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Published in:
Environment International

DOI:
10.1016/j.envint.2022.107544

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

Document license:
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Citation for published version (APA):
Full length article

**Genome-wide alternation and effect of DNA methylation in the impairments of steroidogenesis and spermatogenesis after PM$_{2.5}$ exposure**

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**A R T I C L E   I N F O**

Handling Editor: Adrian Covaci

Keywords:
Male reproductive system
DNA methylene
PM$_{2.5}$
Spermatogenesis

**A B S T R A C T**

The effects of ambient fine particles on male reproductive health have raised widespread concern. The particular underlying mechanisms of the damage remain largely unclear and demand more research in new directions. Previous research has revealed that DNA methylation plays an important role in male reproductive development and is also vulnerable to environmental influences. However, there hasn’t been enough investigation into the involvement of DNA methylation in PM$_{2.5}$-induced male reproductive toxicity. Here, we establish a real-time PM$_{2.5}$ exposure model and revealed that PM$_{2.5}$ exposure could lead to testicular dysfunction including spermatogenesis impairment and steroid hormone dysfunction. In particular, the decrease in the testicular global level of 5-methylcytosine (5mC) indicated a possible association of DNA methylation with testicular injury induced by PM$_{2.5}$ exposure. Further genome-wide methylation analysis revealed genomic hypomethylation of testicular DNA and identified more than 1000 differentially methylated regions in both CAP and UA versus FA, indicating that PM$_{2.5}$ exposure, even low-dose, could modulate the testicular methylome. Furthermore, integrated analysis of methylome and transcriptome identified some key methylated genes and networks, which may be involved in spermatogenesis and synthesis of steroid hormone. The testicular methylation levels of key genes especially Cyp11a1 and P450 increased, and their consequent reduced expression may impair the testosterone and sperm production process. Our research provides fundamental knowledge as well as novel insights into the possible involvement of DNA methylation in PM$_{2.5}$-induced male reproductive harm.

1. Introduction

Ambient fine particulate matter (PM$_{2.5}$) continues to pose a substantial threat to human health worldwide (Markozannes et al., 2022). PM$_{2.5}$ and its adsorbed hazardous components can affect the gas exchange within the lungs, even escape into the bloodstream and eventually cause a variety of adverse health outcomes (Kim et al., 2015). Much research has been conducted to investigate the adverse effects of PM$_{2.5}$ on respiratory and cardiovascular systems (Pimpin et al., 2018; Wang et al., 2021). There has been emerging research that has drawn great attention to the reproductive toxicity of PM$_{2.5}$. Male fertility may be impacted by PM$_{2.5}$, which can also lower sperm quality, according to epidemiological findings (Carré et al., 2017; Huang et al., 2019). Multiple toxicological investigations in the laboratory have also shown that PM$_{2.5}$ has an adverse influence on the male reproductive system (Qiu et al., 2021; Zhou et al., 2019).

Identifying the underlying mechanisms of PM$_{2.5}$-induced male reproductive toxicity might help us better comprehend the dangers of...
PM$_{2.5}$. According to the available data, it is believed that PM$_{2.5}$ can directly lead to oxidative stress (Wei et al., 2018), DNA damage (Yauk et al., 2008), whole-body inflammation (Zhou et al., 2019), mitochondrial dysfunction (Shi et al., 2021), and eventually lead to male reproductive toxicity. Although these studies have made some progress in understanding the mechanisms of PM$_{2.5}$ harm to male reproductive, the specific underlying mechanisms largely remain unknown, necessitating more research in new directions. Epigenetic modification has a significant impact on many pathological processes (Han and Huang, 2021). Epigenetic change has been considered the most sensitive indicator of environmental effects in recent years, and the epigenetic genome is a significant target of environmental factors-induced modification (Meehan et al., 2018). As one of the most important epigenetic processes, DNA methylation is important in male reproductive development and is susceptible to environmental factors (Ma et al., 2018; Rotondo et al., 2021). According to some reports, abnormal sperm parameters and infertility are linked to alterations in DNA methylation (Santi et al., 2016; Tang et al., 2018). However, related research on the function of DNA methylation in PM$_{2.5}$-induced male reproductive damage has been insufficient so far. Recently, the development of “omics” technology can help researchers better understand the transcriptional regulatory mechanisms of environmental reproductive damage (Subramanian et al., 2020). Whole-genome bisulfite sequencing (WGBS) provides information at single-base resolution (Sun et al., 2015), which can comprehensively reflect the methylation status of specific active genes, making it an ideal tool for studying DNA methylation-mediated molecular mechanisms. To our best knowledge, there are still no genome-wide studies associated with PM$_{2.5}$-induced testicular DNA methylation change and it is still unclear which specific pathways and genes will be altered by epigenetic modifications in testes.

In this study, we established a real-time PM$_{2.5}$ exposure mouse model to assess the impact of PM$_{2.5}$ exposure-induced male reproductive damage. This research intended to profile the methyloxic landscape, uncover DNA methylation alterations, and identify some key methylated genes and networks of testes after PM$_{2.5}$ exposure through genome-wide methylome and transcriptome analysis.

2. Methods

2.1. Animal and ambient PM$_{2.5}$ exposure protocol

Male C57BL/6 mice aged 8 weeks, free of any specific pathogens were purchased from the Vital River Laboratory (Vital River, China). Mice were given basic mouse chow, distilled water, and a suitable environment. One week of acclimation is permitted before exposure. The Laboratory Animal Welfare and Ethics Care of the Third Medical University has approved this study for research ethics (AMU-WEC20193430). The animals were subjected to ambient PM$_{2.5}$ as reported in the earlier research to stimulate human exposure (Qiu et al., 2021; Yariwake et al., 2021). PM$_{2.5}$ exposure strategy and experimental groups were shown in Fig. 1A. Briefly, three groups of mice were randomly assigned: filtered air group (FA), unfiltered air group (UA), and concentrated ambient PM$_{2.5}$ group (CAP). The FA group was given ambient air that had been filtered by a highly efficient particulate air filter, whereas the UA group was given ambient air that had not been filtered. An enrichment system for PM$_{2.5}$ (Beijing Huironghe Technology Co., Ltd, China) was used on mice exposed to CAP, located in Tangshan city, Northern China. This device could concentrate PM$_{2.5}$ 6–10 times more than the ambient. Considering animal welfare and instrument maintenance, the exposure protocol was 6 h/day, 5 days/week, for a total of 8 weeks (November 2019-January 2020; coal burning period), with all mice being exposed at the same time (Zhou et al., 2019). Real-time concentrations of PM$_{2.5}$ were determined using an aerosol monitor (TSI Instrument Co., Ltd, USA). Quartz filter membranes (Whatman, UK) were used to collect PM2.5 particles. The National Institute of Measurement and Testing Technology (Chengdu, China) next examined the membranes for water-soluble inorganic ions, polycyclic aromatic hydrocarbons (PAHs), and metal elements.

2.2. Testicular pathological and spermatogenetic parameters analysis

Blood was obtained from the orbital plexus of mice. The testes and epididymis were weighed and one was fixed in Bouin’s solution for
morphological analysis and the other was snap-frozen in liquid nitrogen and then stored at -80 °C. According to standard procedures, the fixed tissues were followed by hematoxylin and eosin (H&E) staining. Then, the spermatogenetic parameters were analyzed (Ahmed and de Rooij, 2009). Briefly, pictures of all testicular tissues were evaluated by the pathologists in a double-blind way (Qiu et al., 2021). Round tubules in stage VII were assessed for average diameter, epithelial height, and the number of Sertoli cells, spermatogonia, pachytene spermatocytes, round spermatids, and total germ cells as reported in the research.  

2.3. Sperm count and motility analysis  
The epididymis was placed in 0.8 mL of Ham’s F-12 Nutrient Mixture (Gibco, USA) and then 2 cuts were made in cauda to allow the sperm to free up. Sperm counts and motility were analyzed using a computer-assisted sperm assay system (Siuplus, China). A minimum of 500 sperm per sample was analyzed.  

2.4. Reproductive hormones assessment  
Normal endocrinology is important for the male reproductive system, with steroid hormones playing an important role in spermatogenesis (Rehman et al., 2018). Testosterone (T), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme Link Biotechnology Co., Ltd., China). Testicular samples from 7 mice per group were randomly selected for testicular hormone evaluation and the levels were normalized by protein concentrations.  

2.5. RNA, DNA, and protein extraction  
Testicular RNA, DNA, and protein were isolated using Trizol (Invitrogen, USA), Bio-Tek DNA Kit (Omega, Georgia, USA), and BCA protein assay kit (Beyotime, Shanghai, China), respectively. Samples for the above extraction experiments were obtained from the same testicular tissues. Testicular samples used to extract DNA/RNA for sequencing were randomly selected from each group.  

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)  
The qRT-PCR assay was performed using the GoTaq® qPCR Master Mix (Promega, USA) and the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories) according to standard procedures. The mRNA expression was calculated by \(2^{-\Delta\Delta CT} \). The primers used in this study were listed in Table S1.  

2.7. 5mC determination  
After quantification of DNA, methylation analysis of the same amount of genomic DNA was performed using MethylFlash™ Methylated DNA Quantification Kit (Epigentek, USA).  

2.8. Whole-genome bisulfite sequencing (WGBS)  
According to the TrueMethyl Seq Kit’s instructions (TrueMethyl Seq Kit, CEGX, Cambridge Epigenetix Limited), the library was prepared. To ensure representative samples and avoid the effects of biological variation (Assfalg et al., 2020; Churchill and Oliver, 2001), two libraries were constructed for each group, each library containing pooled testicular DNA from four mice. Phosphokinase and DNA polymerase were present at the end repair stage, which followed the fragmentation. To determine the oxidative conversion rate, control DNA was spiked. The repaired DNA was then added to the tailing and purified using magnetic beads before being denatured and oxidized to create bisulfite-sequencing libraries. The DNA was oxidized, then transformed with sodium bisulfite. After that, TaqI enzymes were used to amplify and qualify the digestion control. Finally, the libraries were amplified, sequenced, and filtered. The cleaned reads were mapped back to the mouse genome (mm10) using BSMAP software version 2.73 (Xi and Li, 2009). The ratio of methylated cytosine to total cytosines at a specific location was used to determine methylation levels. For subsequent analysis, only cytosines in a CpG context with adequate sequencing depth were chosen. Metilene was used to identify differentially methylated regions (DMR) with default parameter settings (Jühling et al., 2016). DMRs must meet the conditions as differentially methylated regions with a length greater than 50 bp. An initial cut-off of 10 % differential methylation was set for statistical significance.  

2.9. Transcriptome sequencing  
The sequencing libraries were constructed using the NEBNext Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA). The quality of libraries was normalized and evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and then sequenced on an Illumina sequencing platform. Raw data in the fastq format were processed for quality control. Paired clean reads were aligned to the reference genome using Hisat2 v2.0.5 and then the read counts mapped to each gene were calculated using Htsq-counter. The reads are then mapped to the FPKM of each gene. The Deseq2 R software (1.16.1) was used to conduct differential expression analysis, with a threshold of \( P < 0.05 \).  

2.10. Bioinformatic analysis  
IPA software was used to analyze the pathways, diseases and biological function, and networks in our study (Thomas and Bonchev, 2010). Promoter-linked DMR-associated genes (PDMGs) in CAP versus FA were selected for the IPA analysis. Meanwhile, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed by DAVID (version v2022q1). All DMRs-associated genes (DMGs) in both CAP and UA versus FA, and promoter-linked DMR-associated genes (PDMGs) in CAP versus FA were selected to input the above enrichment analysis. Gene Set Enrichment Analysis was conducted based on differentially expressed genes between CAP and FA. \( P \) value \(< 0.05 \) was defined as significant in all of the enrichment analyses.  

2.11. Western blot  
The extracted proteins were quantified using a BCA protein assay kit (Beyotime, China). Membranes were immunoblotted and followed by incubation with corresponding secondary antibodies (Abcam, Cambridge, UK). The blots were detected by chemiluminescence and quantified by ImageJ software. Primary antibodies were as followed: anti-CYP1A1 (1:1000; Bioss, Beijing, China), anti-PAX8 (1:1000; HUABIO, Hangzhou, China), and anti-β-actin (1:1000; Cell Signaling Technology, Boston, USA).  

2.12. Statistical analysis  
Data were expressed as means ± SEM. The association analysis was analyzed using the Pearson method by R software (version 3.5.3). The differences among more than 2 groups were analyzed using one-way ANOVA followed by Tukey’s post hoc test using Graphpad Prism (version 9; USA). \( P \) value \(< 0.05 \) was considered statistically significant. The exact times, conditions, and sample size of each experiment were described in the figure legends.
3. Results

3.1. PM$_{2.5}$ exposure characterization

As depicted in Fig. 1A, male mice were exposed to either FA, UA, or CAP in the dynamic whole-body exposure system for 8 weeks. PM$_{2.5}$ concentration of the CAP group fluctuated as the real-time ambient PM2.5 level changed (Fig. 1B). Since PM$_{2.5}$ is a mixture of several components, an assessment of its composition is necessary. NO$_3^-$, SO$_4^{2-}$, NH$_4^+$, and Cl$^-$ are the most abundant water-soluble inorganic ions in PM$_{2.5}$, as shown in Fig. 1C. The top abundant PAHs components were benzo(b)fluoranthene (BbF), benzo(a)anthracene (BaA), fluoranthene (FLT), pyrene (PYR), chrysene (CHR), benzo(a)pyrene (BaP), indeno (123-c,d)pyrene (IPY), benzo(g,h,i)perylene (BPE), benzo(k)fluoranthene (BkF), and anthracene (ANT). The most abundant metals were Zn, Pb, and Mn (Fig. 1D).

![Fig. 2. PM$_{2.5}$ exposure leads to the spermatogenesis impairment](image)

(A) The representative pathological images of testes. Triangle, interrupted basement membrane. Black arrowhead, vacuolization. (B-C) Spermatogenic parameters including seminiferous tubules diameter (B) and epithelial height (C) were measured in stage VII seminiferous tubules ($n=15$). (D-H) The counts of Sertoli cells (D), spermatogonia (E), pachytene spermatocytes (F), round spermatids (G), and total germ cells (H) per stage VII seminiferous tubule ($n=15$). (I-L) The ratio of spermatogonia/Sertoli cells (I), pachytene spermatocyte/Sertoli cells (J), round spermatid/Sertoli cells (K), total germ cells/Sertoli cells (L) ($n=15$). One-way ANOVA followed by Tukey’s multiple comparisons. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with FA-treated group.
3.2. PM$_{2.5}$ exposure induced sperm quality decline and the spermatogenesis damage

After PM$_{2.5}$ exposure, the final body weight, absolute organ weight, and organ coefficients of mice were not altered significantly among the three groups (Fig. S1). However, our previous study has reported a significant decrease in sperm quality after concentrated PM$_{2.5}$ exposure in mice (Shi et al., 2021). To define the cause of sperm quality decline, a histological examination of the testis was performed. The FA group showed normal seminiferous tubules and germ cells were arranged normally. Slight disorganization of the spermatogenetic cells and vacuolation were found in the UA group. For testes of the CAP group, vacuolar degeneration and interrupted basement membrane were found in some of the seminiferous tubules (Fig. 2A). In addition, the spermatogenetic parameters of seminiferous tubules were evaluated to further define the effects of PM$_{2.5}$ exposure on spermatogenesis. Seminiferous tubule diameter showed a decreasing trend in the PM$_{2.5}$-exposed groups but with no significant difference (Fig. 2B). Compared with the FA group, epithelial height was reduced markedly in the CAP group (Fig. 2C). The counts of Sertoli cells in the CAP and UA groups were not significantly influenced (Fig. 2D). The numbers of spermatogonia in these seminiferous tubules showed a decreasing trend (Fig. 2E). In contrast, pachytestine spermatocytes, the average numbers of total germ cells, and round spermatids per seminiferous tubule were significantly reduced in the CAP group (Fig. 2F-2H). Further, these effects of CAP exposure on germ cells remained significant after normalization with the number of Sertoli cells (Fig. 2I-L). When comparing mice between the FA and UA groups, we found no significant differences in spermatogenetic characteristics (Fig. 2B-2I).

3.3. PM$_{2.5}$ exposure induced testicular steroid hormones dysfunction

To define if reproductive endocrine was interrupted by PM$_{2.5}$, we first assessed testosterone, FSH, and LH levels in serum by ELISA. Serum testosterone levels were significantly reduced in the CAP group, but there was no statistically significant difference in the UA group (Fig. 3A). Meanwhile, the UA and CAP groups had considerably higher serum LH levels than the FA group (Fig. 3B). However, no significant differences in FSH were observed among the FA, UA, and CAP groups (P greater than 0.05, Fig. 3C). T/LH ratios were found to be significantly lower in all PM$_{2.5}$-exposure groups (Fig. 3D), which could indicate the functional capacity of the Leydig cell to a certain extent (Muerkoster et al., 2020). Consistent results were also found in the testis tissue (Fig. 3E-3H).

3.4. PM$_{2.5}$ exposure induced genome-wide hypomethylation in the testis

Primarily, we evaluated whether PM$_{2.5}$ exposure could cause changes in the global 5-methylcytosine (5mC) level of testicular DNA. The level of testicular 5mC was significantly reduced in the CAP group, and the UA group showed a declining tendency with no statistical significance, compared with the FA group respectively (Fig. 4A). The correlation between the global level of 5mC and representative reproductive outcomes indicated that DNA methylation was likely involved in the testicular injury induced by PM$_{2.5}$ exposure (Table S2). Subsequently, we aimed to profile the methylomic landscape and uncover DNA methylation alterations on isolated testes following PM$_{2.5}$ exposure at a single-nucleotide resolution using the WGBS technique (Fig. 4B). Adequate DNA with good quality was sequenced and used for further analysis. In total, about 90 Gb of raw bases were obtained across samples and all of the bisulfite conversion rates were higher than 99 %, indicating integrity and accuracy. What is more, 17.7–18.3 million CG sites were available for measuring the methylation variation in all samples with at least 10 × coverage (Table S3). We then compared the global methylation rates among the three groups according to the mean rate derived from WGBS libraries. Consistent with the previous detection, the global DNA methylation rate showed a decreasing tendency in PM$_{2.5}$ exposure groups compared with the control (Fig. 4C). Even though the comparison of methylation levels in CpG cytosines showed similar genomic features, the distribution of average methylation levels

![Fig. 3. PM$_{2.5}$ exposure induces testicular endocrine dysfunction](image-url)
Fig. 4. PM$_{2.5}$ exposure alters methylation patterns in testes (A) Global 5-methylcytosine (5mC) level of testicular DNA detected by ELISA. (B) Schematic illustration of the genomic methylation sequencing. (C) Global DNA methylation levels of all CpG sites across the genome in testes by WGBS. (D) The genomic features of relative density of 5mC within function genomic region mm10. (E) Identification of DMRs in PM$_{2.5}$-exposure groups versus FA. (F and H) The depiction of the significant DMRs along the chromosomes in CAP (F) and UA (H), respectively. (G and I) The pie graphs show the genomic location (promoter, 5utr, exon, intron, 3utr, intergenic) of identified DMRs in CAP (G) and UA (I), respectively (upper panel, hyper-DMRs; lower panel, hypo-DMRs). (J) Enrichment analysis of the biological process for DMRs-associated genes (DMGs). Groups are shown at the bottom. The color of the dot indicates the $P$ value, and the size of the dot is the enrichment score in the given biological process. ANOVA followed by Tukey’s post hoc test. **$P < 0.01$. 
of CpG in genomic regions showed that the CAP and UA groups presented a hypomethylation paradigm trend compared with the control (Fig. 4D).

3.5. Features of differentially methylated regions (DMRs) identified in testes after PM$_{2.5}$ exposure

Next, we attempted to determine the methylation differences that might be relevant to PM$_{2.5}$-induced reproductive disorders by calculating the differentially methylated regions (DMRs) among the FA, UA, and CAP groups under the CG context. Applying a false discovery rate (FDR) < 0.05 and CpG number $\geq$ 5 returned 1374 DMRs including 661 significant hypermethylated DMRs and 713 hypomethylated regions in the testes of CAP compared with control (Fig. 4E). At the same strict threshold, we identified 1420 DMRs between the UA and FA group, of which 643 was hypermethylated and 777 was hypomethylated (Fig. 4E). Depicting the DMRs along the chromosomes confirmed a preponderance of hypomethylation with the amount of hypo-DMRs being higher than hyper-DMRs in most chromosomes in the CAP and UA group (Fig. 4F and 4H). In addition, these DMRs were widespread across the genome and were mostly located on autosomes and fewer located on sex chromosomes. Notably, chromosome 5 (chr5) had the largest number of DMRs (97 DMRs in CAP versus FA and 110 DMRs in UA versus FA). Subsequently, we defined feature sets spanning sub-typed by the location of the DMRs. As reported in other genome-wide methylation studies (Colwell et al., 2021), the genomic location of identified hyper and hypo-DMRs showed that the majority of DMRs both in CAP and UA groups were located in intergenic and intron regions (Fig. 4G and 4I). Based on the above analysis, these comparisons among PM$_{2.5}$ exposure and control samples identified more than 1000 DMRs, revealing profound differences in gene methylation, indicating that even low-dose PM$_{2.5}$ exposure could modulate the methylome of testes.

![Fig. 5. Characterization of PM$_{2.5}$-induced changes in promoter-associated DMRs](image-url)
3.6. Functionally associated genes based on DMRs

To determine whether any particular biological processes were being selectively altered in their DNA methylation as a result of PM$_{2.5}$ exposure, we then annotated all DMRs-associated genes (DMGs) and conducted ontology analysis. The DMGs both in CAP and UA versus FA enriched in many biological processes such as calcium ion transport and cell migration, which might be involved in the PM$_{2.5}$-induced reproductive function damage (Fig. 4J). Considering DNA methylation in gene promoter regions important for gene transcriptional regulation, we then set our sights on DMRs within gene promoter regions. We obtained 88 promoter-linked DMRs (93 genes) in CAP compared with the FA group (Table S4). Promoter-linked DMR-associated genes (PDMGs) in PM$_{2.5}$-exposure groups were partially presented in Fig. 5A. To understand the biological relevance of these PDMGs in the testes, we performed a functional enrichment analysis. Diseases and biological functions analysis from IPA showed that spermatogenesis was ranked at the top of the list in these PDMGs of CAP versus FA (Table S5). Similar to the IPA analysis, for the most associated biological process based on the ranking of -log10p, synaptonemal complex assembly, meiotic cell cycle, spermatogenesis, and fertilization were listed in the top related functions (Fig. 5B). Meanwhile, the bubble diagram suggested that the genes in the spermatogenesis pathway were more likely to be hypermethylated than hypomethylated in testes after PM$_{2.5}$ exposure (Fig. 5B). As displayed in the Manhattan plot, Meioc, Pnldc1, Tacc2, Olfr279, and Tex11 were ranked at the top of the -log10 FDR value (Fig. 5C). Among them, Meioc, Pnldc1, and Tex11 were reported to be related to spermatogenesis (Nishimura et al., 2018; Yu et al., 2021; Abby et al., 2016), with Meioc loci being significantly hypermethylated in the PM$_{2.5}$-exposed testes compared with controls (Fig. 5D, Table S4).
3.7. PM$_{2.5}$ exposure disrupted transcriptomic profiles of testes

As the testicular DNA methylome has been changed by PM$_{2.5}$ exposure, it is necessary to study whether the transcriptional profiles have been affected. To access possible testicular dysregulation of gene transcription as a result of PM$_{2.5}$ exposure, RNA sequencing (RNA-seq) was conducted on testes (Fig. 6A). Transcriptome analysis showed 92 and 285 differentially expressed genes in CAP and UA compared with FA-exposed testes, respectively (Fig. 6B). Pathway analyses were used to identify the significant pathways associated with the DEGs in CAP, revealing that the top affected pathways were renin-angiotensin system, cortisol synthesis and secretion, steroid hormone biosynthesis, endocrine and other factor-regulated calcium reabsorption, and ovarian steroidogenesis (Fig. 6C). Consistently, GSEA analysis also showed significant downregulation of steroid hormone biosynthetic process (Fig. 6D). This pathway is closely related to the observed steroid hormone dysfunction caused by PM$_{2.5}$ exposure, so we focused on this pathway for further analysis. Cyp11a1, Cyp17a1, and Hsd3b1 are key genes in this pathway and have an important role in testosterone production (Fig. 6E). As shown in the heat maps (Fig. 6F), the mRNA levels of these genes were significantly lower in CAP and UA compared to the FA group, spermatogenesis-related genes were also drastically altered in PM$_{2.5}$-exposed groups. Collectively, PM$_{2.5}$ exposure disturbed transcriptomic profiles of testes and altered the steroidogenesis and spermatogenesis-associated genes in mice testes.

3.8. Integrated analysis of methylome and transcriptome to identify key methylated genes associated with PM$_{2.5}$ exposure

To identify the potential relationship between DNA methylation changes with gene expression as a result of male PM$_{2.5}$ exposure, an integrated analysis of methylation and transcriptomic profiles was then performed. Among the genomic locations, only the promoter’s methylation level was negatively correlated with gene expression (Table S6, P < 0.001). Meanwhile, methylation of the promoter is currently considered to be the most important for affecting transcription (Lev Maor et al., 2015), so we decided to focus on the DMRs located in the promoter region. We identified the differentially expressed genes associated with DMRs within promoters (methylation change $\geq 0.1$, P < 0.01, nCGp $\geq 3$). The integrative analysis of PDMGs and DEGs revealed some interesting candidate genes for study. As shown in the heat maps of the DNA methylation and expression level (Fig. 7A), the decreasing expression of most genes was associated with an increased methylation level. We found 11 well-documented markers of steroid biogenesis and spermatogenesis, the molecular types of which mainly included enzymes (Cyp11a1, Hsd11b1, and Fads2) and transcription regulators (Pax8 and Myc). The network of these genes constructed by IPA was depicted in Fig. 7B and the description of the identified key genes was shown in Table S7. According to the network, genes involved in Aryl hydrocarbon receptor (Myc and Ahr), STAT3 pathway (Myc and Fgfr2), WNT/j- catenin signaling (Wnt4 and Nras2), and pregnenolone biosynthesis (an intermediate process in the synthesis of testosterone; Cyp11a1) were identified. These methylation-regulated genes and pathways were closely linked to abnormal morphology of the reproductive system, synthesis of steroid hormone, and spermatogenesis (Fig. 7B). These methylation-regulated genes and networks were likely to mediate the male reproductive toxicity of PM$_{2.5}$. We then examined the transcriptional levels of these genes through qRT-PCR. The expression of steroidogenesis-related genes (Cyp11a1 and Wnt4) was significantly reduced in the CAP group (Fig. 7C). Altered transcript levels of Cyp11a1 may be directly related to the observed testosterone decline. The expression level of spermatogenesis-related genes (Pax8 and Myc) in the CAP group was also significantly lower than in the FA group (Fig. 7D). Notably, Pax8 was the one of most significantly decreased candidate genes in the CAP and UA groups compare with FA (Fig. 7D). Therefore, Cyp11a1 and Pax8 were mainly selected for further study. As shown in Fig. 7E-F, methylation levels of Cyp11a1 and Pax8 were visualized, showing that methylation of the representative CpG sites within DMRs in their promoter regions was elevated in PM$_{2.5}$-exposed testes compared to the FA group. We further examined the protein expression of Pax8 and Cyp11a1 and found the down-regulation of their protein levels in the CAP group, with no significance in the UA group, compared with the control respectively (Fig. 7G-H).

4. Discussion

In this study, a mouse model of PM$_{2.5}$ exposure, simulating the daily human exposure mode, was established to assess the effects of PM$_{2.5}$-induced male reproductive damage. The results indicated that PM$_{2.5}$ exposure could induce testicular steroidogenesis and spermatogenesis impairments in mice. We further profiled the methylomic landscape and uncovered DNA methylation alterations of testes after PM$_{2.5}$ exposure through genome-wide methylome analysis. According to the integrative analysis of methylome and transcriptome, DNA methylation-associated genes and networks might be involved in spermatogenesis and steroidogenesis impairment as a result of PM$_{2.5}$ exposure.

The association between PM$_{2.5}$ exposure and male reproductive toxicity has raised extensive public concerns. Investigating the effects of PM$_{2.5}$ exposure on male reproductive damage is critical to preventing diseases and maintaining public health. The PM$_{2.5}$ exposure procedure was designed to take into account environmental exposure levels and previous studies (Yang et al., 2019; Zhou et al., 2019). Given the exposure design of 6 h per day, 5 days/week (Qiu et al., 2018), the applied PM$_{2.5}$ concentration in the UA chamber during this period was 10.67 $\mu$g/m$^3$ when calculating for 24-h average, while the CAP group’s 24-hour average was 86.36 $\mu$g/m$^3$. This level of the UA group (10.67 $\mu$g/m$^3$) was roughly comparable to the WHO standard of 10 $\mu$g/m$^3$. Meanwhile, PM$_{2.5}$ level in the CAP averaged 86.36 $\mu$g/m$^3$ during a 24-hour period, which was very common in the most polluted regions according to the state of the global air (Lim et al., 2020). Carrying out a study of this level might make sense in this situation for those who live in places with a lot of PM$_{2.5}$ pollution. The damaging effects of PM$_{2.5}$ may vary depending on the components it adsorbs. In this study, we found that PM$_{2.5}$ was rich in PAHs, metals, and water-soluble ions, of which PAHs and metals have been widely shown to be damaging to male reproduction (Baralić et al., 2022; Ling et al., 2016). Among the constituents, especially benzo[a]anthracene and high molecular weight PAHs (Chen et al., 2021) and metals including Cd, Pb, and As (Kumar and Sharma, 2019; Baralić et al., 2022; López-Botella et al., 2021) have been reported to be correlated with male reproductive health including spermatogenesis and reproductive hormones dysfunction, indicating a reference for risk assessment of PM$_{2.5}$ and prevention of sensitive populations.

Spermatogenesis is a multistep process that happens within the spermatogenic epithelium of the testis and includes three stages: spermatogonia mitosis, spermatocyte meiosis, and spermatozoa maturation (Krausz and Riera-Escamilla, 2018). Environmental factors may cause a reduction in male fertility by disrupting this process. The results of the spermatogenic parameters in this study are consistent with previous studies (Qiu et al., 2018), which indicates that spermatogenesis abnormalities may be one of the major adverse effects induced by concentrated PM$_{2.5}$ exposure. In addition, androgens play a role in spermatogenesis. Testosterone is a key androgen generated mostly by testicular Leydig cells (Christin-Maitre and Young, 2022). Another discovery was that the level of testosterone was considerably reduced in both circulating and testicular tissues after PM$_{2.5}$ exposure, which may lead to an increase in LH in a negative feedback manner. Hypothalamus-pituitary–gonadal axis (HPG axis) is vital to regulating reproductive hormones (Jin and Yang, 2014). Hypothalamic inflammation due to PM$_{2.5}$ exposure has been reported to affect the production of gonadotropin-releasing hormone (GnRH), which will affect the release of FSH and cause the decline of FSH level (Qiu et al., 2018). Therefore,
Fig. 7. Integration of WGBS and transcriptomic profiles reveals dysregulation of candidate epigenetic genes and networks in PM$_2.5$-induced testicular injury. (A) The heat maps of promoter methylation and expression levels of candidate functional genes related to steroid biosynthesis and spermatogenesis. (B) Network of candidate genes involved in reproductive functions. (C) Validation of the mRNA expression levels of candidate steroidogenesis-linked genes in testes by real-time PCR ($n = 6$). One-way ANOVA followed by the Tukey test for multiple comparisons is used. (D) Validation of the mRNA expression levels of candidate spermatogenesis-linked genes in testes by real-time PCR ($n = 6$). Data are expressed as the mean ± SEM. One-way ANOVA with Tukey post hoc test. (E) Methylation profiles of representative CpGs within DMRs of Cyp11a1. Two-way ANOVA with Tukey post hoc test. (F) Methylation profiles of representative CpGs within DMRs of Pax8. Two-way ANOVA with Tukey post hoc test. (G) The protein expression levels of candidate genes in testes by western blot analysis. (H) Proteins quantitative analysis of CYP11A1 and PAX8 ($n = 6$). One-way ANOVA with Tukey test for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with FA-treated group.
no apparent change in FSH might be attributed to the combined factors of HPG axis suppression and testicular injury. Notably, although the UA group was not affected in terms of spermatogenesis, there were moderate differences in steroid hormone levels, suggesting that low-dose PM$_{2.5}$ could also cause changes in sensitive markers like hormone levels. The reduction in steroid hormone level was also supported by altered steroid hormone synthesis pathways in transcriptomics and decreased testis level expression of testosterone biosynthetic enzymes. In a word, the above data suggested that PM$_{2.5}$ exposure could induce steroid hormone disturbance and spermatogenesis damage, providing compelling evidence for male reproductive toxicity.

The impact of environmental factors on epigenome is one of the current research hotspots (Schädarsurengin and Steger, 2016). The global level of DNA methylation observed in this study was consistent with previous research (Li et al., 2019), which may be caused by the imbalance between DNA methylation and demethylation. The causes of DNA methylation changes especially the expression levels of DNA methyltransferases and ten-eleven translocation enzymes after PM$_{2.5}$ exposure awaits further study. In addition, several current studies have only established the association between PM$_{2.5}$ exposure and global methylation level, which could not comprehensively reflect the methylation status of specific active genes, limiting further molecular mechanisms. To our best knowledge, this is the first whole-genome study to analyze the DNA methylation differences of the testis by PM$_{2.5}$ exposure. The results showed that PM$_{2.5}$ altered the DNA methylation of the testis, presenting hypomethylation patterns in genomic distribution. Interestingly, although some serious outcomes of the low-dose PM$_{2.5}$ exposure (UA group) have not been discovered, the methylation of testis has changed on a large scale. This might be due to epigenetic changes taking place earlier than pathophysiological changes (Ma et al., 2022). Given that DNA methylation of the promoter is pivotal for gene transcriptional control, we focused on DMRs inside the promoter. The findings revealed that hypermethylated genes were enriched in spermatogenesis-related gene ontology (GO) terms. Meioic and Pnldc1 were at the top of the -log10 FDR list of candidate spermatogenesis-related genes. Meioic has been found to operate as a transcriptional stabilizer in spermatogenesis and meiosis (Abby et al., 2016). Recent research revealed that Pnldc1 mutations might impair piRNA processing during meiosis and spermatogenesis (Nagirnaja et al., 2021). Thus, these genes’ hypermethylation was likely engaged in the process of spermatogenic damage.

Some overlapping genes between PDMGs and DEGs based on combined RNA-seq and WGBS data were further studied. These genes were involved in the STAT3 pathway and WNT/-catenin signaling, which were concerned as the established environmental toxic pathways and were important for spermatogenesis and testosterone production (Nagasawa et al., 2018; Kerr et al., 2014; Takase and Nusse, 2016; Fu et al., 2020). Meanwhile, our previous study has reported the potential function of the Aryl hydrocarbon receptor pathway in spermatogenesis in vitro (Shi et al., 2021). Cyp11a1 (Zhao et al., 2022), and Pdx8 (Sarkar et al., 2019; Wistuba et al., 2007), selected for further analysis, were reported to play important roles in testosterone production and spermatogenesis, respectively. Both mRNA and protein levels of Pdx8 were altered in the CAP group. The mRNA level of Pdx8 in the UA group differed greatly, but its protein level did not change, which may be due to the post-transcriptional regulation in this dose group. In a human investigation, DNA hypermethylation of Pdx8 was shown to be strongly linked to DNA concentration (Houshdaran et al., 2007), suggesting to some extent that altered epigenetic modifications of Pdx8 may be closely related to spermatogenesis. In addition, the other candidate genes identified from the integrated analysis including Wnt4 and Myc also need to be explored in future studies.

The interaction between environmental factors and epigenetics in the field of environment and health research has been getting more and more attention. To date, few genome-wide methylation studies have been performed on the role of DNA methylation in male reproductive impairment induced by PM$_{2.5}$ exposure. This work could constitute a baseline for other future studies focusing on the potential mechanisms of reproductive injury-associated epigenetic regulation caused by environmental pollutants. However, there are also some limitations in this study: First of all, although we identified some genes linked by DNA methylation, we acknowledge that the results are largely descriptive and cannot establish a causal relationship between DNA methylation and transcription. Further restorative experiments and differential expression tests are necessary to be performed. Second, focusing on the methylation of promoter regions is the strategy we have adopted so far, but this has the potential to obscure the in-depth analysis of the methylation after PM$_{2.5}$ exposure. Third, the detection of internal exposure is also important, but due to methodological limitations, we were not able to detect PM$_{2.5}$ in the urine and blood of mice. Fourth, given that the testis tissue consists of a variety of cells, using the whole testis tissue rather than distinguishing different types of cells for sequencing may introduce confounding factors that affect data mining. Future application of single-cell techniques could provide more accurate results to further reveal the underlying mechanisms of PM$_{2.5}$-induced reproductive toxicity. Fifth, since gene expression is regulated by several factors, including DNA methylation as well as several other factors, such as upstream regulators and histone modifications, relying just on DNA methylation does not offer a complete explanation of the process. It is also expected that researchers would look into how DNA methylation interacts with other epigenomic layers.

In conclusion, this study indicated that PM$_{2.5}$ exposure could cause testicular dysfunction including spermatogenesis and steroid hormone disturbance. We determined that PM$_{2.5}$ could alter the methylation of testes, suggesting that DNA methylation is likely to be involved in the process of male reproductive damage caused by PM$_{2.5}$. Integrative analysis of testicular genome-wide DNA methylation and transcriptome identified key genes and pathways related to steroidogenesis and spermatogenesis processes, which may be the DNA methylation-regulated mechanism in male reproductive impairment caused by PM$_{2.5}$. This research might give important information and new perspectives on the underlying epigenetic mechanisms in PM$_{2.5}$-induced male reproductive toxicity.

Credit authorship contribution statement

Zhonghao Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Jiankang Wang: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization. Fuquan Shi: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization. Yingqing Li: Methodology, Data curation, Software. Peng Zou: Methodology, Data curation, Software. Ying Tang: Methodology, Data curation, Software. Chang Liu: Methodology, Data curation, Software. Yimeng Wang: Data curation, Software. Xi Ling: Data curation, Software. Lei Sun: Data curation, Software. Cuiling Liu: Project administration, Supervision, Methodology. Yanshu Zhang: Project administration, Supervision, Methodology. Fei Gao: Formal analysis, Software, Visualization. Lin Ao: Conceptualization, Methodology. Fei Han: Conceptualization, Methodology, Writing – review & editing. Jinyi Liu: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. Jia Cao: Conceptualization, Methodology, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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