis: physiology and pathology. *Baillieres Clin Endocrinol Metab* 1998; 12:35–66.
- Barakat B, O'Connor AE, Gold E, de Kretser DM, Loveland KL. Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction* 2008; 136:345–359.
- Rocha JS, Bonkowski MS, de Franca LR, Bartke A. Effects of mild calorie restriction on reproduction, plasma parameters and hepatic gene expression in mice with altered GH/IGF-I axis. *Mech Ageing Dev* 2007; 128:317–331.
- Rosenfeld RG. Endocrinology of growth. *Nestle Nutr Workshop Ser Pediatr Program* 2010; 65:225–234.
- Shi Z, Ding L, Zhang H, Feng Y, Xu M, Dai J. Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. *Toxicol Lett* 2009; 188:192–200.
- Berenshtein EB, Baquedano MS, Pepe CM, Costanzo M, Saraco NI, Ponzio R, Rivarola MA, Belgorosky A. Role of IGFs and insulin in the human testis during postnatal activation: differentiation of steroidogenic cells. *Pediatr Res* 2008; 63:662–666.
- N'Diaye MR, Sun SS, Fanua SP, Loseth KJ, Shaw Wilgis EF, Crabo BG. Growth hormone receptors in the porcine testis during prepuberty. *Reprod Domest Anim* 2002; 37:305–309.
- Chandrashekar V, Bartke A, Coschigano KT, Kopchick JJ. Pituitary and testicular function in growth hormone receptor gene knockout mice. *Endocrinology* 1999; 140:1082–1088.
- Keene DE, Suescun MO, Bostwick MG, Chandrashekar V, Bartke A, Kopchick JJ. Puberty is delayed in male growth hormone receptor gene-disrupted mice. *J Androl* 2002; 23:661–668.
- Fernie KJ, Shutt JL, Letcher RJ, Ritchie IJ, Bird DM. Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environ Sci Technol* 2009; 43: 2124–2130.
- Huang DS, O'Sullivan AJ. Short-term oral oestrogen therapy dissociates the growth hormone/insulin-like growth factor-I axis without altering energy metabolism in premenopausal women. *Growth Horm IGF Res* 2009; 19:162–167.
- Cocchi D, Tulipano G, Colciago A, Sibilia V, Pagani F, Vigano D, Rubino T, Parolaro D, Bonfanti P, Colombo A, Celotti F. Chronic treatment with polychlorinated biphenyls (PCB) during pregnancy and lactation in the rat: part 1: effects on somatic growth, growth hormone-axis activity and bone mass in the offspring. *Toxicol Appl Pharmacol* 2009; 237:127–136.
- Risbridger GP, Cancilla B. Role of activins in the male reproductive tract. *Rev Reprod* 2000; 5:99–104.
- de Kretser DM, Buzzard JJ, Okuma Y, O'Connor AE, Hayashi T, Lin SY, Morrison JR, Loveland KL, Hedger MP. The role of activin, follistatin and inhibin in testicular physiology. *Mol Cell Endocrinol* 2004; 225:57–64.
- Casagrandi D, Bearfield C, Geary J, Redman CW, Muttukrishna S. Inhibin, activin, follistatin, activin receptors and beta-glycan gene expression in the placental tissue of patients with pre-eclampsia. *Mol Hum Reprod* 2003; 9:199–203.
- Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW, Vale W. Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci U S A* 1987; 84:5082–5086.
- Mellor SL, Ball EM, O'Connor AE, Ethier JF, Cranfield M, Schmitt JF, Phillips DJ, Groome NP, Risbridger GP. Activin betaC-subunit heterodi-

- mers provide a new mechanism of regulating activin levels in the prostate. *Endocrinology* 2003; 144:4410–4419.
43. Butler CM, Gold EJ, Risbridger GP. Should activin betaC be more than a fading snapshot in the activin/TGFbeta family album? *Cytokine Growth Factor Rev* 2005; 16:377–385.
 44. Hu GX, Lin H, Chen GR, Chen BB, Lian QQ, Hardy DO, Zirkin BR, Ge RS. Deletion of the *Igf1* gene: suppressive effects on adult Leydig cell development. *J Androl* 2010; 31:379–387.
 45. Milardi D, Giampietro A, Baldelli R, Pontecorvi A, De ML. Fertility and hypopituitarism. *J Endocrinol Invest* 2008; 31:71–74.
 46. Austin ME, Kasturi BS, Barber M, Kannan K, MohanKumar PS, MohanKumar SM. Neuroendocrine effects of perfluorooctane sulfonate in rats. *Environ Health Perspect* 2003; 111:1485–1489.
 47. Sanderson JT. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicol Sci* 2006; 94:3–21.
 48. Zhao B, Hu GX, Chu Y, Jin X, Gong S, Akingbemi BT, Zhang Z, Zirkin BR, Ge RS. Inhibition of human and rat 3beta-hydroxysteroid dehydrogenase and 17beta-hydroxysteroid dehydrogenase 3 activities by perfluoroalkylated substances. *Chem Biol Interact* 2010; 188:38–43.
 49. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 2008; 22:659–661.
 50. Rhind SM. Anthropogenic pollutants: a threat to ecosystem sustainability? *Philos Trans R Soc Lond B Biol Sci* 2009; 364:3391–3401.