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Research
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Abstract

**Background:** Exposure to air pollutants represented by diesel exhaust PM$_{2.5}$ (DEP) correlates with the decline of semen quality, but the underlying biological mechanism has not been fully understood. In the present study, mice were intratracheally instilled with DEP for around 7 months, and the effects of PM$_{2.5}$ exposure on the spermatogenic process as well as the alterations of testicular gene expression profile were assessed.

**Results:** Our results show that chronic exposure to DEP significantly impairs the fertility of male mice without influencing their libido. Compared with Vehicle-exposed group, the sperm count and motility from DEP-exposed mice were significantly decreased. In addition, immunohistological staining of γH2AX and DMC1, biomarkers for meiotic double strand breaks (DSBs), demonstrated that chronic exposure to DEP comprised the repair of meiotic DSBs, thus disrupts the spermatogenesis. Deep RNA sequencing test shows massive altered expressions of testicular genes including the GnRH signaling pathway.

**Conclusion:** In summary, our research demonstrates that chronic exposure to PM$_{2.5}$ disrupts spermatogenesis through targeting the meiotic recombination, providing a new perspective for the research on the male reproductive system damage caused by air pollution.

**Key Words:** air pollution; diesel exhaust PM$_{2.5}$; spermatogenesis; meiotic arrest
**Introduction**

Epidemiological studies demonstrate a global uncontrolled decline in the semen quality and male fertility rate over the past several decades [1,2]. The reasons for this decline has not yet been well established. As this is a global decline, genetics apparently may not be its main reasons. The male reproductive system is well known to be vulnerable to various environmental stressors. Thus, environmental pollution is believed to probably account for the major decline in the quality of semen. Ambient fine particulate matter (PM$_{2.5}$) is an airborne pollutant that severely threats the global public health. It is estimated that 91% of the world’s population lives in places where air quality exceeds the world health organization (WHO) guideline limits (who.int/airpollution/en). Furthermore, PM$_{2.5}$ levels markedly below the WHO guideline limit may still be harmful to public health [3]. Therefore, any adverse health effect of PM$_{2.5}$ may be a significant threat for the global public health. Notably, epidemiological studies have increasingly shown that exposure to PM$_{2.5}$ correlates with decline in semen quality [4-8], drawing considerable attention to the role of PM$_{2.5}$ pollution in the global increase in male infertility.

Studies in animal models are essential to establish the causal role of PM$_{2.5}$ exposure in the development of male infertility. Since Watanabe and Oonuki showed that inhalation of diesel engine exhaust affects spermatogenesis in growing male rats in 1999 [9], rapidly increasing studies in animal models have also demonstrated various adverse effects of PM$_{2.5}$ exposure on semen quality. The sperm count, motility, and morphology are three most frequently used indexes for assessing the quality of semen. To date, studies in various animal models all demonstrate a decrease in the sperm count and motility, if assessed [9-18]. In contrast, both negative [11,14,16-18] results have been reported regarding the effect of PM$_{2.5}$ exposure on the rate of morphologically abnormal sperm. The integrity of the blood-testis barrier (BTB) is
essential for spermatogenesis. Several studies show that exposure to PM$_{2.5}$ results in a
disruption of the BTB [10-13,16], providing a potential mechanism for the spermatogenic
abnormalities induced by PM$_{2.5}$ exposure. In addition, exposure to PM$_{2.5}$ was shown to evoke
reactive oxygen species (ROS) production [12,17], inflammation [18], and endoplasmic
reticulum (ER) stress [14] in the testes, suggesting that these may also be local mediators for
the spermatogenic abnormalities induced by PM$_{2.5}$ exposure.

Given that the major components of inhaled PM$_{2.5}$ may not enter the systemic circulation, a
mechanism linking PM$_{2.5}$ inhalation to the pathology in the testis is clearly needed.
Nanoparticles and polycyclic aromatic hydrocarbons (PAHs) in PM$_{2.5}$ may enter the systemic
circulation with various efficiencies. Notably, both nanoparticles [19] and PAHs [20] were shown
to disrupt testicular structure and/or function, supporting that the adverse testicular effects of
PM$_{2.5}$ exposure may be mediated by egress of inhaled PM$_{2.5}$. However, the high doses used in
these studies somehow undermine this possibility. The hypothalamic-pituitary-gonadal (HPG)
axis, including gonadotropin-releasing hormone (GnRH) from the hypothalamus, luteinizing
hormone (LH) and follicle-stimulating hormone (FSH) from the anterior portion of the pituitary
gland, and estrogen and testosterone produced by the gonads, is central in maintaining the
homeostasis of the male reproductive system. Watanabe and Oonuki showed that inhalation of
diesel engine exhaust significantly affects the circulating levels of FSH, LH, testosterone, and
estradiol in rats [9]. We recently demonstrated that exposure to concentrated ambient PM$_{2.5}$
(CAP) influences not only circulating FSH and testosterone but also the hypothalamic
expression of GnRH [15]. These studies strongly suggest that the HPG axis may be a crucial
mediator for those adverse testicular effects of PM$_{2.5}$ exposure.

Spermatogenesis is a complicated process that includes the mitotic division that produces
type A (self-renewal of stem cells) or B (committed to spermatocytes) spermatogonia, the

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meiotic division that produces haploid spermatids, and the spermiogenesis that transforms spermatids into sperms. Notably, despite the above-mentioned considerable evidence for the disruption of spermatogenesis by PM2.5 exposure, how PM2.5 exposure affects the spermatogenic process has hardly been investigated, except for the histological analysis of seminiferous tubules [9,15]. Therefore, the present study exploits the mouse model of intratracheal instillation of diesel exhaust PM2.5 (DEP) to examine the effects of PM2.5 exposure on the spermatogenic process and also thoroughly document the alterations of testicular gene expression profile induced by DEP exposure. Our results show that DEP exposure decreased the number of advanced spermatogenic cells but not spermatogonia, paralleled by marked increase in meiotic double strand breaks (DSBs) in pachytene but not leptotene spermatocytes, strongly suggesting that DEP exposure disrupts spermatogenesis through specifically targeting the repair of meiotic DSBs.
Results

Chronic exposure to DEP impairs the male fertility

To examine the effect of chronic DEP exposure on the fertility of males, male C57Bl/6J mice were subject to 6-month intratracheal instillation of vehicle (PBS) or DEP, and then their fertilities were assessed via an 18-day mating with normal female C57Bl/6J mice. As shown in Figure 1A, these sires were continued with their intratracheal instillation of vehicle or DEP throughout this 18-day mating and the following 1-week singly housing. Therefore, they had been exposed to vehicle or DEP for approximately 7 months in total when euthanized. Figures 1B and 1C show that all the sires inseminated their dam during this 18-day mating and that the times taken for Vehicle- or DEP-exposed sires to inseminate their dam were comparable, suggesting that exposure to DEP may not impact the libido of male mice. In contrast, Figure 1D reveals that while 100% Vehicle-exposed sires impregnated their dam during this 18-day mating, 70% DEP-exposed sires only impregnated their dam, revealing that DEP exposure markedly impairs the fertility of male mice. All the dams were sacrificed on day E16.5. The outcomes of these pregnancies are presented in Table 1. The paternal exposure to DEP did not significantly influence the rates of stillbirth and absorption, implantations per dam, live fetuses per dam, live fetus weight, placental weight and uterus weight.

Chronic exposure to DEP impairs the quality of semen

Given the impairment of fertility of male mice by DEP exposure, we further documented the effect of DEP exposure on the male reproductive system. Table 2 reveals that this 6-month exposure to DEP did not significantly influence the weights of mouse body, testis, and epididymis. In contrast, it significantly reduced the count of epididymal sperms (Figure 2B) and
significantly decreased their motility (Figure 2C), strongly suggesting that chronic exposure to DEP disturbs the development and maturation of sperms. However, the morphological analysis of epididymal sperms showed that chronic exposure to DEP did not significantly influence the rate of morphologically abnormal sperms (Figures 2A and 2D).

Chronic exposure to DEP alters the testicular histology

The testis is the organ that produces sperms. Given the reduction in the epididymal sperm count by chronic exposure to DEP, histological analyses were performed on the testes of Vehicle- or DEP-exposed mice to assess the effects of chronic DEP exposure on testicular histology. In agreement with the reduced epididymal sperm count by chronic DEP exposure (Figure 2B), we observed significant increases in Sertoli cell vacuolization (Figures 3A and 3B) and derangement of the cell layers of seminiferous tubules (Figures 3A and 3C) in the testes of DEP-exposed mice versus those of vehicle-exposed mice. Sperms are released in Stage VIII seminiferous tubules; therefore, the proportion of Stage VIII seminiferous tubules somehow represents the sperm production rate. We thus assessed the proportion of Stage VIII seminiferous tubules in these testes. In line with the reduction in the epididymal sperm count by chronic DEP exposure, Figure 3D reveals that DEP- versus Vehicle-exposed mice had significantly reduced proportion of Stage VIII seminiferous tubules in the testes. In contrast, chronic exposure to DEP did not significantly alter the proportion of Stage VII seminiferous tubules (Figure 3E), the most frequently used seminiferous tubules for assessing the spermatogenic parameters.
Chronic exposure to DEP results in a loss of advanced spermatogenic cells in Stage VII seminiferous tubules

To pinpoint the effects of chronic exposure to DEP on spermatogenesis, we further documented the spermatogenetic parameters of the Stage VII seminiferous tubules. Figures 4A-4C show that chronic exposure to DEP did not significantly affect the wall thickness, diameter, and Sertoli cell number of the Stage VII seminiferous tubules. Notably, chronic exposure to DEP significantly reduced the number of total germ cells in the Stage VII seminiferous tubules (Figure 4D). Cell differentiation analysis demonstrated that chronic exposure to DEP did not significantly change the number of spermatogonia (Figure 4E) but significantly reduced the numbers of advanced spermatogenic cells including the pachytene spermatocytes (Figure 4F) and round spermatids (Figure 4G) in the Stage VII seminiferous tubules.

Chronic exposure to DEP impairs the repair of meiotic double strand breaks (DSBs)

During the spermatogenesis, the spermatogonia produces the primary spermatocyte through mitosis, and the advanced spermatogenic cells are in turn produced by the primary spermatocyte through meiosis. Therefore, the chronic DEP exposure-induced loss of advanced spermatogenic cells strongly suggests that chronic DEP exposure disrupts the meiotic progression during spermatogenesis. A major event during meiosis is the reshuffling of the parental genomes through the formation and repair of DSBs, and there is a surveillance mechanism called the recombination checkpoint ensures that all breaks are repaired before a cell starts the meiotic divisions. To determine the effect of chronic DEP exposure on the formation and repair of DSBs during meiosis, the spermatocyte spreads were prepared from the testes of these Vehicle- or DEP-exposed mice and their DSBs were visualized using SYCP3
and γH2AX antibodies. **Figures 5A-5D** demonstrate that chronic DEP exposure did not influence the distribution pattern of γH2AX, the biomarker for meiotic DSBs, in the leptotene and zygotene spermatocytes, but significantly increased the rate of abnormal XY body and other homologous chromosome in the pachytene spermatocytes, indicators for abnormal repair of DSBs. For further analysis, we then staged and examined spermatocyte spreads by immunolocalization of SYCP3/γH2AX, which were used as the markers of different stages of meiotic prophase. As shown in **Figure 5E**, the proportion of spermatocytes in leptotene and zygotene stages in the DEP group increased, while the proportion of those in pachytene and diplotene stages decreased, strongly suggesting that DEP severely interfered with the progression of meiotic prophase.

To verify the effect of chronic DEP exposure on the repair of meiotic DSBs, the spermatocyte spreads were also stained with DMC1, another biomarker for DSBs. **Figures 5F-5H** reveal that chronic exposure to DEP did not influence the number of DSBs in zygotene spermatocytes but significantly increased the number of DSBs in pachytene spermatocytes, corroborating that chronic DEP exposure does not impact the formation of DSBs but impairs their repair and thus may delay the meiotic progression.

To verify whether chronic exposure to DEP disrupts the spermatogenesis through impact on the repair of DSBs, the sections of testes from the Vehicle- and DEP-exposed mice were visualized using γH2AX antibody. **Figure 6A** shows that the γH2AX+ cells in the advanced spermatocytes (cells close to the lumen of seminiferous tubules) were markedly increased in the testes of DEP-exposed mice versus those of Vehicle-exposed mice, strongly supporting the impairment of repair of DSBs and thus disruption of the meiotic progression by chronic DEP exposure. In normal testes such as the PBS-exposed in **Figure 6A**, most γH2AX+ cells are the early spermatogenic cells (those close to the base membrane), and the visualization of γH2AX
facilitates determining the meiotic stages of these early spermatogenic cells: the diffused distribution pattern marked by the yellow arrows in Figure 6A represents the leptotene or zygotene spermatocyte; and the focused distribution pattern marked by the red arrows in Figure 6A represents the pathytene or diplotene spermatocyte. Therefore, we analyzed the meiotic stages of seminiferous tubules using the γH2AX distribution pattern of early spermatogenic cells. Figures 6B-6C show that chronic exposure to DEP significantly decreased the proportion of seminiferous tubules with the leptotene or zygotene spermatocyte but increased the proportion of seminiferous tubules with the pathytene or diplotene spermatocyte, strongly suggesting a delayed transition from zygotene to pathytene induced by chronic DEP exposure.

Chronic exposure to DEP alters the testicular gene expression profile

To thoroughly determine the effect of chronic DEP exposure on the testis, we additionally profiled the testicular gene expression of Vehicle- or DEP-exposed mice through deep RNA sequencing. After the quality control, alignment to the mouse genome, and assembling of transcripts, 18598 transcripts were identified. The volcano plot (Figure 7A) reveals that there are 80 genes differentially expressed in the testes of DEP-exposed mice versus those of Vehicle-exposed mice (p value of FDR < 0.05 and fold change < 0.5 or > 2): 56 genes were under-expressed in the DEP- versus Vehicle-exposed testes, and 24 genes were over-expressed in the DEP- versus Vehicle-exposed testes. The relative expression levels of these differentially expressed genes are presented in Figure 7B. To identify the biological processes that are influenced by the chronic DEP exposure, gene ontology (GO) enrichment analysis using the 80 differentially expressed genes was performed. Figure 7C shows that 8 GO terms were significantly enriched, including our previously identified GnRH signaling pathway.
**Discussion**

The male reproductive system is vulnerable to environmental pollution, and published studies have increasingly demonstrated that it may be targeted by PM$_{2.5}$ exposure. However, the biological mechanism by which PM$_{2.5}$ exposure disrupts the male reproductive system and thus the male fertility has not yet been fully understood. In the present study, we show that chronic exposure to DEP, an important source for ambient PM$_{2.5}$, 1) impaired the fertility of male mice but did not influence their libido; 2) decreased the count and motility of epididymal sperms; 3) compromised the repair of meiotic DSBs and thus the meiotic progression during spermatogenesis; and 4) massively altered the testicular gene expression profile including the GnRH signaling pathway. To our best knowledge, this is the first study showing that PM$_{2.5}$ exposure compromises the male fertility through targeting the repair of meiotic DSBs and thus the spermatogenesis and also the first high-throughput data-based evidence for the implication of the HPG axis in the impairment of male fertility by chronic PM$_{2.5}$ exposure.

Epidemiological studies increasingly demonstrate that exposure to PM$_{2.5}$ inversely correlates with semen quality and male fertility rate [4-8]. The present study corroborates the impairment of semen quality and thus male fertility in a mouse model. The sperm count, motility, and morphology collectively determine the semen quality and thus male fertility. Notably, the present study shows that chronic exposure to DEP decreased the epididymal sperm count and motility but did not increase the rate of abnormal sperm (Figure 2). The lack of effect on the rate of abnormal sperm is supported by our present data showing that chronic exposure to DEP did not increase the rates of stillbirth and absorption (Table 1), and also consistent with our [15] and others’ [9,13,18,20] published data. However, in contrast to the very consistent published data regarding the adverse effects of PM$_{2.5}$ exposure on the sperm count and motility [9,13-
there are several published studies showing that exposure to PM$_{2.5}$ increases the rate of abnormal sperm [14,17,21]. Although the reason for this confliction of published data regarding the effect of PM$_{2.5}$ exposure on the rate of abnormal sperm remains to be determined, it somehow suggests a composition-dependency for this particular effect of PM$_{2.5}$ exposure. This also suggests that PM$_{2.5}$ exposure impact the sperm count, motility and the rate of abnormal sperm probably through different mechanisms.

In the present study, we demonstrate that chronic exposure to DEP did not impact the insemination capacity of male mice neither the time taken for the insemination. To our knowledge, this is the first evidence for that PM$_{2.5}$ exposure decreases the male fertility rate not through impact on the sexual behaviors. Thus, these results add massive support for the crucial role of decreased semen quality in the induction of male infertility by PM$_{2.5}$ exposure.

To date, although there is considerable evidence supporting the adverse effect of PM$_{2.5}$ exposure on the spermatogenesis and thus the semen quality, the biological mechanism remains elusive. The present study demonstrates that chronic exposure to DEP reduced advanced spermatogenic cells but not early stages of germ cells such as spermatogonia in the Stage VII seminiferous tubules. This is consistent with several published studies [9,15]. Given that the advanced spermatogenic cells are produced in turn from the primary spermatocytes through meiosis, these data strongly suggest that exposure to PM$_{2.5}$ disturbs spermatogenesis probably through targeting the spermatogenetic meiosis. The meiotic recombination between homologous chromosomes through the programmed homologous pairing and formation and repair of DSBs is a rich source of diversity in a population, and the meiotic progression is regulated primarily using the recombination checkpoint that monitors meiotic recombination during meiosis and blocks the entry into metaphase I if recombination is not properly processed. The present study shows that chronic exposure to DEP markedly influenced the processing of
meiotic recombination, particularly the repair of meiotic DSBs, as evidenced by both the increased mis-pairing of sex and other chromosomes (Figure 5C,D) and the increased DSBs in pachytene spermatocytes (Figure 5H). The present data showing that chronic exposure to DEP significantly altered the proportion of different stages of seminiferous tubules (Figures 3D, 6B, and 6C) strongly support that the disruption of meiotic recombination by chronic DEP exposure is toxicologically significant. To our best knowledge, this is the first study demonstrating that chronic exposure to PM$_{2.5}$ disrupts spermatogenesis through targeting the meiotic recombination. Because a scientific framework for extrinsic factors to regulate the repair of meiotic DSBs has not yet been established, the present study did not investigate the molecular mechanism by which PM$_{2.5}$ exposure impact the repair of meiotic DSBs. Nonetheless, the present study provides a valuable model to establish the scientific framework for the regulation of repair of meiotic DSBs by extrinsic factors.

Notably, although the initiation of spermatogenic meiosis (the transition from A to A1 spermatogonia) is better known to be regulated by extrinsic factors [22], the present study suggests that chronic exposure to DEP may not impact the initiation of spermatogenic meiosis, as evidenced by the normal formation of DSBs in the leptotene spermatocytes (Figure 5G) of DEP-exposed testes and the normal number of spermatogonia of DEP-exposed testes (Figure 4E). Along with the above-mentioned evidence for the disruption of repair of meiotic DSBs by chronic DEP exposure, these results strongly suggest that the repair of meiotic DSBs is precisely targeted for chronic DEP exposure to impair the spermatogenesis.

The present study is also the first one using high-throughput technique to thoroughly document the effect of chronic DEP exposure on the testicular gene expression profile. Another important finding in the present study is the implication of the HPG axis in the development of adverse effects on the male reproductive system due to exposure to DEP by our testicular gene
expression profiling. Our gene expression profiling analysis showed not only that the Glycoprotein Hormones, Alpha Polypeptide (Cga), the shared alpha subunit of luteinizing hormone (LH) and follicle stimulating hormone (FSH), is the most remarkable under-expressed gene (Figure 7A) but also that the GnRH signaling pathway is one of eight significantly-enriched GO terms (Figure 7C). Targeting the HPG axis for PM$_{2.5}$ exposure to evoke adverse effects on the male reproductive system is consistent with our [15] and others’ [23] published studies. Notably, recent studies showed that PM$_{2.5}$ exposure disrupts the testicular histology and spermatogenesis through a reactive oxygen species (ROS)-dependent mechanism [12,17]. However, our testicular gene expression profiling did not identify any ROS-related genes differentially expressed in DEP- versus Vehicle-exposed testes.

Although the present study provides compelling evidence that long-term exposure to DEP affects the male reproductive system by disrupting first meiosis, it has a range of important limitations. This includes the time and dose-dependent data that we have not provided for any of these adverse effects due to DEP exposure. Another limitation is that this study failed to determine which protein or gene changes in meiosis caused meiosis abnormalities. Of course, this in-depth discussion requires a deeper accumulation of expertise and more sensitive technology. Furthermore, the present study did not provide any data on the causal relationship between meiosis arrest and damage to spermatogenesis. Therefore, it is necessary to conduct additional experiments to determine how the male reproductive system damage via affecting the meiosis process due to DEP exposure.
Methods

Animals

All procedures of this study were approved by the Institutional Animal Care and Use Committee at Fudan University, and all the animals were treated humanely and with regard for alleviation of suffering. C57Bl/6J mice (male, 4-week-old) were purchased from the Animal Center of Shanghai Medical School, Fudan University (Shanghai, China) and were housed in standard cages with a 12-h light/12-h dark cycle with temperatures of 18-25°C and relative humidity of 40-60%. One week of acclimation was allowed before the intratracheal instillation of DEP.

Intratracheal instillation of DEP

DEP was obtained from the National Institute of Standards and Technology (SRM 2975; NIST, Gaithersburg, MD, USA). They were kept away from direct sunlight at 4°C before use. To perform intratracheal instillation, DEP was first suspended in sterile PBS. To minimize aggregation, DEP suspensions were sonicated (Clifton Ultrasonic Bath, Clifton, NJ, USA) for 20 mins on the day of instillation and vortexed for 30s before instillation. The prepared DEP suspensions were then intratracheally instilled to mice as previously described with minor modification. In brief, animals were first anesthetized with 3% of isoflurane and placed supine with extended neck on an angled board. A Becton Dickinson 18 Gauge cannula was then inserted via the mouse mouth into the trachea. DEP suspension (20 µg/50µl in PBS) or PBS only was intratracheally instilled using a sterile syringe followed by 150µl air bolus. The mouse was transferred to a vertical hanging position with its head up for 5 min after the removal of intubation catheter to ensure that the delivered material was maintained in the lung without blocking the airways. The deposition and distribution of instilled material was verified by Evans
Blue (data not shown). PBS or DEP suspension was instilled 3 times/week (on Mondays, Wednesdays and Fridays) for around 7 months.

**Fertility parameters collection**

Male C57Bl/6J mice (5-week-old) were subject to intratracheal instillation of PBS/DEP for 6 months, and their fertilities were assessed via an 18-day mating with normal age-matched female C57Bl/6J mice followed by a week of singly housing. PBS/DEP instillation was continued during the whole treatment period (thus approximately 7 months in total). Vaginal sperm plug was checked twice every day, and the insemination capacity was assessed as the percentage of mated mice showing the presence of sperm plug, time taken for insemination was assessed as the time spend between the start of mating and the earliest presence of sperm plug and the impregnation capacity was assessed as the percentage of pregnant mice after 18-day mating.

**Sperm count and motility analysis**

Sperm counting and motility analysis were conducted as previously described [24]. In brief, the left epididymis was placed in 1ml normal saline, and then 6 deep cuts were made in each cauda with micro-scissors to release sperms into the media at 35°C for 10 min. The suspensions were then filtered with nylon mesh (pore size of 70µm) and stained with Papanicolaou (containing formalin). The numbers and motility of sperm were then analyzed using a CASA system (IVOS II Sperm Analyzer, Hamilton). At least 200 sperms from each sample were counted to assess the abnormal sperm percentage.
Tissue harvesting, testicular pathological analysis and spermatogenetic parameters collection

On the day of experiment, after measurement of their body weight, all the mice were euthanized and their blood was harvested from the orbital venous plexus. Fresh isolated testes, epididymis and seminal vesicles were weighted, fixed in 4% paraformaldehyde for morphological analysis and/or snap-frozen in liquid nitrogen and then stored at \(-80^\circ\text{C}\) for further use.

The testicular histology and spermatogenetic parameters were analyzed as described previously [15]. In brief, freshly separated testicles were fixed in Bouin fixation fluid, dehydrated and then embedded in paraffin. 5 \(\mu\text{m}\)-thick sections were prepared and stained with hematoxylin-eosin. Pictures of all testicular tissues covering two successive sections of each testicle were assessed by a pathologist who was blind to the sample grouping. All the convoluted tubules in each sample section were used to assess Sertoli cell vacuolization, tubules with deranged cell layer, percentage of stage VIII and VII seminiferous tubules. 4 stage VII seminiferous round tubules (the ratio of long to short axis diameter < 1.2) of the first observed field from each sample were used to assess the wall thickness, diameter, Sertoli cell number, total germ cell/Sertoli cells, spermatogonia/Sertoli cells, pachytene spermatocytes/Sertoli cells and round spermatids/Sertoli cells.

Meiotic prophase cell spreading and immunofluorescence staining

Spreads of spermatocytes and immunofluorescence staining were performed as previously described [25]. In brief, testes of these PBS/DEP-exposed mice were placed in hypotonic extracts (50mM sucrose, 17mM sodium citrate, 30mM Tris in pH8.2, 2.5mm DTT, 1mM PMSF in pH8.3 and 5mM EDTA), incubated on ice for 20 minutes and minced in 100mM sucrose. The
spermatocyte spreads were then prepared on a slide and solidified in 1% PFA containing 0.1% Triton X-100. The slides were incubated overnight in a damp room, dried and washed in PBS and water containing Photoflo (Kodak, NY, USA). Samples were first blocked using 10% donkey serum with 3% BSA, and then stained with primary antibodies γH2AX (1:500, Abcam), SYCP3 (1:100, Abcam) or DMC1 (1:100, Abcam) overnight at room temperature. Alexa 488 donkey anti-rabbit (1:500, Molecular Probes) and Alexa 594 goat anti-mouse (1:200, Molecular Probes) were then used as the secondary antibodies. The slides were incubated in darkness for 1 hour at 37°C, washed, mounted with Vecta shield cover slips (Vector Laboratories) and imaged using confocal microscope (Nikon N-STORM).

RNA sequencing and gene ontology (GO) analysis

5 testis samples from each group were collected for the deep RNA sequencing test. The transcriptome libraries were constructed according to the MGIEasy RNA Library Prep Set V3.0 (MGI). For each RNA library, 20 million clean reads were generated by BGISEQ 500(BGI-Shenzhen). After passing the base composition and quality tests, the sequence of adapter, high content of unknown bases and low-quality reads were removed. Differentially expressed genes were identified using cuffdiff with standard parameters and clustered by Genesis using a hierarchical clustering method. Go enrichment was analyzed using Metascape (metascape.org). A hypergeometric test was performed using the default parameters to adjust the p value.

Statistics

All data are expressed as means ± SEMs unless noted otherwise. Statistical tests were performed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni
correction or unpaired student’s t test using GraphPad Prism (version 5; GraphPad Software, La Jolla, CA, USA). The significance level was set at $p < 0.05$.

**Conclusion**

In summary, the present results demonstrate that long-term exposure to DEP impact spermatogenesis by disrupting meiotic prophase and thus impair the male reproductive function. To the best of our knowledge, our study is the first to use meiosis mechanism to analyze the reproductive system damage caused by DEP exposure, which will provide a new idea for the research on the male reproductive system damage caused by air pollution.
List of abbreviations

PM$_{2.5}$, ambient fine particles; CAP, concentrated ambient PM$_{2.5}$; DEP, diesel exhaust PM$_{2.5}$; ANOVA, analysis of variance; DSBs, double strand breaks; WHO, world health organization; BTB, blood-testis barrier; ROS, reactive oxygen species; ER, endoplasmic reticulum; PAHs, polycyclic aromatic hydrocarbons; HPG, hypothalamic-pituitary-gonadal; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone

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Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY, HP and SS acquired the data used in the present study. YX, WY, HP and FT analyzed and interpreted the present results. YX, ZY, RL and WL drafted the manuscript. YW, MX, MY, ST and HK were also major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Ethics approval
Fudan University is an AAALAC accredited institution. All procedures of this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Fudan University, and all the animals were treated humanely and with regard for alleviation of suffering.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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Table 1. Pregnancy outcomes. *p<0.05 versus PBS control, Chi-square test or student t test.

| Index                        | PBS       | DEP       | p value |
|------------------------------|-----------|-----------|---------|
| Stillbirth rate              | 1.12%     | 5.45%     | 0.12    |
| Absorption rate              | 13.48%    | 9.09%     | 0.43    |
| Implantations per dam        | 7.6 ± 0.84| 7.7 ± 1.3 | 0.96    |
| Live fetuses per dam         | 6.9 ± 0.88| 6.7 ± 1.26| 0.9     |
| Fetus weight (mg/fetus)      | 4.43 ± 0.68| 4.6 ± 1.05| 0.9     |
| Placental weight (mg/placenta)| 0.88 ± 0.1| 0.84 ± 0.12| 0.79    |
| Uterus weight (mg)           | 7.71 ± 0.89| 7.68 ± 1.55| 0.99    |
Table 2. Reproductive organ weights of male mice after 6-month intratracheal instillation of PBS/DEP. *p<0.05 versus PBS control, student t test.

| Weight (g)       | PBS         | DEP         | p value |
|------------------|-------------|-------------|---------|
| Body weight      | 29.27 ± 0.3698 | 29.29 ± 0.5804 | 0.9771  |
| Testis           | 0.2018 ± 0.0064 | 0.1834 ± 0.0143 | 0.2734  |
| Epididymis       | 0.0889 ± 0.0028 | 0.0877 ± 0.0021 | 0.7393  |
| Seminal vesicle  | 0.2606 ± 0.0173 | 0.2734 ± 0.0146 | 0.5782  |
**Figure Legends**

**Figure 1. Chronic exposure to DEP impairs the male fertility.**

*Figure 1.* Chronic exposure to DEP impairs the male fertility. **A.** Experimental scheme. **B.** Insemination capacity of male mice after 6-month intratracheal instillation of PBS/DEP. \( n = 10 \) /group, versus PBS, repeated measures chi-square test. **C.** Time taken for insemination. \( n = 10 \) /group, versus PBS, repeated measures Kaplan-Meier survival analysis. **D.** Impregnation capacity. \( n = 10 \) /group, \( p = 0.06 \) versus PBS, repeated measures chi-square test.

**Figure 2. Chronic exposure to DEP impairs the quality of semen.**

**Figure 2.** Chronic exposure to DEP impairs the quality of semen. **A.** Papanicolaou staining images of sperm morphology. **a,** normal sperm; **b-d,** abnormal sperm head; **e-f,** abnormal sperm tail. **B.** Sperm count in epididymis tissue of male mice after 6-month intratracheal instillation of PBS/DEP. \( n = 10 \) /group, \( * p < 0.05 \) versus PBS, student \( t \) test. **C.** Sperm motility of male mice after 7-month intratracheal instillation of PBS/DEP. \( n = 10 \) /group, \( * p < 0.05 \) versus PBS, student \( t \) test. **D.** Abnormal sperm percentage of male mice after 7-month intratracheal instillation of PBS/DEP. \( n = 10 \) /group, \( * p < 0.05 \) versus PBS, student \( t \) test.

**Figure 3. Chronic exposure to DEP alters the testicular histology.**

**Figure 3.** Chronic exposure to DEP alters the testicular histology. **A.** Representative H&E staining images of seminiferous tubule morphology in testis of male mice after 6-month intratracheal instillation of PBS/DEP. Yellow arrow: derangement of the cell layers of seminiferous tubules; Red arrow: Sertoli cell vacuolization. **B.** Proportion of Sertoli cell vacuolization based on H&E images of testis. **C.** Proportion of seminiferous tubules with deranged cell layer. **D.** Percentage of stage VIII seminiferous tubules. **E.** Percentage of stage VII seminiferous tubules. \( n = 10 \) /group, \( * p < 0.05 \) versus PBS, student \( t \) test.

**Figure 4. Chronic exposure to DEP results in a loss of advanced spermatogenic cells in Stage VII seminiferous tubules.**

**Figure 4.** Chronic exposure to DEP results in a loss of advanced spermatogenic cells in Stage VII seminiferous tubules. **A.** Stage VII seminiferous tubule wall thickness. **B.** Stage VII seminiferous tubule diameter. **C.** Sertoli cell number/Stage VII seminiferous tubule. **D.** Total
germ cells/Sertoli cells. E. Spermatogonia/Sertoli cells. F. Pachytene spermatocytes/Sertoli cells. G. Round spermatid/Sertoli cells. *p<0.05 versus PBS, student t test.

**Figure 5. Chronic exposure to DEP impairs the repair of meiotic double strand breaks (DSBs).** A. Double immunofluorescence images of surface-spread chromatin preparations of PBS/DEP-treated mice testes. Synapses of the homologous chromosome were observed by labeling SYCP3(red), a lateral element of the synaptonemal complex, and the initiation and repair of programmed DSB was observed by labeling γH2AX (green). White arrow: autosomal unfinished repair and sex vesicle formation failure. B. Representative double immunofluorescence images of surface-spread chromatin preparations of DEP-treated mice testes with labeling of SYCP3(red) and γH2AX (green). a-d: X and Y chromosomes can’t be paired, and sex vesicle can’t form. C. Percentage of abnormal XY body in pachytene stage cells. D. Percentage of abnormal homologous chromosome in pachytene stage cells. E. Percentage of spermatocytes in different stages. *p<0.05 versus PBS, student t test. F. Double immunofluorescence images of surface-spread chromatin preparations of PBS/DEP-treated mice testes with labeling of SYCP3(red) and DMC1(green). G. Number of DMC1 foci in zygotene stage cells. H. Number of DMC1 foci in pachytene stage cells. *p<0.05 versus PBS, student t test.

**Figure 6. Chronic exposure to DEP disrupts the spermatogenesis through impact on the repair of DSBs.** A. IHC assay with γH2AX specific antibody was performed on 18dpp testes. Yellow arrow: diffused distribution pattern of γH2AX expression represents leptotene/zygotene spermatocytes. Red arrow: focused distribution pattern of γH2AX expression represents pachytene/diplotene spermatocytes. B. Proportion of seminiferous tubules with the leptotene/zygotene spermatocytes. C. Proportion of seminiferous tubules with the pachytene/diplotene spermatocytes. *p<0.05 versus PBS, student t test.
Figure 7. Chronic exposure to DEP alters the testicular gene expression profile. A. Volcano plot of the identified testicular gene expressions. B. Heatmap of differentially expressed genes in the testes of Vehicle/DEP-exposed mice. C. 8 significantly enriched GO terms of the differential genes assessed by gene ontology (GO) analysis. n = 5/group.
Chronic exposure to DEP impairs the male fertility. A. Experimental scheme. B. Insemination capacity of male mice after 6-month intratracheal instillation of PBS/DEP. n = 10/group, versus PBS, repeated measures chi-square test. C. Time taken for insemination. n = 10/group, versus PBS, repeated measures.
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Figure 4
Chronic exposure to DEP results in a loss of advanced spermatogenic cells in Stage VII seminiferous tubules. A. Stage VII seminiferous tubule wall thickness. B. Stage VII seminiferous tubule diameter. C. Sertoli cell number/Stage VII seminiferous tubule. D. Total germ cells/Sertoli cells. E. Spermatogonia/Sertoli cells. F. Pachytene spermatocytes/Sertoli cells. G. Round spermatid/Sertoli cells. n = 10/group, *p<0.05 versus PBS, student t test.

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seminaliferous tubules with the leptotene/zygotene spermatocytes. C. Proportion of seminaliferous tubules with the pachytene/diplotene spermatocytes. n = 10/group, *p<0.05 versus PBS, student t test.

Figure 7

Chronic exposure to DEP alters the testicular gene 599 expression profile. A. Volcano plot of the identified testicular gene expressions. B. Heatmap of differentially expressed genes in the testes of Vehicle/DEP-
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