TOXICOLOGICAL SCIENCES, 162(1), 2018, 318–326

doi: 10.1093/toxsci/kfx261
Advance Access Publication Date: November 20, 2017
Research Article

Exposure to Concentrated Ambient $PM_{2.5}$ Compromises Spermatogenesis in a Mouse Model: Role of Suppression of Hypothalamus-Pituitary-Gonads Axis

Lianglin Qiu,[*,†] Minjie Chen,[*,‡] Xiaoke Wang,[*,†] Xiaobo Qin,[*,§] Sufang Chen,[*,¶] Yingyun Qian,[†] Zhenzhen Liu,[†] Qi Cao,[‖] and Zhekang Ying[*,‡,1]

[*]Department of Medicine Cardiology Division, School of Medicine, University of Maryland, Baltimore, Maryland 21210; [†]School of Public Health, Nantong University, Nantong, Jiangsu 226019, P.R. China; [‡]Department of Environmental Health, School of Public Health, Fudan University, Shanghai 200032, P.R. China; [§]The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi 530021, P.R. China; [¶]Department of Geriatric Endocrinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China; and [‖]Department of Diagnostic Radiology and Nuclear Medicine, School of Medicine, University of Maryland, Baltimore, Maryland 21210

[1]To whom correspondence should be addressed at Department of Medicine Cardiology Division, School of Medicine, University of Maryland, 20 Penn St. HSFII S022, Baltimore, MD 21201. Fax: (410) 328 1048. E-mail: zying@medicine.umaryland.edu;yingzhekang@hotmail.com.

ABSTRACT

Epidemiological studies link ambient fine particulate matter ($PM_{2.5}$) pollution to abnormalities in the male reproductive system. However, few toxicological studies have investigated this potentially important adverse effect of $PM_{2.5}$ pollution. Therefore, in the present study, we analyzed the effects of $PM_{2.5}$ exposure on spermatogenesis and hypothalamic-pituitary-gonadal (HPG) axis in a murine model.Fourteen male C57BL/6J mice were subjected to a 4-month exposure to filtered air or concentrated ambient $PM_{2.5}$ (CAP). Their sperm count, testicular histology, spermatogenic parameters, and the major components of HPG axis were assessed.Exposure to CAP significantly reduced sperm count in the epididymis. This was accompanied by Sertoli cell vacuolization, immature germ cell dislocation, and decreases in pachytene spermatocytes and round spermatids of stage VII seminiferous tubules, suggesting a marked impairment of spermatogenesis in these mice. This impairment of spermatogenesis appeared to be attributable to a suppression of HPG axis subsequent to CAP exposure-induced hypothalamic inflammation, as exposure to CAP significantly increased *TNFα* and *IL1b* mRNA levels and meanwhile decreased gonadotropin-releasing hormone mRNA expression in the hypothalamus. Moreover, CAP exposure significantly reduced circulating testosterone and follicle-stimulating hormone, testicular testosterone and mRNA expression of follicle-stimulating hormone target gene *SHBG* and luteinizing hormone target genes *P450scc*, *17βHSD*, and *StAR*.The present data demonstrate that exposure to ambient $PM_{2.5}$ impairs spermatogenesis in murine model, raising the concern over effects of ambient $PM_{2.5}$ pollution on the male reproductive function.

Key words: ambient fine particulate matter ($PM_{2.5}$); inflammation; hypothalamic-pituitary-gonadal axis; spermatogenesis; male reproductive system.

Ambient fine particulate matter ($PM_{2.5}$) pollution is one of the leading challenges for the global public health. It has been well known to cause various adverse cardiopulmonary effects (Feng *et al.*, 2016). However, while the male reproductive system is known to be susceptible to environmental pollution (Sifakis *et al.*, 2017), the effects of exposure to ambient $PM_{2.5}$ on the male

reproductive system have not yet been systemically investigated. Epidemiological studies have shown that ambient $PM_{2.5}$ levels are positively associated with abnormalities in sperm morphology (Hansen *et al.*, 2009) and negatively associated with sperm count, motility, and testosterone levels (Hammoud *et al.*, 2010; Jurewicz *et al.*, 2015; Zhou *et al.*, 2014). Consistently, intratracheal instillation of ambient $PM_{2.5}$ or inhalation exposure to diesel exhaust significantly increases abnormal sperms and decreases sperm count and testosterone levels in rats (Cao *et al.*, 2015; Watanabe and Oonuki, 1999). Collectively, these studies have suggested that exposure to ambient $PM_{2.5}$ impacts the male reproductive system, warranting further studies to systemically document these effects.

The hypothalamic-pituitary-gonadal (HPG) axis is central in the regulation of development of male reproductive system and spermatogenesis, which mainly includes gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus by GnRH-expressing neurons, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) produced by the anterior portion of the pituitary gland, and estrogen and testosterone produced by the gonads (Corradi *et al.*, 2016). GnRH controls the synthesis and secretion of LH and FSH by the anterior pituitary cells in a periodic pulsatile manner (Corradi *et al.*, 2016; Jin and Yang, 2014; Thackray *et al.*, 2010). Due to its special model of action, circulating GnRH level is very low and is rarely taken as a biomarker of HPG axis activity (Mortimer and Yeo, 1976; Tsutsumi and Webster, 2009). LH in turn controls the biosynthesis of testosterone by the Leydig cells through regulating the expression of various genes involved in testosterone biosynthesis and transportation (Jin and Yang, 2014), and FSH regulates the bioavailability of testosterone through regulation on the expression of sex hormone binding globulin (SHGB) by Sertoli cells (Corradi *et al.*, 2016). In addition, FSH is also crucial for maintaining the homeostasis of microenvironment of spermatogenesis by Sertoli cells (Corradi *et al.*, 2016; Jin and Yang, 2014). As the most downstream component of HPG axis, testosterone is essential for the development of male reproductive system and spermatogenesis. FSH together with testosterone regulates the maturation of sperm cells. Impaired testosterone production has been shown to result in various male reproductive dysfunction such as low sperm count, abnormal sperm morphology and even infertility (Ohlander *et al.*, 2016).

Notably, the HPG axis is well known to be vulnerable to various environmental pollution (Kay *et al.*, 2014; Qiu *et al.*, 2013; Rochester, 2013). However, whether it is targeted by $PM_{2.5}$ exposure has not been systemically investigated. In the present study, male C57Bl/6J mice were exposed to concentrated ambient $PM_{2.5}$ (CAP) or filtered air (FA) using a whole-body exposure system that mimic real-world exposure to air pollution (Ying *et al.*, 2014), and effects of CAP exposure on sperm count, major hormones of HPG axis, testicular histology, and mRNA expression of testosterone biosynthesis genes were analyzed. Our results showed that CAP exposure significantly decreased sperm count, testicular germ cells, and circulating FSH and testosterone levels, and hypothalamic GnRH mRNA levels, strongly supporting that exposure to ambient $PM_{2.5}$ impacts the male reproductive function.

MATERIALS AND METHODS

Institution animals. University of Maryland, Baltimore (UMB) is an AAALAC accredited. All procedures of this study were approved by the Institutional Animal Care and Use Committee (IACUC) at UMB, and all animals were treated humanely and with regard for alleviation of suffering. Fourteen male C57Bl/6J mice (7-week-old) were obtained from Jackson Laboratories (Bar Harbor, Maine). After 1-week-adaption, these mice (7 per group) were subjected to exposure to FA or CAP from May 2016 to September 2016 for a total duration of 4 months (sufficient to induce various adverse cardiopulmonary effects as per our previous studies) (Ying *et al.*, 2009) in a mobile trailer with a 12-h light/12-h dark cycle, temperatures of 18–25 °C, and relative humidity of 40–60%. The mobile trailer was located on the campus of the University of Maryland, Baltimore. Animal exposure and the monitoring of exposure atmosphere and ambient aerosol were performed as previously described using a versatile aerosol concentration enrichment system that was modified for long-term exposures (Ying *et al.*, 2014). The average $PM_{2.5}$ concentrations in the FA and CAP chambers during this 4-month period were 3.6 ± 1.9 and $71.6 \pm 33.2\ \mu g/m^3$, respectively. Given that the exposure protocol comprised exposures for 6 h/day, 5 days/week (no exposure took place during weekends), the average applied $PM_{2.5}$ concentration during this period was $12.8\ \mu g/m^3$, which is slightly above the US National Ambient Air Quality Standard ($12\ \mu g/m^3$) (Agency, 2013), but very common in areas with heavy pollution such as Beijing, China.(Guo *et al.*, 2017) The elemental composition of CAP was determined as previously described (Wang *et al.*, 2017), and presented in Table 1. All mouse euthanasia and tissue collection were performed on the day next to the last exposure.

Sperm counting and weighing of testis and epididymis. The sperm counting and weighing of testis and epididymis were conducted as previously described (Qiu *et al.*, 2016). Briefly, freshly isolated testes and epididymis were weighted and then normalized to the animal's bodyweight to obtain the organ coefficients of testis and epididymis. For sperm counting, the left epididymis was placed in 1-ml normal saline then 6 deep cuts were made in each cauda with microscissors to release sperm into the media at 35 °C for 10 min. Subsequently, the suspension was filtered with nylon mesh (pore size: 70 μm). After staining with 0.25% eosin Y (containing 0.5% formalin), the numbers of sperm were counted by using a Neubaur counting chamber and corrected by normalizing to the epididymal weight, which was expressed as 10^6 per epididymal weight.

Testicular pathological analysis and spermatogenetic parameters. The testicular histology and spermatogenetic parameters analysis were conducted as previously described (Qiu *et al.*, 2013). Briefly, fresh isolated testes were fixed in Bouin fixation fluid and then processed for dehydration, paraffin embedding. About 5-μm thick paraffin sections were made and stained with hematoxylin-eosin. Pictures covering all the testicular tissues of 2 consecutive sections of each testes ($n = 7$ per group) were taken and analyzed by a pathologist who was blind to the grouping of samples as previously described (Ahmed and de Rooij, 2009; Creasy, 1997). Briefly, all round tubes (the ratio of long- to short-axis diameter < 1.2, approximately 200 per section) were identified. Of them, all tubes at the VII stage (approximately 20 per section) were further identified as previously described (Ahmed and de Rooij, 2009), and subjected to assessments of average diameter, epithelial height, numbers of germ cells, and Sertoli cells. The tubule diameters of these round seminiferous tubules (stage VII) were remeasured as previously defined (Li *et al.*, 2012). In the same tubule, height of seminiferous epithelium (from the membrane base to the tubular lumen in 2 different areas) was assessed. The crude testicular cells count (nuclear count) of the germ cells (spermatogonia, pachytene

Table 1. The Average Concentration of Elements Detected in PM2.5

Element	Mean ± SD (ng/m^3)	Percentage (%)
Na	35.14 ± 19.66	6.45
Mg	8.62 ± 4.81	1.58
Al	25.93 ± 12.67	4.76
Si	78.53 ± 39.39	14.42
P	1.00 ± 0.50	0.18
S	249.91 ± 183.42	45.88
K	20.38 ± 11.57	3.74
Ca	39.30 ± 19.86	7.21
Ti	3.55 ± 2.09	0.65
V	0.26 ± 0.15	0.05
Cr	0.28 ± 0.15	0.05
Mn	1.19 ± 0.75	0.22
Fe	47.19 ± 30.10	8.66
Ni	0.65 ± 0.40	0.12
Cu	1.45 ± 0.93	0.27
Zn	13.77 ± 6.82	2.53
As	0.19 ± 0.10	0.03
Se	0.13 ± 0.06	0.02
Br	8.42 ± 5.22	1.55
Sr	0.24 ± 0.13	0.04
Ag	0.55 ± 0.19	0.10
Sn	1.25 ± 0.53	0.23
Ba	1.66 ± 1.16	0.31
Ce	0.24 ± 0.25	0.04
Pr	0.30 ± 0.13	0.05
Er	0.98 ± 0.75	0.18
Lu	1.33 ± 1.19	0.24
W	0.41 ± 0.26	0.08
Ir	0.11 ± 0.04	0.02
Pt	0.20 ± 0.09	0.04
Au	0.22 ± 0.14	0.04
Hg	0.34 ± 0.05	0.06
Tl	0.11 ± 0.04	0.02
Pb	0.93 ± 0.32	0.17

spermatocytes, and round spermatids) and Sertoli cells in stage VII tubules were recorded (Abercrombie, 1946; Ahmed and de Rooij, 2009; Marchlewska *et al.*, 2011). Subsequently, the true count was determined after the crude count values were corrected with the respective nuclear diameter of each cell type and the section thickness using a corrected formula, true count = crude count × section thickness/(section thickness + nuclear diameter of cells) (George *et al.*, 1996; Marchlewska *et al.*, 2011). The numbers of Sertoli cells were expressed as true counts of Sertoli cells per seminiferous tubule, and the numbers of germ cells were expressed as true counts of each germ cell per Sertoli cell (germ cell/Sertoli cell ratios).

Immunofluorescence analysis. Immunofluorescence analysis was conducted as previously described (Qiu *et al.*, 2016). Briefly, testicular samples were fixed Bouin fixation fluid and then processed for dehydration, paraffin embedding. About 5-μm thick paraffin sections were made and incubated with goat serum (containing 0.5% Triton X-100) followed by incubating with the primary antibodies including Tra98, CREB1, and F-actin at 4 °C overnight. After washing, the sections were incubated with fluorescent-labeled secondary antibodies for 40 min at a dark place. The localizations of these proteins in seminiferous tubules were detected by a confocal fluorescence microscopy (Zeiss, Jena, Germany). Two slides (each including 3 sections) per testis ($n = 7$ per group) were analyzed and quantitated. The quantitation results and 1 representative image were presented.

Enzyme-linked immunosorbent assay. Serum and testicular hormones including LH, FSH, testosterone (T), and estradiol (E2) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (LH, CSB-E12770m, CUSABIO, Maryland; FSH, LH-E10075MU, LIUHEBIO, Wuhan, China; T, IB79174, Minnesota; E2, ES180S-100, CALBIOTECH, California) according to the manufacturer directions. The sensitivities of the assay were 0.5 mIU/ml for LH, 0.064 mIU/ml for FSH, 0.066 ng/ml for T and10 pg/ml for E2, respectively. The inter- and intraexperiment coefficients of variation were <15% for LH, 5% for FSH, 6.5% and 11.3% for T, and 5% for E2, respectively. No replicates were used for the assessments of reproductive hormones in serum and testicular samples ($n = 7$ per group) due to the limited amount of each sample.

Real-time PCR. Primers in this study were designed using Primer Premier 5.0 software based on GenBank sequence of target genes including androgen receptor (AR), cytochrome P450 CHOL side-chain cleavage enzyme (P450scc), cytochrome P450 aromatase (P450arom), cytochrome P450 family 17α (CYP17α), 17β-hydroxysteroid dehydrogenase (HSD17β), 3β-hydroxysteroid dehydrogenase (HSD3β), steroidogenic acute regulatory protein (StAR), sex hormone binding globulin (SHGB), GnRH, and glyceraldehydes 3 phosphate dehydrogenase (GAPDH) and synthesized by Sigma-Aldrich (Saint Louis, Missouri), which was listed in Table 2. Total RNA was isolated from testicular or hypothalamic tissues of animals described above with TRIzol (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. RNA was reverse-transcripted in cDNA using a Revert Aid First Stand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Quantitative polymerase chain reaction (qPCR) was performed as previously described using a lightcycler 480 (Roche, Mannheim, Germany) (Qiu *et al.*, 2013). Reactions were carried out using a FastStart Universal SYBR Green Master (ROX) system (Roche, Indianapolis, Indiana). All qPCRs for the relative gene expression in the hypothalamic and testicular samples ($n = 7$ per group) were performed in triplicate, and the specificity of the PCR products was confirmed using melting curve analyses. Relative gene expression levels, normalized to GAPDH expression, were calculated as $2^{-\Delta\Delta Ct}$. Gene expression levels in the treatment group are expressed as a percentage compared with the control groups.

Statistical analysis. The results are expressed as mean ± SD. Statistical significance was assessed using t or χ^2 test by SPSS 17.0 software (Chicago, Illinois). Values of $p < .05$ were considered statistically significant.

RESULTS

Exposure to CAP Reduces Sperm Count in the Epididymis

To examine the effects of chronic exposure to ambient $PM_{2.5}$ on the male reproductive system, we exposed male C57Bl/6J mice to FA or CAP for 4 months. During the exposure period, the average $PM_{2.5}$ concentrations in the FA and CAP chambers were 3.6 ± 1.9 and 71.6 ± 33.2 μg/m^3, respectively. The elemental composition of CAP was presented in Table 1, showing a relatively high ratio of Na/Al (reflecting a more contribution by the marine source than the crustal source and consistent with the geographic proximity of the study site to the ocean) and a relatively

Table 2. The Primers of the Analyzed Genes

Gene	Forward Primer	Reverse Primer
GAPDH	5′-TGAACGGGAAGCTCACTGG-3′	5′-TCCACCACCCTGTTGCTGTA-3′
AR	5′-CTGGGAAGGGTCTACCCAC-3′	5′-GGTGCTATGTTAGCGGCCTC-3′
P450scc	5′-AGGTCCTTCAATGAGATCCCTT-3′	5′-TCCCTGTAAATGGGGCCATAC-3′
P450arom	5′-ATGTTCTTGGAAATGCTGAACCC-3′	5′-AGGACCTGGTATTGAAGACGAG-3′
CYP17α	5′-GCCCAAGTCAAAGACACCTAAT-3′	5′-GTACCCAGGCGAAGAGAATAGA-3′
HSD17β	5′-ACTTGGCTGTTCGCCTAGC-3′	5′-GAGGGCATCCTTGAGTCCTG-3′
HSD3β	5′-CCTCCGCCTTGATACCAGC-3′	5′-TTGTTTCCAATCTCCCTGTGC-3′
StAR	5′-ATGTTCCTCGCTACGTTCAAG-3′	5′-CCCAGTGCTCTCCAGTTGAG-3′
SHGB	5′-TCTGCTGTTGCTACTACTGATGC-3′	5′-GGGCCATTGCTGAGGTACTTA-3′
GnRH	5′-AGCACTGGTCCTATGGGTTG-3′	5′-GGGGTTCTGCCATTTGATCCA-3′
TNFα	5′-TTCCGAATTCACTGGAGCCTCGAA-3′	5′-TGCACCTCAGGGAAGAATCTGGAA-3′
IL1β	5′-ACGGACCCCAAAAGATGAAG-3′	5′-TTCTCCACAGCCACAATGAG-3′
IL6	5′-ATCCAGTTGCCTTCTTGGGACTGA-3′	5′-TAAGCCTCCGACTTGTGAAGTGGT-3′

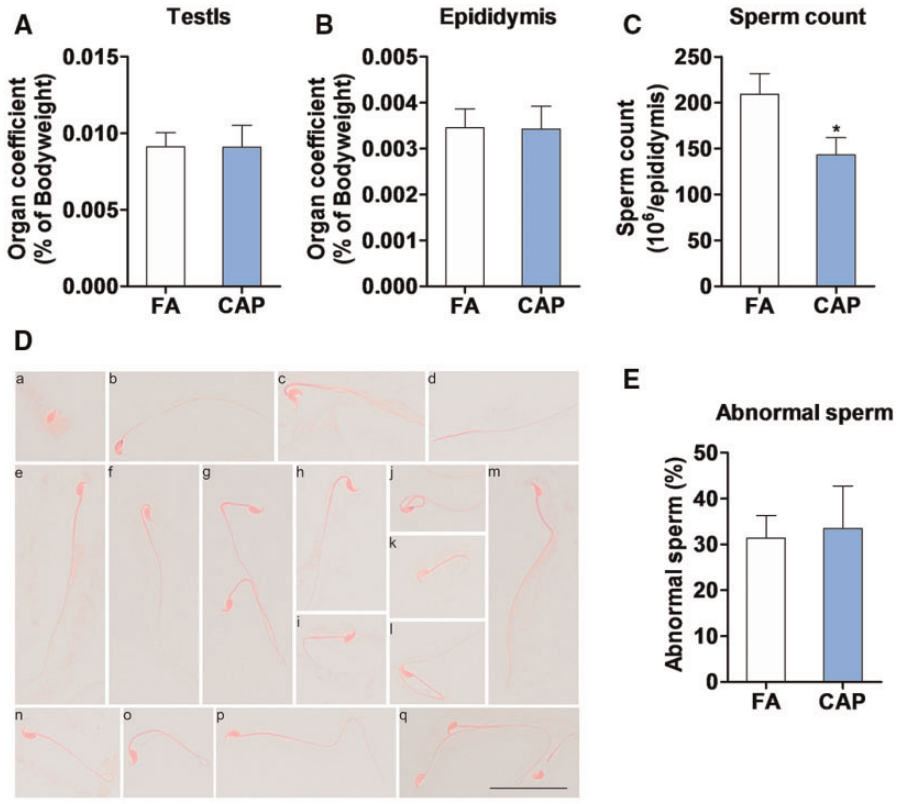

Figure 1. The effects of CAP exposure on sperm count and organ coefficients. A, Organ coefficient of testis. B, Organ coefficient of epididymis. C, Sperm count. D, Abnormal sperm morphology. A–D, Abnormal sperm head; e: normal sperm; f–m: abnormal sperm; n–q: abnormal sperm tail. E, Normal sperm rate. Bar: 20 μm. The data are expressed as the mean ± SD. $n = 7$ per group. *$p < .05$, compared with FA-treated group.

high sulfur (suggesting that the study site was most strongly affected by secondary aerosols) (Huang *et al.*, 1999; Wang *et al.*, 2017). Figures 1A and 1B demonstrate that this chronic exposure to CAP did not significantly change the weights of testis and epididymis. Consistent with previous studies (Cao *et al.*, 2015; Watanabe and Oonuki, 1999), exposure to CAP significantly decreased the sperm count in the epididymis (Figure 1C), strongly supporting that exposure to ambient $PM_{2.5}$ may impair the male reproductive system. To further document the effects of CAP exposure on the male reproductive system, we next analyzed the morphology of epididymal sperms. Unexpectedly, we did not observe any significant difference in the occurrence of abnormal sperm between FA- and CAP-exposed animals (Figs. 1D and 1E).

CAP Exposure Alters Mouse Testicular Histology

To delineate the mechanism whereby exposure to CAP reduces sperm count in the epididymis, the testes from these FA- or CAP-exposed mice were subjected to pathological assessments. Although no any marked abnormality in the structure and

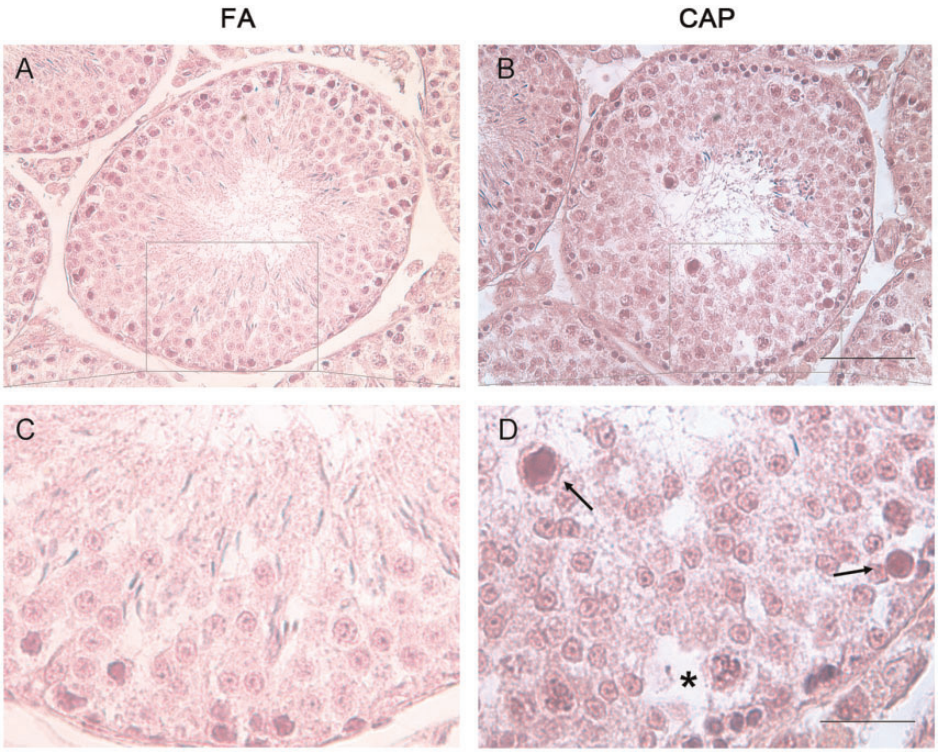

Figure 2. The effects of CAP exposure on testicular histology. A, A stage VII seminiferous tubule morphology in testis in control group. B, A stage VII seminiferous tubule morphology in testis in CAP-treated group. C, The magnification image of the selected area from (A). D, The magnification image of the selected area from (B). Black arrow head: immature germ cells. Asterisk: Sertoli cell vacuolization. $n = 7$ per group. Bar in the upper panel: 100 μm. Bar in the lower panel: 20 μm.

morphology of testis was observed in FA-exposed controls (Figs. 2A and 2C), we observed a significant increase in Sertoli cell vacuolization (0 in 7 FA-exposed mice vs 3 in 7 CAP-exposed mice, $p < .05$, χ^2 test), derangement of the cell layers (0 in 7 FA-exposed mice vs 4 in 7 CAP-exposed mice, $p < .05$, χ^2 test), and dislocated immature germ cells in the lumens of seminiferous tubules (0 in 7 FA-exposed mice vs 3 in 7 CAP-exposed mice, $p < .05$, χ^2 test) in CAP-exposed animals (Figs. 2B and 2D).

CAP Exposure Reduces Pachytene Spermatocytes and Round Spermatids

To define the effects of CAP exposure on spermatogenesis, we assessed the spermatogenetic parameters of the stage VII seminiferous tubules. Figures 3A–C show that exposure to CAP did not significantly influence their diameter, epithelial height, and the ratio of seminiferous tubules diameter/epithelial height. The count of Sertoli cells and spermatogonias in these seminiferous tubules was also comparable between FA- and CAP-exposed animals (Figs. 3D and 3E). In contrast, the average numbers of pachytene spermatocytes and round spermatids in each stage VII seminiferous tubule were significantly decreased in CAP-exposed animals (Figs. 3F and 3G). To confirm these effects of CAP exposure on pachytene spermatocytes and round spermatids, we normalized these results using the number of Sertoli cells. Figures 3J and 3K recapitulate these significant decreases in pachytene spermatocytes and round spermatids by chronic exposure to CAP.

CAP Exposure Reduces Germ Cells

To confirm the effects of CAP exposure on spermatogenesis, the number of germ cells in the stage VII seminiferous tubules was assessed by immunofluorescence using antibodies against Tra98, a specific marker of premeiotic germ cells (Yamaguchi *et al.*, 2013), and CREM1, a specific marker of postmeiotic germ cells (Nantel *et al.*, 1996). As shown in Figure 4, chronic exposure to CAP significantly decreased both premeiotic (Figs. 4A and 4B) and postmeiotic germ cells (Figs. 4C and 4D).

CAP Exposure Decreases Circulating Testosterone and FSH Levels

Reproductive hormones are central in the regulation of spermatogenesis (Mizrachi and Auchus, 2009). To test if CAP exposure impact spermatogenesis through alteration of reproductive hormone production, we assessed their levels in the plasma and testes. Figures 5A and 5B reveal that exposure to CAP significantly decreased plasma testosterone and FSH levels. In contrast, it did not significantly alter plasma estradiol level (Fig. 5C). Consistent with its effects in plasma, exposure to CAP reduced testicular testosterone and FSH levels (Figs. 5D–F). Due to the limited sensitivity of ELISA kit, we failed to determine the LH levels in the plasma and testis.

CAP Exposure Alters Testicular Expression of Genes in Testosterone Biosynthesis

To determine how CAP exposure reduces testosterone biosynthesis, we assess the testicular expression of AR, P450scc, CYP17α, P450arom, 17βHSD, 3βHSD, StAR, and SHBG mRNA. As shown in Figure 6, exposure to CAP significantly decreased the testicular mRNA levels of P450scc, 17βHSD, StAR, and SHBG (Figs. 6B, 6E, 6G, and 6H) but not AR, CYP17α, P450arom, and 3βHSD (Figs. 6A, 6C, 6D, and 6F).

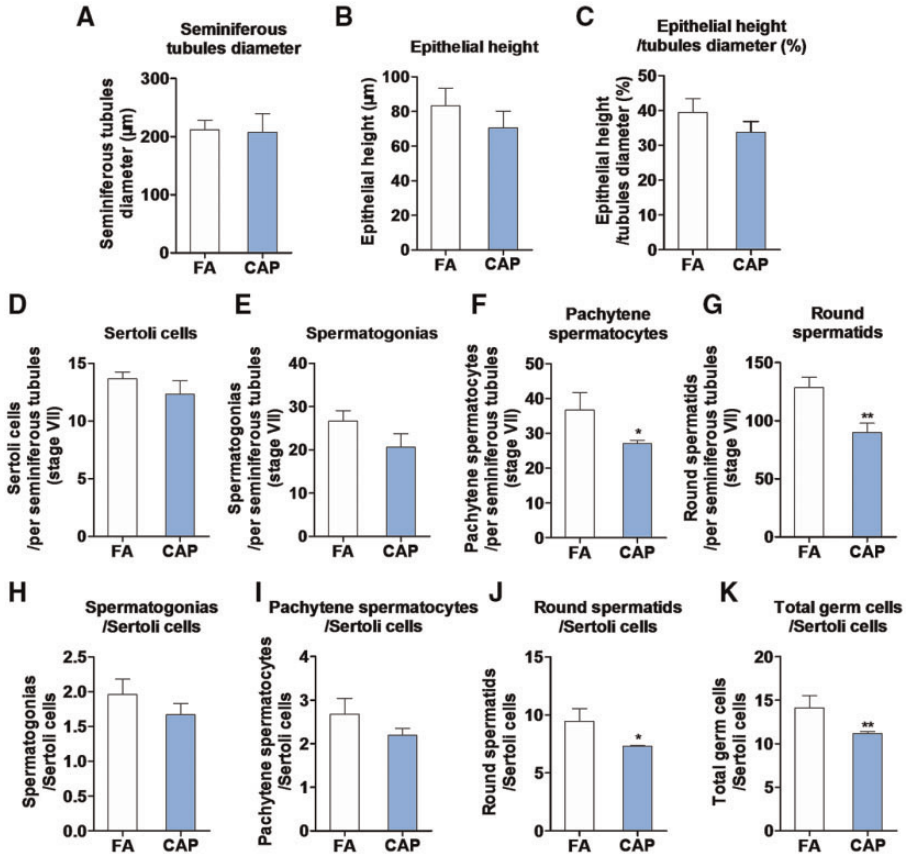

Figure 3. The effects of CAP exposure on spermatogenetic parameters. A, Seminiferous tubules diameter. B, Epithelial height. C, Ratio of seminiferous tubules diameter/epithelial height. D, The number of Sertoli cell per stage VII seminiferous tubule. E, The number of spermatogonias per stage VII seminiferous tubule. F, The number of pachytene spermatocyte per stage VII seminiferous tubule. G, The number of round spermatid per stage VII seminiferous tubule. H, The ratio of spermatogonias/Sertoli cell per stage VII seminiferous tubule. I, The ratio of pachytene spermatocyte/Sertoli cell per stage VII seminiferous tubule. J, The ratio of pachytene round spermatid/Sertoli cell per stage VII seminiferous tubule. K, The ratio of total germ cell/Sertoli cell per stage VII seminiferous tubule. The data are expressed as the mean ± SD. $n = 7$ per group. *$p<.05$, **$p<.01$, compared with FA-treated group.

CAP Exposure Alters Hypothalamic Expressions of GnRH and Proinflammatory Cytokines

Inflammation is one of the putative mechanisms that mediate the development of extrapulmonary effects due to exposure to ambient $PM_{2.5}$ (Feng *et al.*, 2016). Inflammation contributes to the impairment of centre neuron and its related functions. Whether long-term exposure to ambient $PM_{2.5}$ develops the inflammation in hypothalamus and further impacts the secretion of the important hormone, GnRH, the hypothalamic expression levels of proinflammatory cytokines, IL-1β, IL-6, and TNFα, and GnRH were analyzed by real-time PCR. Figure 7 shows that exposure to CAP significantly increased hypothalamic levels of IL-1β and TNFα mRNA while decreased that of GnRH.

DISCUSSION

Male reproductive system is particularly susceptible to environmental pollutants (Sifakis *et al.*, 2017). However, despite that ambient $PM_{2.5}$ pollution is one of the most concerning risk factors for global public health, its effects on male reproductive system have rarely been investigated. In the present study, we systemically examined the effects of exposure to CAP on the murine male reproductive system. The main findings include that 1) exposure to CAP significantly reduced sperm count in the epididymis; 2) this decrease in sperm count was paralleled by reduction in testicular germ cells, particularly pachytene spermatocytes and round spermatids; 3) exposure to CAP significantly decreased testosterone levels in the plasma and testes, which was accompanied by decreases in plasma FSH levels and testicular expression of testosterone synthesis and function-related genes including P450scc, 17βHSD, StAR, and SHBG; 4) exposure to CAP significantly increased TNFα and IL-1β mRNA but decreased GnRH mRNA in the hypothalamus; 5) exposure to CAP did not increase testicular expression of IL-1β, IL-6, and TNFα (data not show). These data collectively suggest that long-term exposure to ambient $PM_{2.5}$ may impair spermatogenesis and thus male reproductive function.

Sperm counting is one of the most important assessments to evaluate male fertility (Lu *et al.*, 2014). The present data have demonstrated that exposure to CAP remarkably reduced mouse epididymal sperm count. This is consistent with previous studies showing that intratracheal instillation of ambient $PM_{2.5}$ or inhalation exposure to diesel exhaust significantly increases abnormal sperms and decreases sperm count and testosterone level in rats (Cao *et al.*, 2015; Watanabe and Oonuki, 1999). As the present study used whole-body inhalation exposure system that mimic real-world exposure to air pollution (Wang *et al.*, 2017; Ying *et al.*, 2014), the present data thus provide more compelling evidence for the impairment of male reproductive function by exposure to ambient $PM_{2.5}$.

In the present study, we also reveal that the decreased sperm count in CAP-exposed mice is paralleled by a decrease in

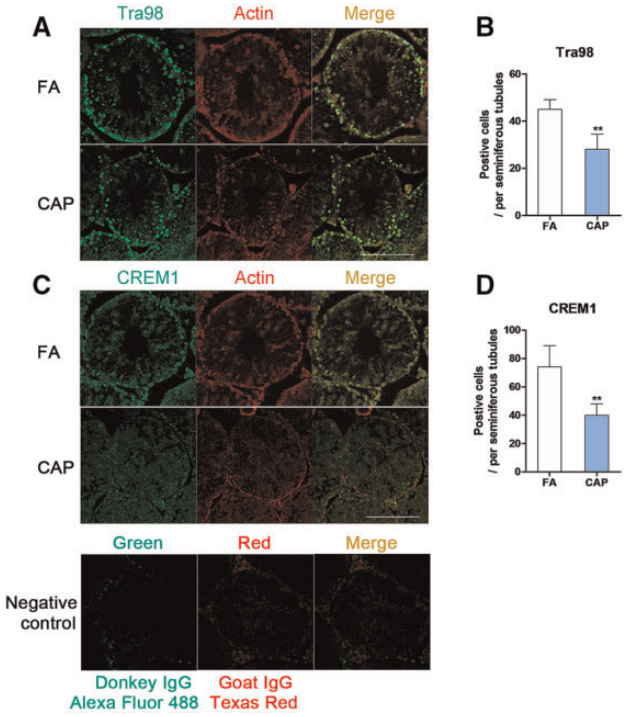

Figure 4. The effects of CAP exposure on count of Tra98 or CREM1 positive cells. A, Tra98 positive cells in seminiferous tubule. B, The count of Tra98 positive cells per seminiferous tubule. C, CREM1 positive cells in seminiferous tubule. B, The count of CREM1 positive cells per seminiferous tubule. The data are expressed as the mean ± SD. $n = 7$ per group. Bar: 100 μm. $^{**}p<.01$, compared with FA-treated group.

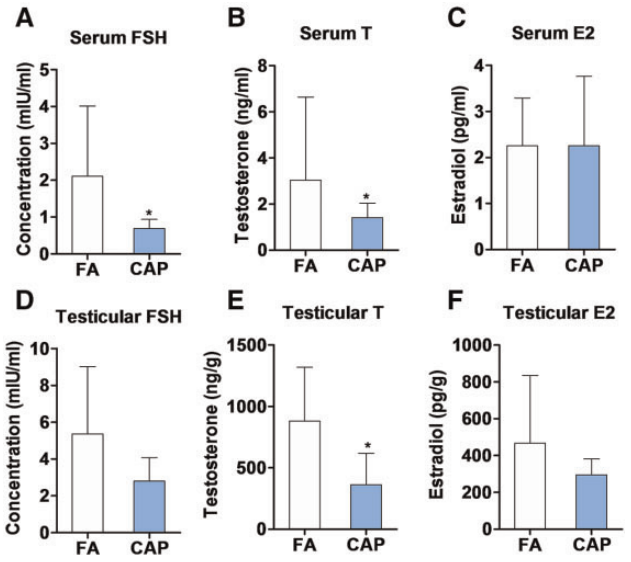

Figure 5. The effects of CAP exposure on reproductive related hormones. A, Serum FSH. B, Serum T. C, Serum E2. D, Testicular FSH. E, Testicular T. F, Testicular E2. The data are expressed as the mean ± SD, $n = 7$ per group. $^{*}p<.05$, compared with FA-treated group.

testicular germ cells. This impairment of spermatogenesis in CAP-exposed mice is corroborated by the present data showing that exposure to CAP significantly decreased pachytene spermatocytes and round spermatids but not spermatogonias. Since diesel exhaust is one of the major sources for ambient $PM_{2.5}$ (Jaiprakash and Habib, 2017), these present data are somehow consistent with previous studies showing that inhalation exposure to diesel exhaust significantly reduces epididymal sperm count and impairs spermatogenesis in rats (Watanabe and Oonuki, 1999). Taken together, these data suggest that the ambient $PM_{2.5}$ exposure-induced decrease in sperm count is at least partly attributable to defect in spermatogenesis.

Another important finding in the present study is that exposure to CAP remarkably decreases testicular and circulating testosterone. This decrease in testosterone is also supported by the decreased testicular expression of testosterone biosynthesis enzymes including P450scc, 17βHSD, and StAR. As spermatogenesis is primarily regulated by testosterone (Chao and Page, 2016; O'Hara and Smith, 2015), these data not only consolidate the finding that exposure to CAP impairs spermatogenesis but also strongly suggest that this impairment of spermatogenesis may be mediated primarily by the reduction in testosterone biosynthesis.

Testosterone is one of the major components of HPG axis (Corradi *et al.*, 2016), and the pituitary hormone LH is crucial for the expression of testosterone biosynthesis enzymes. Although we failed to determine the LH levels in the mouse plasma and testis in the present study, the remarkable decreases in the testicular expression of P450scc, 17βHSD, and StAR mRNA strongly suggest that exposure to CAP may indeed decrease the LH level. Moreover, the present data show that exposure to CAP markedly decreases circulating and testicular levels of FSH, another critical component of HPG axis in the pituitary. This reduction in FSH is corroborated by the decrease of testicular expression of the best known FSH target gene, SHBP. The hypothalamus is the control center of the HPG axis, which controls the biosynthesis and release of pituitary hormones (eg, LH and FSH) primarily through GnRH (Ubuka *et al.*, 2013; Voliotis *et al.*, 2017). Consistent with the effects on pituitary hormones and spermatogenesis, the present study showed that CAP exposure also significantly reduced hypothalamic expression of GnRH. Interestingly, our data additionally demonstrated that this decreased expression of GnRH is accompanied by increased hypothalamic expression of proinflammatory cytokines, TNFα, and ILβ1. Given that local inflammation has been shown to suppress hypothalamic functions (Gabuzda and Yankner, 2013), these data have strongly suggested that CAP exposure may suppress the GnRH mRNA expression and thus HPG axis probably through induction of inflammation in the hypothalamus.

Systemic inflammation is widely believed to mediate extrapulmonary effects due to exposure to ambient $PM_{2.5}$ (Feng *et al.*, 2016). However, the present study reveals that inflammatory biomarkers including IL-1β, IL-6, and TNFα mRNA in the testis was not increased. Therefore, these data somehow disprove the implication of systemic proinflammatory mediators in the CAP exposure-induced male reproductive system impairment, whereas underscore the role of suppression of HPG axis.

Although the present study provides compelling evidence that exposure to ambient $PM_{2.5}$ influences the male reproductive system through suppression of HPG axis, it has a number of important limitations. These include the fact that we have not provided any data on the time- and dose-dependency of these adverse effects due to exposure to CAP. Another limitation is the fact that the present study failed to determine the levels of some critical components of HPG axis, in particular LH. This will require more sensitive techniques such as radioactive immunoassay. Moreover, the present study did not provide any data regarding the cause-effect relationship between suppression of HPG axis and impairment of spermatogenesis. Additional experiments using genetically modified mouse models is thus

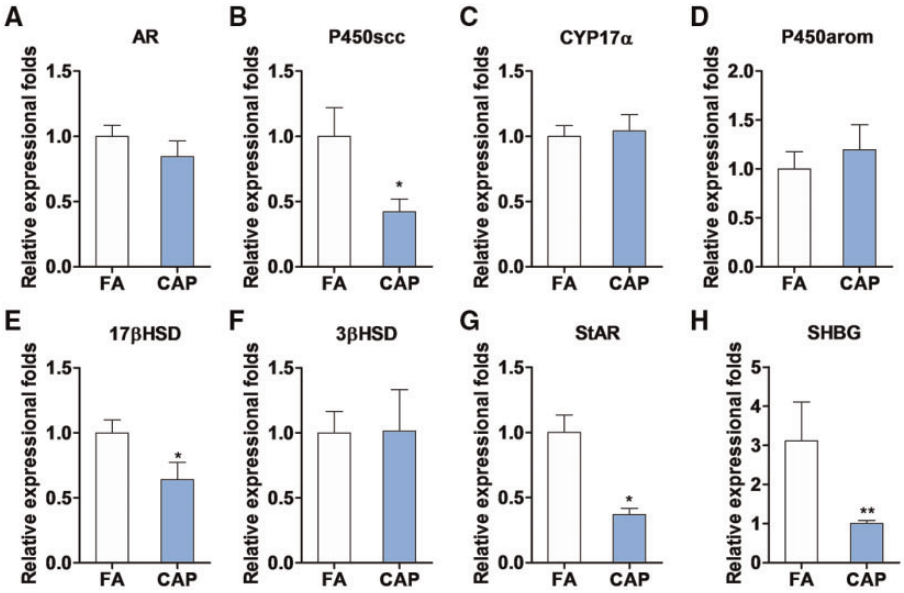

Figure 6. The effects of CAP exposure on the expressions of testosterone synthesis and function related genes. A, AR. B, P450scc C, CYP17α. D, P450arom. E, 17βHSD. F, 3βHSD. G, StAR. H, SHBG. The data are expressed as the mean ± SD. $n = 7$ per group. $^{*}p<.05$, $^{**}p<.01$, compared with FA-treated group.

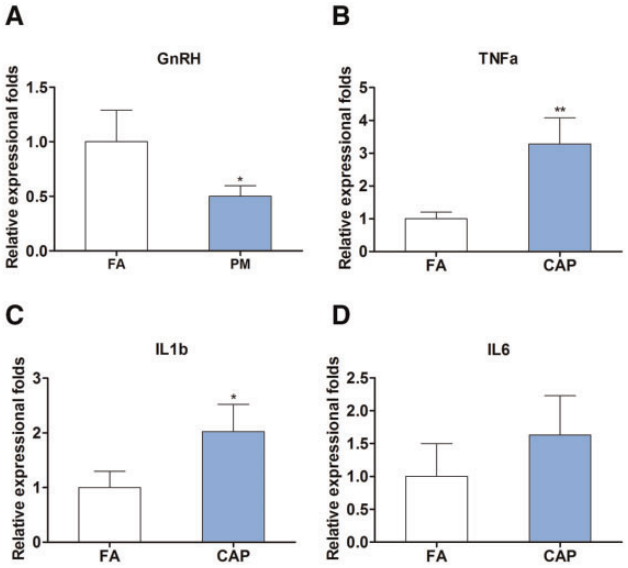

Figure 7. The effects of CAP exposure on the hypothalamic mRNA expressions of GnRH and inflammatory biomarkers. A, TNFα. B, IL-1β. C, IL-6. The data are expressed as the mean ± SD. $n = 7$ per group. $^{*}p<.05$, compared with FA-treated group.

warranted to confirm the role of HPG axis in the mediation of impairment in the male reproductive system due to exposure to ambient $PM_{2.5}$.

CONCLUSION

In summary, the present results demonstrate that long-term exposure to CAP impact spermatogenesis and thus impair the male reproductive function.

FUNDING

National Institutes of Health (R01ES024516 to Z.Y.), American Heart Association (13SDG17070131 to Z.Y.), National Natural Science Foundation of China (Grant No. 81302452 to L.Q., 81270342 to Z.Y., 81500216 to M.C., 21407082 to X.W.), and Scientific Research Starting Foundation for The Doctoral Researcher of Nantong University (No. 14B14).

ACKNOWLEDGMENTS

We thank Dr Gang Chen and Yihua Qiu for their helpful suggestion to this study. The authors declare no conflict of interest.

REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239–247.

Agency, U. S. E. P. (2013). National ambient air quality standards for particulate matter. Final rule. *Fed. Regist.* **78**, 3086–3287.

Ahmed, E. A., and de Rooij, D. G. (2009). Staging of mouse seminiferous tubule cross-sections. *Methods Mol. Biol.* **558**, 263–277.

Cao, X. N., Yan, C., Liu, D. Y., Peng, J. P., Chen, J. J., Zhou, Y., Long, C. L., He, D. W., Lin, T., and Shen, L. J. (2015). Fine particulate matter leads to reproductive impairment in male rats by overexpressing phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. *Toxicol. Lett.* **237**, 181–190.

Chao, J. H., and Page, S. T. (2016). The current state of male hormonal contraception. *Pharmacol. Ther.* **163**, 109–117.

Corradi, P. F., Corradi, R. B., and Greene, L. W. (2016). Physiology of the hypothalamic pituitary gonadal axis in the male. *Urol. Clin. North Am.* **43**, 151–162.

Creasy, D. M. (1997). Evaluation of testicular toxicity in safety evaluation studies: The appropriate use of spermatogenic staging. *Toxicol. Pathol.* **25**, 119–131.

Feng, S., Gao, D., Liao, F., Zhou, F., and Wang, X. (2016). The health effects of ambient PM2.5 and potential mechanisms. *Ecotoxicol. Environ. Saf.* **128**, 67–74.

Gabuzda, D., and Yankner, B. A. (2013). Physiology: Inflammation links ageing to the brain. *Nature* **497**, 197–198.

George, V. K., Li, H., Teloken, C., Grignon, D. J., Lawrence, W. D., and Dhabuwala, C. B. (1996). Effects of long-term cocaine exposure on spermatogenesis and fertility in peripubertal male rats. *J. Urol.* **155**, 327–331.

Guo, H., Cheng, T., Gu, X., Wang, Y., Chen, H., Bao, F., Shi, S., Xu, B., Wang, W., Zuo, X., *et al.* (2017). Assessment of PM2.5 concentrations and exposure throughout China using ground observations. *Sci. Total Environ.* **601–602**, 1024–1030.

Hammoud, A., Carrell, D. T., Gibson, M., Sanderson, M., Parker-Jones, K., and Peterson, C. M. (2010). Decreased sperm motility is associated with air pollution in Salt Lake City. *Fertil. Steril.* **93**, 1875–1879.

Hansen, C., Luben, T. J., Sacks, J. D., Olshan, A., Jeffay, S., Strader, L., and Perreault, S. D. (2009). The effect of ambient air pollution on sperm quality. *Environ. Health Perspect.* **118**, 203–209.

Huang, S. L., Rahn, K. A., and Arimoto, R. (1999). Testing and optimizing two factor-analysis techniques on aerosol at Narragansett, Rhode Island. *Atmos. Environ.* **33**, 2169–2185.

Jaiprakash, and Habib, G. (2017). Chemical and optical properties of PM2.5 from on-road operation of light duty vehicles in Delhi city. *Sci. Total Environ.* **586**, 900–916.

Jin, J. M., and Yang, W. X. (2014). Molecular regulation of hypothalamus-pituitary-gonads axis in males. *Gene* **551**, 15–25.

Jurewicz, J., Radwan, M., Sobala, W., Polańska, K., Radwan, P., Jakubowski, L., Ulańska, A., and Hanke, W. (2015). The relationship between exposure to air pollution and sperm disomy. *Environ. Mol. Mutag.* **56**, 50–59.

Kay, V. R., Bloom, M. S., and Foster, W. G. (2014). Reproductive and developmental effects of phthalate diesters in males. *Crit. Rev. Toxicol.* **44**, 467–498.

Li, Y., Wang, M., Piao, F., and Wang, X. (2012). Subchronic exposure to arsenic inhibits spermatogenesis and downregulates the expression of ddx3y in testis and epididymis of mice. *Toxicol. Sci.* **128**, 482–489.

Lu, J. C., Huang, Y. F., and Lu, N. Q. (2014). Computer-aided sperm analysis: Past, present and future. *Andrologia* **46**, 329–338.

Marchlewska, K., Kula, K., Walczak-Jedrzejowska, R., Oszukowska, E., Orkisz, S., and Slowikowska-Hilczer, J. (2011). Triiodothyronine modulates initiation of spermatogenesis in rats depending on treatment timing and blood level of the hormone. *Mol. Cell. Endocrinol.* **341**, 25–34.

Mizrachi, D., and Auchus, R. J. (2009). Androgens, estrogens, and hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* **301**, 37–42.

Mortimer, C. H., and Yeo, T. (1976). Gonadotrophin-releasing hormone. *J. Clin. Pathol.* **s1–7**, 46–54.

Nantel, F., Monaco, L., Foulkes, N. S., Masquilier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M., and Sassone-Corsi, P. (1996). Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature* **380**, 159–162.

O'Hara, L., and Smith, L. B. (2015). Androgen receptor roles in spermatogenesis and infertility. *Best Pract. Res. Clin. Endocrinol. Metab.* **29**, 595–605.

Ohlander, S. J., Lindgren, M. C., and Lipshultz, L. I. (2016). Testosterone and male infertility. *Urol. Clin. North Am.* **43**, 195–202.

Qiu, L., Qian, Y., Liu, Z., Wang, C., Qu, J., Wang, X., and Wang, S. (2016). Perfluorooctane sulfonate (PFOS) disrupts blood-testis barrier by down-regulating junction proteins via p38 MAPK/ATF2/MMP9 signaling pathway. *Toxicology* **373,** 1–12.

Qiu, L. L., Wang, X., Zhang, X. H., Zhang, Z., Gu, J., Liu, L., Wang, Y., Wang, X., and Wang, S. L. (2013). Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentration of bisphenol A. *Toxicol. Lett.* **219**, 116–124.

Rochester, J. R. (2013). Bisphenol A and human health: A review of the literature. *Reprod. Toxicol.* **42**, 132–155.

Sifakis, S., Androutsopoulos, V. P., Tsatsakis, A. M., and Spandidos, D. A. (2017). Human exposure to endocrine disrupting chemicals: Effects on the male and female reproductive systems. *Environ. Toxicol. Pharmacol.* **51**, 56–70.

Thackray, V. G., Mellon, P. L., and Coss, D. (2010). Hormones in synergy: Regulation of the pituitary gonadotropin genes. *Mol. Cell. Endocrinol.* **314**, 192–203.

Tsutsumi, R., and Webster, N. J. (2009). GnRH pulsatility, the pituitary response and reproductive dysfunction. *Endocr. J.* **56**, 729–737.

Ubuka, T., Son, Y. L., Bentley, G. E., Millar, R. P., and Tsutsui, K. (2013). Gonadotropin-inhibitory hormone (GnIH), GnIH receptor and cell signaling. *Gen. Comp. Endocrinol.* **190**, 10–17.

Voliotis, M., Garner, K. L., Alobaid, H., Tsaneva-Atanasova, K., and McArdle, C. A. (2017). Gonadotropin-releasing hormone signaling: An information theoretic approach. *Mol. Cell. Endocrinol.* doi: 10.1016/j.mce.2017.07.028 [Epub ahead of print].

Wang, X., Chen, M., Zhong, M., Hu, Z., Qiu, L., Rajagopalan, S., Fossett, N. G., Chen, L. C., and Ying, Z. (2017). Exposure to concentrated ambient PM2.5 shortens lifespan and induces inflammation-associated signaling and oxidative stress in Drosophila. *Toxicol. Sci.* **156**, 199–207.

Watanabe, N., and Oonuki, Y. (1999). Inhalation of diesel engine exhaust affects spermatogenesis in growing male rats. *Environ. Health Perspect.* **107**, 539–544.

Yamaguchi, S., Hong, K., Liu, R., Inoue, A., Shen, L., Zhang, K., and Zhang, Y. (2013). Dynamics of 5-methylcytosine and 5-hydroxymethylcytosine during germ cell reprogramming. *Cell Res* **23**, 329–339.

Ying, Z., Xu, X., Bai, Y., Zhong, J., Chen, M., Liang, Y., Zhao, J., Liu, D., Morishita, M., and Sun, Q. (2014). Long-term exposure to concentrated ambient PM2.5 increases mouse blood pressure through abnormal activation of the sympathetic nervous system: A role for hypothalamic inflammation. *Environ. Health Perspect.* **122**, 79–86.

Ying, Z., Yue, P., Xu, X., Zhong, M., Sun, Q., Mikolaj, M., Wang, A., Brook, R. D., Chen, L. C., and Rajagopalan, S. (2009). Air pollution and cardiac remodeling: A role for RhoA/Rho-kinase. *Am. J. Physiol. Heart. Circ. Physiol.* **296**, H1540–H1550.

Zhou, N., Cui, Z., Yang, S., Han, X., Chen, G., Zhou, Z., Zhai, C., Ma, M., Li, L., Cai, M., *et al.* (2014). Air pollution and decreased semen quality: A comparative study of Chongqing urban and rural areas. *Environ. Pollut.* **187**, 145–152.