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PM2.5 exposure impairs sperm quality through testicular damage dependent on NALP3 inflammasome and miR-183/96/182 cluster targeting FOXO1 in mouse

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ABSTRACT

Exposure to ambient fine particular matter (PM2.5) has been clearly associated with male reproductive disorders. However, very limited toxicological studies were carried out to investigate the potential mechanisms underlying the PM2.5-induced sperm quality decline. In the present study, we established a real time whole-body PM2.5 exposure mouse model to investigate the effects of PM2.5 on sperm quality and its potential mechanisms. Sixty male C57BL/6 mice were randomly subjected to three groups: filtered air group, unfiltered air group and concentrated air group. Half of the mice from each group were sacrificed for study when the exposure duration accumulated to 8 weeks and the rest of the mice were sacrificed when exposed for 16 weeks. Our results suggested that PM2.5 exposure could induce significant increases in circulating white blood cells and inflammation in lungs. PM2.5 exposure induced apparently DNA damages and histopathologic changes in testes. There were significantly decreased sperm densities of mice, which were paralleled with the down-regulated testosterone levels in testes tissue of mice after exposure to PM2.5 for 16 weeks. The numbers of motile sperms were decreased and sperms with abnormal morphology were increased after PM2.5 exposure in a time-depended and dose-depended manner. PM2.5 exposure significantly increased the expression of the major components of the NACHT, LRR and PYD domains-containing protein3 (NALP3) inflammasome, accompanied by the increased expression of miR-183/96/182 targeting FOXO1 in testes. The present data demonstrated that sperm quality decline induced by PM2.5 could be partly explained by the inflammatory reaction in testes which might be a consequence of systemic inflammation. The molecular mechanism was depended on the activation of NALP3 inflammasome accompanied by miR-183/96/182 targeting FOXO1 in testes.

1. Introduction

Infertility has become a global public health threat and it was estimated to affect 8–12% of reproductive-aged couples worldwide (Ombelet et al., 2008). Male factor was considered to make contribution more than 50% (Mascarenhas et al., 2012). Many studies in recent years indicated that sperm quality has been considerably decreased over the past several decades (Merzenich et al., 2010) in many areas of the world but the etiology was currently unknown. Possible factors included unhealthy lifestyle, tobacco smoking, excessive alcohol consumption, radiation exposure as well as chronic exposure to hazardous chemicals have been verified to be related with decreases of sperm quality (Gabrielsen and Tanrikut, 2016). Air pollution exposure has gained more interest because its potential side effects on sperm quality (Najafpour et al., 2018; Nassan et al., 2018). Particulate matter in the respirable range, especially fine particles with diameters less than 2.5 μm (PM2.5), was of particular interest because they could penetrate into blood carrying multiple heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Gao et al., 2018; Lu et al., 2017). Heavy metals has been shown to induce adverse effects on mammalian reproductive system (Egbowon et al., 2016; Li et al., 2016; Mancuso et al., 2018) and PAHs could disturb balance of the endocrine system to affect the spermatogenesis process (Han et al., 2010; Yang et al., 2017).

Few epidemiological studies have been carried out to examine the link between PM2.5 exposure and sperm alterations. In an ecological study in Salt Lake City, Utah, PM2.5 was found to be associated with

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reduced sperm motility on two months after exposure, however, there was no correlation between semen parameters and PM2.5 values recorded 1, 3 and 4 months previously (Hammoud et al., 2010). A cross-sectional study conducted in Taiwan indicated that every increment of 5 μg/m^3 in 2-year average PM2.5 was significantly associated with a decrease of 1.29% normal sperm morphology rate and a 26% increase risk of having the bottom 10% sperm normal morphology (Lao et al., 2018). There was also suggestive evidence of an association between additional PM2.5 exposure and increased sperms with immature chromatin, indicating impaired sperm quality among male participants recruited from infertility clinics (Radwan et al., 2016). In China, fertility rates were significantly decreased by 2.0% per 10 mg/m^3 increment of PM2.5 based on PM2.5 maps (2009–2010) (Xue and Zhang, 2018). However, there also had some conflicting results published. The results from a Healthy Men Study suggested that PM2.5 exposure at levels below the National Ambient Air Quality Standards of USA in 2009 (35 μg/m^3 for 24-h average, a transition limit implemented form 2006 to 2012) were not associated with statistically significant decrements in sperm outcomes (Hansen et al., 2010). Besides, no change was found in sperm number of young men after exposure to periods of elevated air pollution (Selevan et al., 2000). The inconsistence of these findings may due to the differences in the air pollution mix and sources in multiple geographic locations, or the different characteristics of recruited subjects, given that participants of subfertile couples attending infertility clinics for diagnostic purposes may differ from the general population. Furthermore, large number of confounders like temperature, age, lifestyle, time of sexual abstinence, semen sample collection, individual precise exposure assessment could also pose big challenges in observing the correct relation between PM2.5 exposure and semen quality (Hammoud et al., 2010; Lao et al., 2018).

Besides the epidemiological evidences, several toxicological studies in animals had provided preliminary evidences that decreased male reproductive capacity was caused by PM2.5. After PM2.5 exposure via intratracheal instillation for seven weeks in rats, the integrity of blood-testis barrier was destroyed through excessive ROS-mediated autophagy, and then resulted in the degraded sperm quality and triggering a low conception rate (Wei et al., 2018). In the previous study, a whole-body exposure murine model was applied to document impacts of PM2.5 on male reproductive system. Their results suggested a possible underlying mechanism of concentrated ambient PM2.5-induced spermatogenesis damage related with the suppression of hypothalamus-pituitary-gonads axis (Qiu et al., 2018). These studies provided suggestive evidences of an association between PM2.5 and male reproductive capacity. However, spermatogenesis is a complex process which requires not only regular testes function but also proper hormones stimulation. Large numbers of genes in the signal pathways were involved in this process (Biswas et al., 2018). Up to now, the mechanism details that PM2.5 induced spermatogenesis damage were not known clearly. Therefore, further studies will still warrant revealing the possible mechanism of decreased sperm quality induced by PM2.5.

Some studies have verified that macrophages, an important responsor in the innate immune system, which also being a key member in the testis tissue, might be activated and secrete cytokines promoting inflammation (Bekki et al., 2016). Fine particulate matter administered by oropharyngeal aspiration was discovered not only accumulate in the lung but also penetrate the pulmonary barrier and travel into other organs, including the brain, liver, spleen, kidney and testis (Li et al., 2017a, 2017b). The systemic inflammation was evidenced by significantly increased pro-inflammatory cytokines triggered in diverse tissues after chronic PM2.5 exposure (Ying et al., 2015). Inflammation within the male reproductive tract have detrimental effects on reproduction, which usually manifest as reduced androgen production, lowered sperm counts and temporary loss of fertility (Carlsen et al., 2003). It is not difficult to understand these negative consequences, since the inflammatory process is fundamentally destructive in nature. In previous study, PM2.5 could exaggerate (NACHT, LRR and PYD domains-containing protein3) NALP3 inflammasome in brain and heart (Carlsen et al., 2003). As an essential part of the innate immune, NALP3 was a global sensor of cellular damage and its activation could subsequently trigger the activation of caspase1, recruitment of ASC and the maturation of interleukin-1β (IL-1β). IL-1β was one of the principle regulators in inflammation-related diseases, which would in tune promote the inflammation process (Jo et al., 2016). The inflammation process could be activated by oxidative stress and reactive oxygen species (ROS) generation (Heid et al., 2013; Sorbara and Girardin, 2011). Forkhead box protein O1 (FOXO1) is a vital transcriptional factor which coordinates various cellular responses, involving its function of oxidative stress control through regulating a serious of antioxidases (Goto et al., 2008). FOXO1 could also act as a pivotal intermediary of the spermatogonial stem cell maintenance and differentiation (Goertz et al., 2011). Take into account the importance of FOXO1 in male fertility and recent researches demonstrating NALP3 expression in damaged-mammalian testes (Goertz et al., 2011; Minutoli et al., 2016), it is hypothesized that FOXO1 and NALP3 inflammasome pathway might involve in PM2.5 associated adverse effects on sperm quality.

Here a real time whole-body PM exposure mouse model located at Shijiazhuang, China, from December 1st, 2017 to March 25th, 2018 were established. Sperm quality, testicular histology, testicular testosterone levels, NALP3 inflammasome expression, expressions of FOXO1 and its up-stream regulation microRNAs, miR-183/96/182 were evaluated. Our results showed that PM2.5 exposure was associated with sperm dysfunction, which molecular mechanism might be attributed to testicular damage dependent on FOXO1 and the NALP3 inflammasome pathway.

2. Materials and methods

2.1. Animals and treatment

Sixty male C57BL/6 mice (6-week-old) were obtained from Vital River Laboratory (Beijing Vital River Laboratory Animal Technology Co., Ltd, Beijing, China). The procedures of the study were approved by the Institution Animal Care and Use Committee (IACUC). All mice used in this study were treated humanely under good laboratory practice conditions, free access to drinking distilled water and commercial standard pellet diet were provided. After one week of acclimatization, mice were weighed and randomly divided into three groups (filtered air group, the unfiltered air group and the concentrated air group). Filtered air-exposed mice received ambient air filtered by a high-efficient particulate air filter which was added to remove particles in the air stream. Unfiltered air-exposed mice were housed in identical chambers and exposed to ambient air transported through a pipeline. Mice exposed to concentrated air was performed with a PM2.5 concentration enrichment system (Beijing Huironghe Technology Co., Ltd, Beijing, China) located at the Hebei Medical University, Shijiazhuang, China. This system could make PM2.5 6 ~10 folds concentrated compared with the ambient. The real-time concentrations of PM2.5 in chambers and the outdoor air were constantly measured by aerosol monitor (TSI instrument Co., Ltd, Minneapolis, USA), and the distribution of the particulate matters was measured by ultraviolet aerodynamic particle sizer (TSI instrument Co., Ltd, Minneapolis, USA). Mice were subjected to exposure to filtered air, unfiltered air or concentrated air from December 1st, 2017 to March 25th, 2018 for a total duration of 16 weeks. The exposure protocol was 6 h/d (from 9:00 a.m. to 15:00 p.m.), 7 days/week and the mice from different groups were exposed at exactly the same time. Humidities and temperatures of the exposure chambers during exposure period were also monitored and recorded continuously. Moreover, the air quality index (AQI), particulate matter with particle size below 10 μm (PM10), PM2.5, sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO) and ozone (O_3) levels of ambient air in Shijiazhuang were recorded daily according to the data

published in the website of Ministry of Ecology and Environment of People's Republic of China (www.zhb.gov.cn.hjzl). Levels of gaseous pollutants (SO_2, NO_2, NO and O_3) in chambers and outdoor air were measured with monitors contemporaneously. At January 27th, 2018, when the exposure period accumulated to 8 weeks, 10 mice were chosen randomly from each group and sacrificed. The rest mice were continued to expose until March 25th, 2018.

The animal use protocol was reviewed and approved by the Laboratory Animal Ethical and Welfare Committee of Hebei Medical University, Shijiazhuang, China. Approval No. is IACUC-Hebmu-20170163. Body weights of mice were measured and recorded once a week. Mice were anesthetized by pentobarbital sodium and sacrificed the day after the last exposure. Blood samples were collected from abdominal aorta. Organs including lungs, testes and epididymides were removed and weighed. Organ coefficients were calculated by normalizing the absolute weights to the corresponding body weights of mice.

2.2. Blood cell counts

Blood sample of each mouse was collected from the abdominal aorta, and then counted the numbers of red blood cells, platelets, white blood cells and their subtypes (lymphocytes, monocytes, eosinophils, basophils and neutrophils,) within half an hour after the draw. Measurements were performed using an automatic hematological analyzer (TECOM Technology Co., Ltd, Nanchang, China).

2.3. Pathological examinations of lungs and testes

Left lungs and testes of mice were isolated and fixed with 4% paraformaldehyde, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Then tissues were cut into 5 μm sections and mounted on slides, de-paraffinized, rehydrated, and stained with hematoxylin and eosin. Sections were examined and photos were taken under the light microscope (Olympus Optical Co., Ltd, Tokyo, Japan) at both 100 and 200 magnifications for the lung or 200 and 400 magnifications for the testes.

2.4. Comet assay

Alkaline Comet assay was used to detect DNA damages of isolated testicular cells of six mice from each group. In brief, isolated testicular cells suspended in PBS (1×10^7 cells/mL) were mixed with 0.5% low–melting point agarose and layered onto slides precoated with 1% normal melting point agarose. After lysis at 4 °C overnight, electrophoresis was then carried out for 20 min at 25 V, 300 mA, and slides were neutralized with neutral buffer for 3 times. Slides were visualized under fluorescence microscope after ethidium bromide staining. The DNA integrities of 200 cells per slide at least were analyzed using comet assay software project (CASP) software (CASP version1.2.3b1, Biolauching Technologies Co., Ltd, Beijing, China). The olive tail moment (OTM value) was used as measure to evaluate DNA damage.

2.5. Evaluation of testicular testosterone

Forty milligrams testis tissue was homogenized by a high throughput tissue grinder (Ningbo Scientz Biotechnology Co., Ltd, Ningbo, China) at 4 °C with 0.5 mL enzyme-linked immunosorbent assay (ELISA) buffer provided in the commercially available ELISA kit (Cayman Chemical Co., Ann Arbor, USA) and supernatant obtained after centrifugation was applied for the ELISA assay according to the manufacturer's protocol. The sensitivity of the assay was 6 pg/mL. The inter-experiment and intra-experiment coefficients of variation were < 8.8% and 7.9%, respectively. Six mice from each group were used for analyses of testosterone. Each mouse was performed in triplicate for intra-experiments. Testosterone content was calculated according to the formula in the protocol and normalized by dividing the testicular tissue weight.

2.6. Measurements of sperm count, sperm motility and sperm morphology

In order to assess the sperm motility, left epididymis was dissected and sperms were collected and prepared as described by literature with a small revision (Elangovan et al., 2006). In brief, one caudal epididymis was placed in Modified HEPES (MH) medium, which containing 120 mM NaCl, 2 mM KCl, 1.2 mM $MgSO_4{\cdot}7H_2O$, 0.36 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, and 1.1 mM sodium pyruvate as well as penicillin (100 IU/mL) and streptomycin (100 μg/mL). The cauda of epididymis was incised with a pin and incubated at 37 °C for 10 min in a 5% CO_2 incubator. Sperms were collected and 5 μL of the sperm suspension was applied for sperm motility and count assessment using a computer-assisted sperm assay (CASA) with a sperm motility analyzer (Beijing Weili New Century Science & Tech. Deve. Co., Ltd, Beijing, China). The following parameters of sperm motility were evaluated: total sperm motility (%), percentage of sperm moving forward (NP%), percentage of sperm moving not forward (RP%), curve-line velocity (VCL), straight-line velocity (VSL) and beat frequency. Eight mice from each group were applied for the analyses. For each sample, at least 500 sperms were analyzed. Sperm concentration was expressed as $\times 10^6$/mL. Out of the sperm concentration and sperm motility, 20 μL of the sperm suspension was mounted on the slide glass and fixed with absolute methanol for 10 min and later visualized by 1% eosin staining. The prepared slides were examined to determine the morphological abnormalities under oil immersion. One thousand sperms per animal at least in four different areas of the smear were evaluated according to the literature (Kruger et al., 1988).

2.7. Immunofluorescence assay

Testes from 5 mice per group were fixed in 4% paraformaldehyde, dehydrated in increasing concentrations of ethanol, embedded in paraffin and sectioned at 5 μm thickness. Sections were deparaffinized and rehydrated, and antigen retrieval was performed by microwaving the sections for 3 × 3 min in 50 mM Tris-HCl (pH 10). Non-specific antibody binding was blocked in PBS containing 5% bovine serum albumin (BSA) and 10% normal goat serum for 1 h at room temperature, after which sections were incubated with anti-caspase1 (1:200 with 1% BSA/PBS) primary antibody (EarthOx, LLC, San Francisco, USA) at 4 °C overnight. Then sections were washed with PBS and incubated with fluorescent conjugated secondary antibody, Dylight549 goat anti-rabbit IgG (MultiScience Biotechnology Co., Ltd, Hangzhou, China, 1:200 with 1% BSA/PBS) for 1 h at room temperature. After washing thrice with PBS, 2 μg/mL DNA-specific fluorochrome 4, 6-diamino-2-phenylindole (DAPI) was used for nuclei staining. After 3 washes with PBS, the slides were mounted with glycerol and cover slips. Slides were observed and images were taken under fluorescence using an Olympus BX51T microscope.

2.8. Western blot

Fifty milligram testis tissue in 700 μL radio immunoprecipitation assay (RIPA) lyses buffer was homogenized by a high throughput tissue grinder at 4 °C and then centrifuged at 13,000*g* with a tabletop ultracentrifuge (Shanghai Likang Disinfection High-Tech Co., Ltd, Shanghai, China) for 10 min at 4 °C. Protein content in the supernatant was quantified by a BCA kit (Thermo Fisher Scientific Inc., Waltham, USA). Samples were denatured in sodium dodecyl sulfate (SDS) buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) at 75 °C for 15 min. Equal amount of protein was separated by SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Merck & Co., Inc, New Jersey, USA). After being blocked for 3 h at room temperature with Tris-buffered saline containing Tween20 (TBST) buffer (150 mM NaCl,

20 mM Tris-HCl pH 7.4, 0.1% Tween20) containing 5% nonfat milk, blots were then incubated with specific primary antibodies including NALP3 (Abways Biotechnology Co., Inc, Shanghai, China, 1:1000 diluted with 1 × TBST), ASC (Novus Biologicals Co., Ltd, San Diego, USA, 1:1500 diluted with 1 × TBST), pro-caspase1 (Abcam Inc., Cambridge, UK, 1:1000 diluted with 1 × TBST), caspase1 (EarthOx, LLC, San Francisco, USA, 1:1000 diluted with 1 × TBST), IL-1β (EarthOx, LLC, San Francisco, USA, 1:1500 diluted with 1 × TBST), GAPDH (EarthOx, LLC, San Francisco, USA, 1:2000 diluted with 1 × TBST) and FOXO1 (Cell Signaling Technology, Boston, USA, 1:3000 diluted with 1 × TBST) at 4 °C overnight. After washing three times with TBST, the goat-anti-rabbit secondary antibody (HuaAn Biotechnology Co., Ltd, Hangzhou, China) were incubated at 37 °C in an incubator for 1 h, then followed by additional three-times washing and the membranes were detected by Western blot ECL Kit (Affinity Biosciences Co., Ltd, Cincinnati, USA). The protein bands of the blots were observed and taken pictures by automatic chemiluminescence image analysis system (Tanon Science & Technology Co., Ltd, Shanghai, China) and then analyzed by the gel-pro32 software (Roper Technologies, Inc., Sarasota, USA). Three mice per group were used to perform western blot analyses and each mouse was performed in triplicate.

2.9. qRT-PCR

Total RNA was extracted by Trizol (Invitrogen Co., Carlsbad, USA) and isolated RNA was quantified by Nano-100 microspectrophotometer (Allsheng instrument Co., Ltd, Hangzhou, China). Two micrograms RNA was used for cDNA synthesis using miRNA OneScript™ cDNA Synthesis Kit (Applied Biological Materials Inc, Richmond, Canada) according to the manufacturer's protocol. Stem-loop primer for miR96 was GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACA GCAAA. Stem-loop primer for miR182 was GTCGTATCCAGTGCAGGG TCCGAGGTATCCGCACTGGATACGACCGGTGT. Stem-loop primer for miR183 was GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACAGTGAA. Gotaq® qPCR Mix (Promega Biotech Co., Ltd, USA) was used to quantify the levels of microRNAs. The reaction conditions were set as follows: 10 min at 95 °C, 45 cycles of 15 s at 95 °C, 1 min at 60 °C, followed by a melting curve (60–95 °C) to rule out the presence of artifacts. The specificity of microRNA amplification was evaluated by melting curve analysis. The results were normalized against the expression of endogenous control U6 snRNA. Each reaction was performed in triplicate. Relative quantification of each gene expression was calculated according to the $2^{-\Delta\Delta CT}$ methodology using the Biorad software tool Genex-Gene Expression MacroTM. Real-time PCR was performed on FQD-96A real time-PCR instrument (Bioer Technology Co., Ltd, Hangzhou, China). All the primers were obtained from Sangon, China. The microRNA-specific qRT-PCR primer pairs were as follows:

Genes	Sense (5′ →3′)	Anti-sense (3′ →5′)
U6	GTGCTCGCTTCGGCAGCA	GGAACGCTTCACGAATTTGC
miR96	GCGTTTGGCACTAGCACATT	AGTGCAGGGTCCGAGGTATT
miR182	GCGTTTGGCAATGGTAGAACTC	AGTGCAGGGTCCGAGGTATT
miR183	CGCGTATGGCACTGGTAGAA	AGTGCAGGGTCCGAGGTATT

2.10. Statistical analysis

All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, USA). Comparisons of data between multiple groups were analyzed using one-way analysis of variance (ANOVA) and comparisons between two groups were analyzed with unpaired Student't *t*-test. Differences were considered significant at $P < 0.05$. Data were expressed as the means ± SD and figures were generated using Graphpad Prism 7.0 software.

3. Results

3.1. The evaluation of exposure parameters

During exposure, average concentrations of PM2.5 in the atmosphere of Shijiazhuang were 100.19 μg/m^3 and 99.51 μg/m^3 respectively for 8 weeks and 16 weeks. Only 8 days in the 8 weeks duration and 15 days in the 16 weeks duration had a PM2.5 concentration lower than the 24 h average concentration limit (GB3095-2012, China). Average concentrations of PM10 in the ambient air were 159.93 μg/m^3 and 159.70 μg/m^3 respectively for 8 weeks and 16 weeks durations (Supplemental Table S1). Concentrations of SO_2, NO_2, CO, O_3 in chambers were verified to be consistent with that in the outdoor air (Supplemental Fig. S1A-B).

The average concentrations of PM2.5 were 94.84 μg/m^3 and 900.21 μg/m^3 for 8 weeks exposure in unfiltered air chamber and concentrated air chamber, respectively. Whereas, the calculatedly accumulated exposure doses (Davel et al., 2012) were 1327.76 and 12,602.94 μg/m^3, respectively. For 16 weeks exposure, average concentrations of PM2.5 were 86.78 μg/m^3 and 671.87 μg/m^3 whereas the calculatedly accumulated exposure doses were 2429.84 and 18,812.36 μg/m^3, respectively. Although there were minor fluctuations, temperatures and humidities in the different chambers were all in the optimum ranges suitable for mice. Meanwhile, the comparable temperatures and humidities made a foundation for the good comparability in different groups (Supplemental Table S2).

Particulate matters in the unfiltered air chamber had relatively wider size-ranges. As shown in Supplemental Fig. S1C, 90.46% particular matters had aerodynamic diameters less than 2.5 μm and nearly 60% particulate matters had aerodynamic diameters in the range of 0.523–1 μm. Diameter analysis of particle size in the concentrated chamber showed that more than 99% particulate matters were less than 2.5 μm and 75.03% of them had cutoff sizes ranging from 0.523 to 1 μm.

3.2. Effects of PM2.5 on systemic inflammation, lungs and testes damages

Final body weights, absolute organ weights and organ coefficients of mice were summarized in Table 1. The body weights of mice were not affected by PM2.5 exposure for both of 8 weeks and 16 weeks ($P > 0.05$). Although absolute lung weights of mice exposed to unfiltered air and concentrated air presented to be a little higher than mice exposed to filtered air, the changes did not have statistical significance ($P > 0.05$). Both for 8 weeks and 16 weeks exposure, concentrated air caused significant increases in lung coefficients ($P < 0.05$) compared with the filtered air. Similar to the changes of lung coefficients, statistically significant increases of testis coefficients were found in the concentrated air group both for the 8 weeks and 16 weeks exposure periods ($P < 0.05$).The epididymis coefficients or the absolute weights of testes and epididymides were not statistically changed ($P > 0.05$).

As shown in Fig. 1A1–A8, for both 8 weeks and 16 weeks exposure periods, notably increased white blood cell counts were observed in the unfiltered air group and the concentrated air group. Statistically significant increased ($P < 0.05$) lymphocyte and neutrophil counts were found in mice exposed to concentrated air for both the 8 weeks and 16 weeks. Monocyte counts of mice after unfiltered air and concentrated air exposure for 16 weeks were significantly higher than filtered air ($P < 0.05$). As for eosinophils, basophils and platelets, significantly increases ($P < 0.05$) of cell counts were only observed in mice after concentrated air exposure for 16 weeks. Red cell counts did not show any changes after PM2.5 exposure.

As shown in Fig. 1B, there were generally intact pulmonary alveoli, but notable inflammatory cell infiltration around the bronchiole in a dose and time dependent manner in lung of mice after PM2.5 exposure. Besides, slight alveoli hemorrhage and obvious thickening of the

Table 1
Effects of PM2.5 exposure on the absolute weights of lungs, testes, epididymides and coefficients of these organs of mice.

Exposure duration	Groups	Final body weight (g)	Absolute lung weight (g)	Lung coefficient (mg/g b.wt)	Absolute testis weight (g)	Testis coefficient (mg/g b.wt)	Absolute epididymis weight (g)	Epididymis coefficient (mg/g b.wt)
8 weeks	Filtered air	24.22 ± 1.17	0.18 ± 0.03	7.89 ± 1.50	0.20 ± 0.03	8.06 ± 1.08	0.069 ± 0.009	2.83 ± 0.34
	Unfiltered air	22.95 ± 1.29	0.19 ± 0.03	8.25 ± 1.53	0.19 ± 0.02	8.51 ± 0.99	0.070 ± 0.008	3.06 ± 0.37
	Concentrated air	23.13 ± 1.31	0.20 ± 0.04	9.68 ± 2.38*	0.21 ± 0.03	9.11 ± 0.92*	0.073 ± 0.014	3.22 ± 0.64
16 weeks	Filtered air	24.76 ± 2.29	0.18 ± 0.03	7.58 ± 1.41	0.20 ± 0.02	8.07 ± 0.82	0.079 ± 0.011	3.21 ± 0.50
	Unfiltered air	24.64 ± 1.96	0.19 ± 0.02	8.94 ± 1.78	0.20 ± 0.01	8.21 ± 0.86	0.076 ± 0.009	3.14 ± 0.43
	Concentrated air	23.70 ± 1.23	0.20 ± 0.04	9.95 ± 1.15#	0.21 ± 0.03	9.09 ± 0.85##/§§	0.077 ± 0.017	3.25 ± 0.61

Lung coefficient: lung weight/body weight. Testis coefficient: testis weight/body weight. Epididymis coefficient: epididymis weight/body weight. Values were expressed as mean ± SD. n = 10 per group.
* $P < 0.05$, compared with mice exposed to filtered air for 8 weeks.
# $P < 0.05$, compared with mice exposed to filtered air for 16 weeks.
$P < 0.01$, compared with mice exposed to filtered air for 16 weeks.
§§ $P < 0.01$, compared with mice exposed to unfiltered air for 16 weeks.

alveolar septum were presented in lungs for both 8 weeks and 16 weeks exposure periods. There was bronchiolar epithelial hyperplasia in concentrated-air-exposed mice for 8 weeks, whereas the focal sloughing of bronchial epithelial cells was found in mice exposed for 16 weeks.

No visible changes of testes were found by the unaided eye, as shown in Supplemental Fig. S2. Histopathological examination of the testes from mice (Fig. 2) in filtered air group showed normal seminiferous tubules and conventional arrangement of the spermatogenetic cells, including spermatogonia, spermatocytes, and spermatids. Sertoli cells rested on the basement membrane regularly. Leydig cells were a component of interstitial tissues. Testes of mice exposed to unfiltered air for 8 weeks revealed a reduced relative total number of seminiferous tubules and increased interstitial space. The basement membrane was intact in general and the spermatogenic cells showed regular arrangement. However, there was obvious increase of sertoli cell vacuolation and such disorder was exacerbated in the concentrated air group. Slight disorganization and reduction in the thickness of the spermatogenetic cells were also found in the testes of mice after concentrated air exposure. Less sperms were produced in the lumen in concentrated air group compared with the filter air group. For testes in mice exposed to unfiltered air for 16 weeks, the basement membrane was slightly interrupted, the spermatogenetic cells of seminiferous tubules appeared disorganized and spermatogenesis was reduced. Vacuolation and pyknotic spermatogenetic cells were also found in some of the

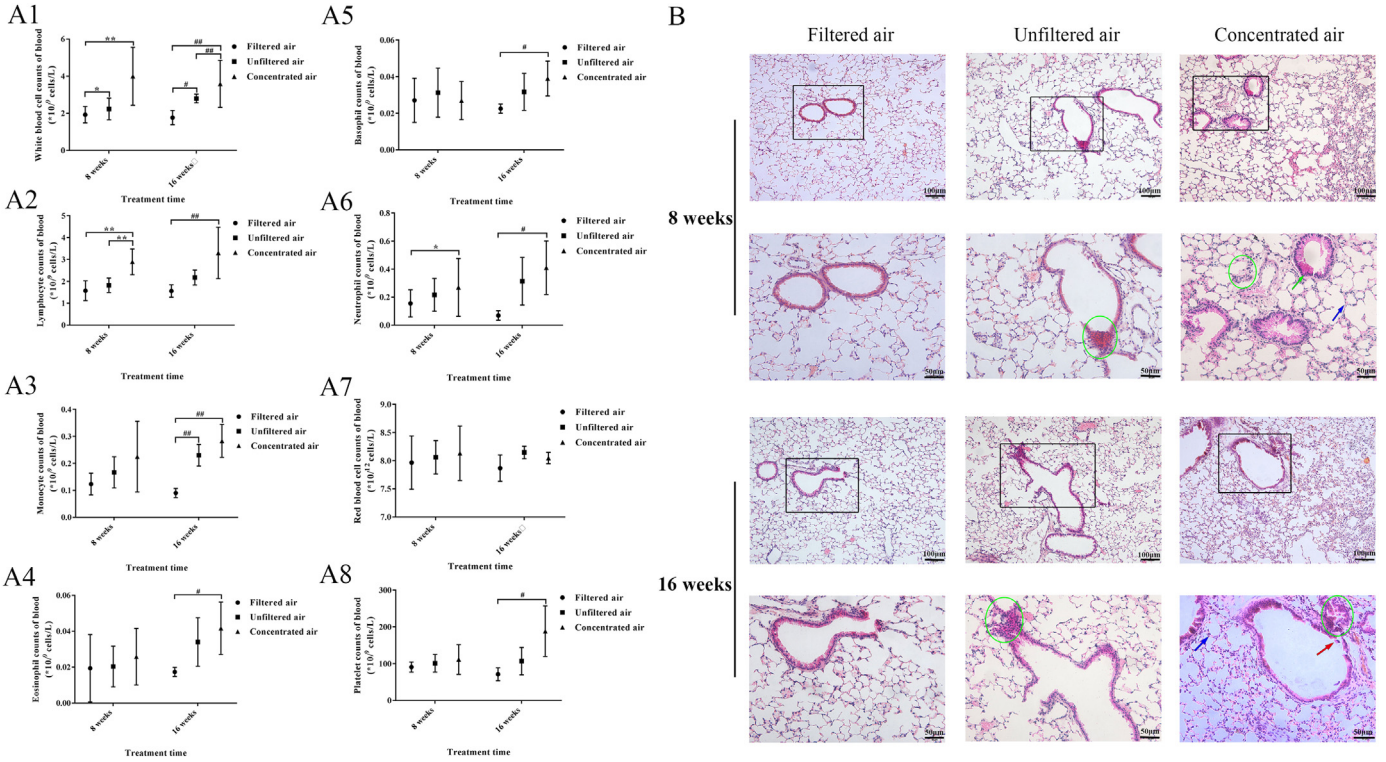

Fig. 1. Effects of PM2.5 on systematic inflammation and histopathological changes of lungs in mice. **A** The changes of blood cell counts after PM2.5 exposure. **A1-A8** Counts for white blood cells, lymphocytes, monocytes, eosinophils, basophiles, neutrophils, red blood cells and platelets of mice. n = 10 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 weeks. $^{**}P < 0.01$, compared with mice exposed to filtered air group for 8 weeks. $^{\#}P < 0.05$, compared with mice exposed to filtered air for 16 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air for 16 weeks. **B** Representative histopathological images of lungs in mice. Green cycles indicate inflammatory cell infiltration. Green arrow indicates hypertrophy of the bronchial epithelium. Blue arrows indicate thickening of the alveolar septae. Red arrow indicates focal sloughing of bronchial epithelial cells. Scale bar for upper panel of 8 weeks or 16 weeks: 100 μm; scale bar for lower panel of 8 weeks or 16 weeks: 50 μm. n = 3 per group.

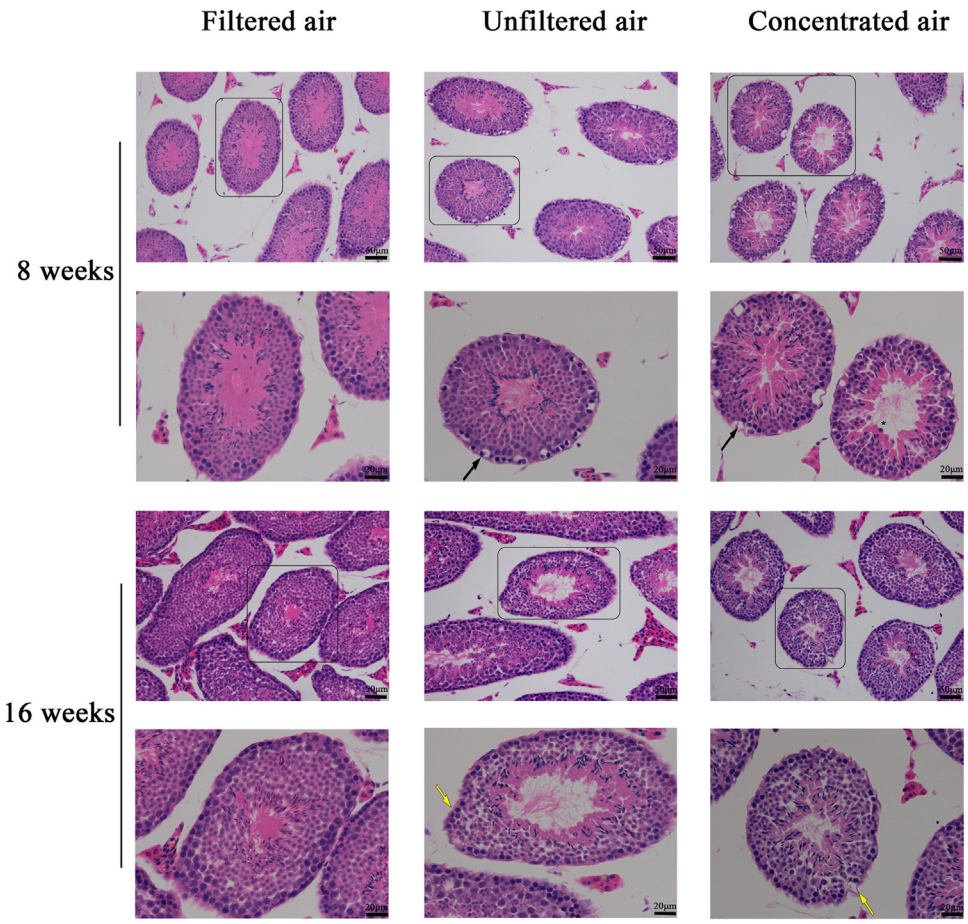

Fig. 2. Pathological changes of testes in mice after PM2.5 exposure. Black arrow head: sertoli cell vacuolization. Yellow arrow head: interrupted basement membrane. Asterisk: immature germ cells. Scale bar for upper panel of 8 weeks or 16 weeks: 50 μm; scale bar for lower panel of 8 weeks or 16 weeks: 20 μm. n = 3 per group.

seminiferous tubules and the spermatogenetic cells showed shrinkage of their cytoplasm resulting in enlargement of the intercellular spaces. Histopathological changes in the testes of concentrated air group was similar to that of the unfiltered air group, but turned up a bit more severe. The inflammatory cell infiltration under the tunica vaginalis of testes in mice exposed to concentrated air for 16 weeks was found.

Comet assay was applied to determine DNA damages of testicular cells induced by PM2.5 exposure. Compared with the filtered air group, significant increases of DNA damages (OTM values) were observed in mice exposed to concentrated air for 8 weeks and 16 week, respectively ($P < 0.05$) (Supplemental Fig. S3). In comparison with the filtered air group, no significant increases of OTM values were observed in the unfiltered air group.

3.3. Effects of PM2.5 on male reproductive function of mice

Testosterone was one of the most important reproductive hormones which played a vital role in spermatogenesis. We assessed the testosterone levels in testis tissues to define if its levels were interrupted by PM2.5. As shown in Fig. 3A, the testosterone levels were not altered after PM2.5 exposure for 8 weeks either in the unfiltered air group or the concentrated air group. While exposure to unfiltered air or concentrated air for 16 weeks led to significant decreases in testicular testosterone levels ($P < 0.05$). In comparison with 8 weeks, the testosterone levels had a significant decrease in mice after unfiltered air exposure for 16 weeks ($P < 0.05$). Consistent with the effect in the unfiltered air group, there was also a significant decrease of testosterone in the concentrated air group for 16 weeks when compared with that of the 8 weeks ($P < 0.05$).

As shown in Fig. 3B, sperm densities of mice were markedly decreased in the unfiltered air group and concentrated air group at 16 weeks exposure duration when compared with the filtered air group ($P < 0.05$). Fig. 3C1 showed that adverse sperm motility changes were caused by PM2.5 in a dose-dependent manner. Particularly, the percentages of motile sperm of mice with unfiltered air exposure for 16 weeks decreased significantly ($P < 0.05$) when compared with mice exposed to filtered air. And the percentages of motile sperm of mice exposed to concentrated air had significant decreases ($P < 0.05$) compared with mice exposed to filtered air for both 8 weeks and 16 weeks exposure durations. Analyses of PR%, NP%, VSL, VCL and beat frequency were shown in Fig. 3C2-C6. For 8 weeks, no significant difference was found in PR% between the filtered air and unfiltered air group, nor did the difference found in the mice exposed to concentrated air. But for 16 weeks, PR% of mice in unfiltered air group and concentrated air group presented a significant decrease when compared to the parallel filtered air-exposed mice ($P < 0.05$). Likewise, NP% was significantly lower in concentrated air group than the filtered air when exposed for 8 weeks ($P < 0.05$). For 16 weeks, NP% of mice in unfiltered air group and concentrated air group had significantly decreased when compared with the parallel filtered air mice ($P < 0.05$). Apparently, changes were also observed in other motility parameters like VSL, VCL and beat frequency. Although there were no obvious changes of VCL for those groups exposed for 8 weeks, VSL and beat frequency of mice in the concentrated air group was significantly lower ($P < 0.05$) than that of the filtered air group and the decreases presented in a dose-dependent manner. When the exposure duration extended to 16 weeks, sperms of mice exposed to concentrated air showed significant decreases both in the VCL and the VSL compared with mice in the filtered air ($P < 0.05$). Beat frequency of concentrated air-exposed mice decreased significantly when compared with the mice exposed to filtered air. For mice under the 16 weeks exposure, the significant decrease of beat frequency of sperms was also found in the

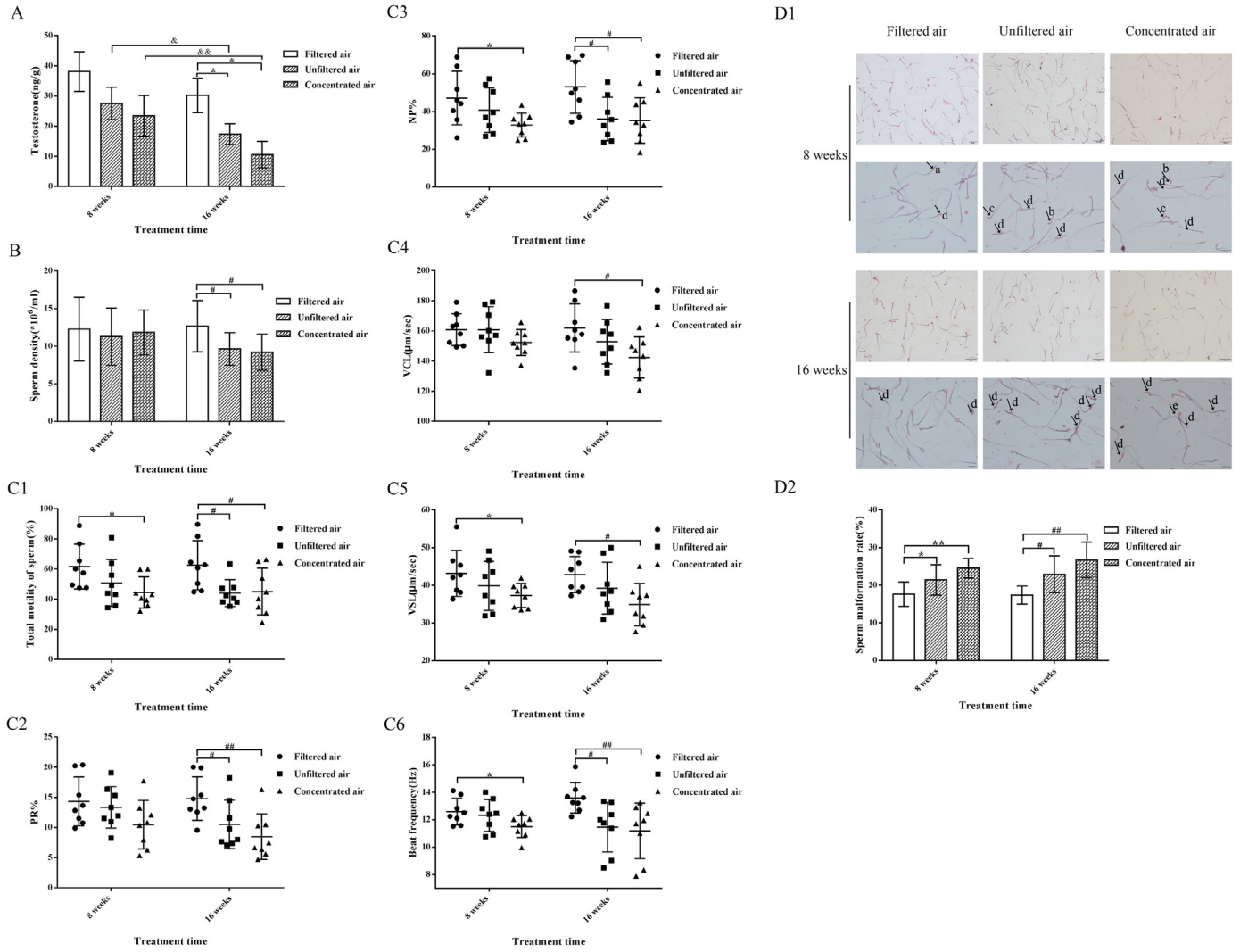

Fig. 3. Effects of PM2.5 on testicular function and sperm quality of mice. **A** Effects of PM2.5 exposure on testicular testosterone contents. n = 6 per group. **B** Sperm density changes after exposed to PM2.5. n = 8 per group. **C** Sperm motility changes of mice after exposed to PM2.5. **C1** Total motility of sperm. **C2** NP% of sperm. **C3** PR% of sperm. **C4** Beat frequency. **C5** VCL of sperm. **C6** VSL of sperm. n = 8 per group. **D** Morphological changes of sperm after exposed to PM2.5. **D1** Representative sperm microphotographs of mice exposed to PM2.5 for 8 or 16 weeks. a, banana head; b, hookless sperm; c, pin head; d, folded tail; e, double heads. Bars for upper panel of 8 weeks or 16 weeks were: 50 μm; bars for lower panel of 8 weeks or 16 weeks were: 20 μm. **D2** Sperm malformation rates of mice after exposed to PM2.5. n = 8 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 weeks. $^{**}P < 0.01$, compared with mice exposed to filtered air for 8 weeks. $^{\#}P < 0.05$, compared with mice exposed to filtered air for 16 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air for 16 weeks. $^{\&}P < 0.05$, when compared mice in the unfiltered air group for 8 weeks with that for 16 weeks exposure duration. $^{\&\&}P < 0.01$, when compared mice in the concentrated air group for 8 weeks with that for 16 weeks exposure duration.

unfiltered air group ($P < 0.05$).

Sperms with abnormal morphology were observed in unfiltered air group and concentrated air group. Moreover, the number of abnormal sperms significantly increased in mice from the unfiltered air group and concentrated air group compared with mice in the filtered air group for 8 weeks exposure in a dose-dependent manner (Fig. 3D1-D2). More obvious abnormalities were found in mice exposed for 16 weeks ($P < 0.05$). However, no significant exposure time-dependent changes were noticed between mice exposed to the same air condition. Sperms in the caudal epididymis were morphologically classified into three types and frequency of each of these types was compared. As shown in Table 2, head, midpiece and tail abnormality rates in each group at both exposure time points were calculated. The main malformations of sperms occurred in tail or head abnormality. Hockless-head and pin-head being the most head abnormality types, followed by banana-shaped-head and big-head sperm, double head was the least (data not shown). Tail abnormality was characterized by folded tail. Multiple-

Table 2
Effects of PM2.5 on rates of different sperm malformation types.

Exposure duration	Groups	Head abnormality rate (%)	Midpiece abnormality rate (%)	Tail abnormality rate (%)
8 weeks	Filtered air	3.88 ± 1.69	0.94 ± 0.31	9.77 ± 2.22
	Unfiltered air	3.87 ± 1.23	1.51 ± 0.46	10.81 ± 1.91
	Concentrated air	8.33 ± 1.52*	1.59 ± 0.36	10.33 ± 1.52
16 weeks	Filtered air	4.43 ± 1.70	1.54 ± 1.23	12.72 ± 1.78
	Unfiltered air	4.52 ± 1.65	2.33 ± 1.48	12.26 ± 2.38
	Concentrated air	8.43 ± 3.96#	4.12 ± 1.38#	14.77 ± 2.75

Values were expressed as mean ± SD. n = 8 per group.
* $P < 0.05$, compared with mice exposed to filtered air for 8 weeks.
# $P < 0.05$, compared with mice exposed to filtered air for 16 weeks.

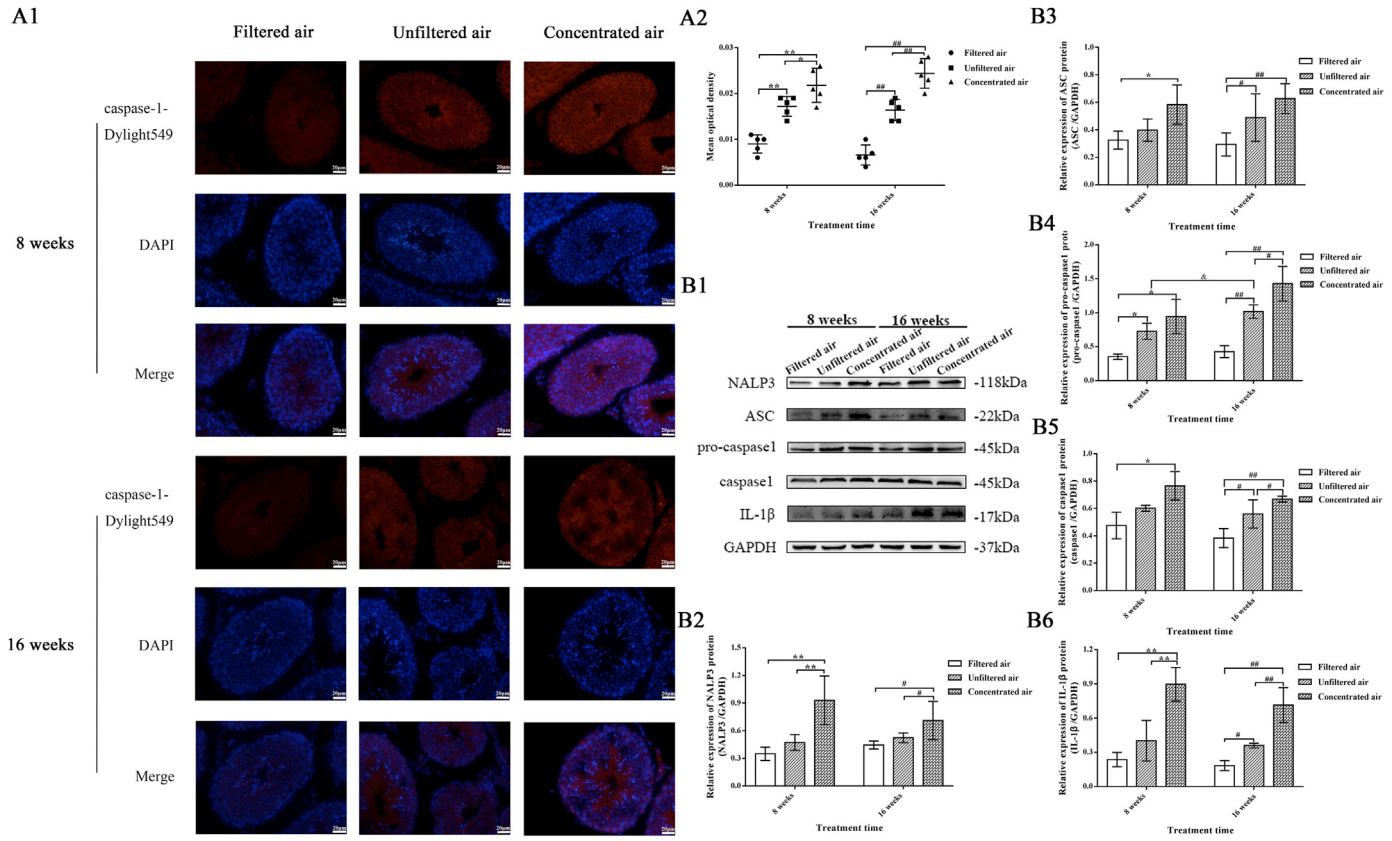

Fig. 4. Effects of PM2.5 on NALP3 inflammasome pathway activation in testes of mice. **A** Alternation of caspase1 expression in testes of mice after PM2.5 exposure. **A1** Representative images of caspase1 expression in different treatment groups. The bar represents 50 μm. **A2** The levels of caspase1 expression in different treatment groups measured by densitometry analyses. n = 5 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 weeks. $^{**}P < 0.01$, compared with mice exposed to filtered air group for 8 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air group for 16 weeks exposure duration. **B** The NALP3, ASC, pro-caspase1, caspase1 and IL-1β protein expressions in testes of mice after PM2.5 exposure. **B1** Representative protein bands of NALP3, ASC, pro-caspase1, caspase1, IL-1β and their corresponding GAPDH in Western blot. **B2-B6** Quantitative analyses for NALP3, ASC, pro-caspase1, caspase1 and IL-1β protein expressions which were normalized to GAPDH. n = 3 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 weeks. $^{**}P < 0.01$, compared with mice exposed to filtered air for 8 weeks. $^{\#}P < 0.05$, compared with mice exposed to filtered air for 16 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air for 16 weeks. $^{\&}P < 0.05$, when compared mice in the unfiltered air group for 8 weeks with that for 16 weeks exposure duration.

tails was rarely observed. Compared with mice in the filtered air group, no overt abnormalities were observed in midpiece or tail abnormality rates of sperm in the unfiltered air group and concentrated air group for 8 weeks. However, the frequency of sperm with head abnormalities was significantly higher in the concentrated air group when compared with the filtered air group and unfiltered air group ($P < 0.05$). As for the 16 weeks, abnormality frequency of the head in concentrated air group were about 2-folds higher than that of the filtered air group ($P < 0.05$). Sperm midpiece abnormality rate of mice exposed to concentrate air for 16 weeks was apparently increased than that of the filtered air group or unfiltered air group ($P < 0.05$).

3.4. Effects of PM2.5 on expressions of the NALP3 inflammasome and the miR-183/96/182 targeting FOXO1 pathway in testes

From the results of immunofluorescence (Fig. 4A1-A2), caspase1 was seen to be slightly expressed in testes of mice in the filtered air group. Caspase1 expression in the unfiltered air group and concentrated air group were markedly stronger than that of the filtered air group. At both of the 8 and 16 weeks time points, the expressions of caspase1 were significantly increased in the unfiltered air group and concentrated air group compared to the filtered air ($P < 0.05$), and expression of caspase1 in the concentrated air group was significantly higher than that of the unfiltered air group ($P < 0.05$). In the present study, we investigated whether the NALP3 signal pathway played a vital role in the testicular damage induced by PM2.5 exposure. There were good dose-response relationships in expressions of the related proteins. As shown in Fig. 4B1-B6, protein expressions of NALP3, ASC, pro-caspase1, caspase1 and IL-1β were all up-regulated upon concentrated air exposure both for 8 and 16 weeks exposure durations when compared with the filtered air exposure group at the parallel time points. Similarly, expression levels of pro-caspase1, caspase1 and IL-1β in the unfiltered air group were significantly increased ($P < 0.05$) when compared with the filtered air group for 16 weeks exposure duration. But for 8 weeks exposure duration, statistically significant increase ($P < 0.05$) was only observed in the pro-caspase1 expression in the unfiltered air group when compared with the filtered air group. Meanwhile, expressions of NALP3, pro-caspase1 and IL-1β in testes of the concentrated air group were markedly higher ($P < 0.05$) than that of the unfiltered air group for both 8 and 16 weeks exposure durations. For ASC expression, significant increase ($P < 0.05$) was only observed in the concentrated air group compared with the unfiltered air group for 16 weeks exposure duration. And for pro-caspase1, the expression level in mice exposed to unfiltered air group for 16 weeks was markedly higher ($P < 0.05$) than that for 8 weeks. Densitometry value analyses were performed at least in triplicate for each independent experiment.

Expression of FOXO1 had similar changes with the NALP3 inflammasome. As shown in Fig. 5A1-A2, for both exposure durations, the FOXO1 levels were up-regulated in the unfiltered air group and concentrated air group, compared with the filtered air group ($P < 0.05$) and the highest expression level turned up in the concentrated air group. And when compared with the unfiltered air group, expression of

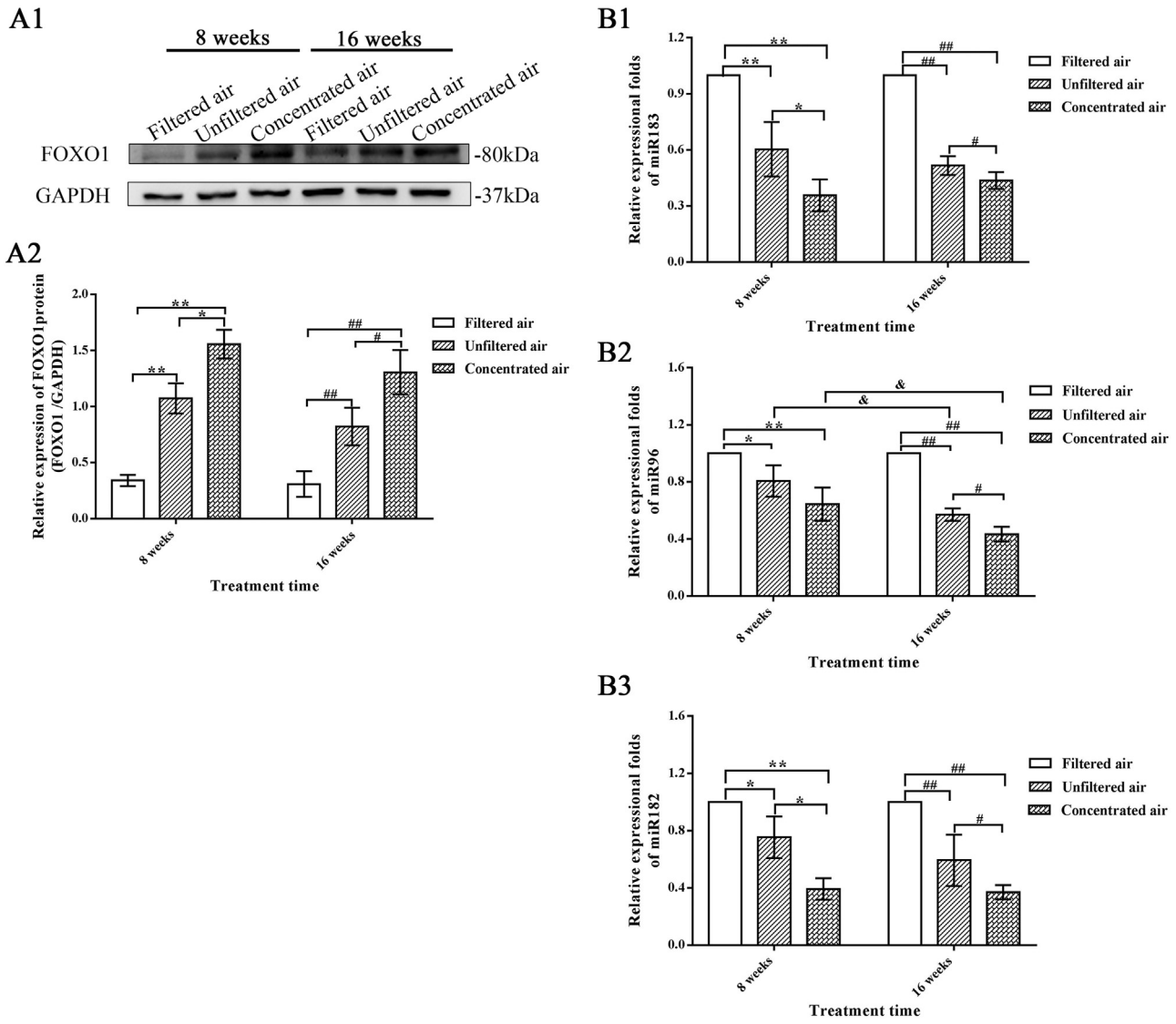

Fig. 5. Effect of PM2.5 on expressions of FOXO1 and miR-183/96/182 in testes of mice. **A** FOXO1 protein expressions in testes of mice after PM2.5 exposure. **A1** Representative protein bands of FOXO1 and the corresponding GAPDH in Western blot. **A2** Quantitative analysis for FOXO1 expression which was normalized to GAPDH. n = 3 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 week. $^{**}P < 0.01$, compared with mice exposed to filtered air for 8 weeks. $^{\#}P < 0.05$, compared with mice exposed to filtered air for 16 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air for 16 weeks. **B** Expressions of miR183, miR96 and miR182 in testes of mice after PM2.5 exposure. Values of gene expressions expressed as fold changes to the respective control samples. B1-B3 Quantitative analyses for miR183, miR96 and miR182 expressions. n = 3 per group. $^{*}P < 0.05$, compared with mice exposed to filtered air for 8 week. $^{**}P < 0.01$, compared with mice exposed to filtered air for 8 weeks. $^{\#}P < 0.05$, compared with mice exposed to filtered air for 16 weeks. $^{\#\#}P < 0.01$, compared with mice exposed to filtered air for 16 weeks. $^{\&}P < 0.05$, when compared mice in the unfiltered air group or concentrated air group for 8 weeks with that for 16 weeks exposure duration.

FOXO1 in the concentrated air group was significantly increased for both exposure durations.

As shown in Fig. 5B1-B3, results revealed that miR182, miR96 and miR183 were significantly down-regulated in the unfiltered and concentrated air group compared with the filtered air group both for 8 and 16 weeks exposure durations ($P < 0.05$). Furthermore, when compared with the unfiltered air group, the miR-183/96/182 cluster levels in the concentrated air group were significantly decreased for both the 8 and 16 weeks exposure durations (both $P < 0.05$). Both dose and time-dependent decreases were found in the expression of miR96 ($P < 0.05$).

4. Discussion

As a ubiquitous contaminant among population, about 32% of the reported total deaths in China were associated with PM2.5 in 2013 (Fang et al., 2016). The overall health burden attributable to PM2.5 was expected to increase by 2030 (GBD MAPS Working Group, 2016). Numerous studies (Lepeule et al., 2012; Pope et al., 2011) have provided evidences that PM2.5 exposure could contribute to a series of adverse health outcomes. Most literatures mainly paid attention to the adverse effects of PM2.5 on respiratory (Lepeule et al., 2012; Pope et al., 2011) or cardiovascular systems (Madrigano et al., 2013; Tillett, 2012). Recently, there was elevating interests in the PM2.5-induced reproductive dysfunction, especially in the male sperm quality (Hansen et al., 2010; Santi et al., 2018).

A study of epidemiology of infertility in China indicated that about 17.2% couples of reproductive age in Hebei province suffered from infertility (Zhou et al., 2018). Such rate was higher than that of other provinces (Fujian < 8%, Neimogol 10.2%, Beijing 11.3%, Anhui 14.5%, Heilongjiang 15.6%) (Zhou et al., 2018). Shijiazhuang, the capital of Hebei province, is located at the foot of Taihang mountain and

surrounded by mountains in the west and north. It was winter during the exposure and winter is the most polluted season in the north of China, including Shijiazhuang. In winter, the closed terrain hinders the northwest wind and limits the dispersion of atmospheric pollutant, thus leading to the heavily PM2.5 suffering. The data published in the website of Ministry of Ecology and Environment of People's Republic of China showed the average PM2.5 concentration at Shijiazhuang was 99.51 μg/m^3 from December 1st, 2017 to March 25th, 2018. This concentration was more than 2-folds higher than the Chinese National Ambient Air Quality Standard (NAAQS, 35 μg/m^3). In this case, carrying out the study of high PM2.5 exposure level would make sense for population residing in areas with heavy PM2.5 pollution. In the present study, a real-time PM2.5 exposure mouse model was established at Shijiazhuang to evaluate the adverse effects of PM2.5 on reproductive system. During the total exposure duration (December 1st, 2017 to March 25th, 2018), PM2.5 concentration in the unfiltered air chamber was 87 μg/m^3, lower than PM2.5 concentration in the parallel ambient air measured by aerosol analyzer (136.6 μg/m^3). This difference might be due to the pipelines blocking. The average concentration of PM2.5 in the concentrated air group chamber was 672 μg/m^3. Given that the exposure design comprised exposures for 6 h/day and 7 days/week, the average applied PM2.5 concentration during this period was 21.75 μg/m^3 for 24-h average in the unfiltered air chamber from December 1st, 2017 to March 25th, 2018, which almost equaled to the daily average PM2.5 concentration limit of the WHO air quality goal (25 μg/m^3). The average PM2.5 concentration was 672 μg/m^3 in concentrated air chamber. That was an astonishing PM2.5 level much higher than other studies. However, it equaled to 168 μg/m^3 when calculating for 24-h average. Such level was common in heavily-polluted cities like Shijiazhuang. We also recorded the hazardous gases including SO_2, NO_2, CO and O_3 in ambient air from December 1st, 2017 to March 25th, 2018. The consistency of these parameters between the ambient air and the exposure chamber was confirmed. O_3 was a very reactive secondary toxic pollutant and was implicated as a sperm toxicant (Sokol et al., 2006). CO and NO_2 were negatively associated with testosterone level (Radwan et al., 2016) and SO_2 could disturb the BTB (blood-testis barrier) structure (Zhang et al., 2016). In the present study, all scenario factors between each group were comparable except the differences of PM2.5 concentration, making it an appropriate model to evaluate the effects induced by PM2.5.

PM2.5 has complex constituents including heavy metals and PAHs. Heavy metals, such as cadmium, lead and mercury, had been demonstrated to be negatively associated with male reproductive potential (Ghaffari and Motlagh, 2011; Jeng et al., 2015). PAHs exposure was also indicated to be linked with increased sperm DNA damages or even sperm dysfunction (Han et al., 2010; Jeng and Yu, 2008). Although the heavy metals and PAHs were well-known reproductive toxicants, PM2.5 effects on sperm quality were still controversial. In the previous study, exposure to high level of PM2.5 (about 20 times higher than the maximum cut-off proposed for human health maintenance) was found to be directly related to total sperm number (Santi et al., 2016). However, no association was found between PM2.5 and sperm concentration in another epidemiological study, which might because of the relatively low level of air pollution (14 μg/m^3 for 24-h average) (Hansen et al., 2010). What's more, a positive relationship between PM2.5 exposure (26 μg/m^3 for 24-h average) and sperm concentration was found, that seems unexpected because there's no biologically reasonable explanation for protective effects. The author speculated that the slight increase in sperm concentration might be a compensatory phenomenon. The low dose exposure might stimulate an increase in levels of FSH and LH, which could act as spermatogenesis stimulator (Lao et al., 2018). In the present study, notably sperm density decline was observed in mice exposed to unfiltered air and concentrated air for 16 weeks. This result was supported by human studies suggesting a potential threshold effect (29 μg/m^3) for the infertility effect of PM2.5 (Xue and Zhang, 2018). Sperm motility analysis was an initial and essential test in the evaluation for male fertility. We used CASA to evaluate the quality of sperm motion and found a negative correlation between sperm motility with PM2.5 exposure. This data was similar to several previous conclusions which demonstrated that people exposed to higher level of air pollution were prone to have fewer motile sperms (Guven et al., 2008; Hammoud et al., 2010). Accounting for the spermatogenetic cycle involves the division of primitive spermatogonial stem cells into subsequent germ cells. The total duration of spermatogenesis of mice is amount to approximately 35–39 days (Guven et al., 2008; Hammoud et al., 2010). As a consequence, 8 weeks was a sufficient duration to encompass the entire period of spermatogenesis and was speculated to detect changes on any stage of the spermatogenesis when using semen measures as the biologic end-point (Selevan et al., 2000). Although no significant decrease could be observed in PR%, NP % or VCL for 8 weeks exposure, their decline trend may suggest that PM2.5 exposure do have side effect on sperm motility. VSL was determined to be related to pregnancy rates in subfertile couples and might be a bio-indicator of the fertilizing ability of human sperm (Garrett et al., 2003). VSL of mice was notably decreased in concentrated air chamber might partly strengthen the evidences that ambient air pollution did detrimental effects on male fertility (Lafuente et al., 2016). Besides, lowest motility of sperm turned out in mice exposed to concentrated air, which indicated a dose-dependent sperm damage. Moreover, mice exposed to PM2.5 for 16 weeks (112 days) had more obvious decline in sperm motility in comparison to mice exposed for 8 weeks, although this difference was not obvious, it may also indicate a time course-dependency.

Sperms were matured in the epididymis and generalized in the testis. Testis lesions could lead to biochemical dysfunction of sperm (Hong et al., 2015). Comet assay in our present study revealed increased DNA damage of testicular cells in the concentrated air group. Testicular DNA damage may be the possible origin of sperm DNA damage (Rajpurkar et al., 2002; Zhang et al., 2018) and our results made a further understanding in the mechanism of PM2.5-induced reproductive impairment. Furthermore, as reported in various studies (Rajpurkar et al., 2002; Zhang et al., 2018), DNA fragmentation may alter sperm morphology and motility. Increased rate of abnormal sperm was also found in the PM2.5 exposure groups, generally in consistent with the DNA damage. Additionally, we evaluated the types of sperm abnormality and sperms with head abnormality seemed to be positively related to PM2.5 exposure. Sperms with head abnormality have been demonstrated to result in infertility in some cases (Wolff et al., 1976).

Spermatogenesis is contingent upon hormones and growth factors acting through endocrine and paracrine pathways (Wolff et al., 1976). It is well established in various experimental models that testosterone is the key endocrine stimulus of spermatogenesis (Duan et al., 2014; Huhtaniemi et al., 2015). As the primary male sex hormone, testosterone is an anabolic steroid which plays a key role in the development of male reproductive tissues such as testis and prostate as well as promoting secondary sexual characteristics (Mooradian et al., 1987). The largest amounts of testosterone (> 95%) of male are produced by Leydig cells located in the testis. Decreased testosterone may be an indicator of the Leydig cell impairment (Sarti et al., 2011). There were findings suggested that smoking leads to a secretory dysfunction of the Leydig cells (Yamamoto et al., 1998). Rats inhaled cigarette smoke prone to have a lower testosterone level (Park et al., 2012). Previous study has shown that carbon black and titanium dioxide nanoparticles could be uptaken by Leydig TM3 cells and induce cytotoxicity (Komatsu et al., 2008). In the present study, although we failed to find remarkable changes of Leydig cells through the testicular pathology, the significant dose-dependent decrease of testosterone level might somehow indicate the Leydig cell damage after PM2.5 exposure.

Currently, evidences reported were still limited and very few data were available regarding the biological mechanisms underlying these toxicological effects. Local inflammation in the male reproductive tract (testis, epididymis) was harmful to sperm production. Account for the

spermatogenesis dysfunction induced by PM2.5 exposure, and compelling evidences suggesting inflammatory reactions happened in multiple tissues after PM2.5 exposure (Chen et al., 2018; Liu et al., 2014). The signaling platforms known as inflammasome have emerged as crucial initiators of inflammation in response to diverse pathogen- and host-derived danger signals (Janowski and Sutterwala, 2016). PM2.5 exposure suppressed the hypothalamus-pituitary-gonads axis through induction of inflammation in the hypothalamus. However, mRNA expressions of pro-inflammatory cytokines in the testis didn't change significantly (Janowski and Sutterwala, 2016). But in the present study, post-transcriptional expression of IL-1β in testis was significantly increased both in the unfiltered air and concentrated air group for both 8 and 16 weeks exposure durations. The more severe outcomes in our study might be attributed to the higher PM2.5 exposure levels. PM2.5 concentrations in chambers were about 2-folds and 13-folds higher in the unfiltered air and the concentrated air than the previous study, respectively (Qiu et al., 2018). The outcomes were also in consistent with the decreased testosterone levels in present study due to previous evidence showing that IL-1 was a potent inhibitor of luteinizing hormone/human choriogonadotropin-induced androgen formation by Leydig cells (Qiu et al., 2018). IL-1β maturation relies on caspase1, a cleaved form of pro-caspase1. And the activation of pro-caspase1 to its cleaved form results from the recruitment of the ASC regulated by the pattern recognition receptor NALP3. Data in the present study strongly suggested the impairment of sperm quality might be mediated by testicular damage through the NALP3 pathway. What's more, obviously increased expression of caspase1 was also shown in the immunofluorescence assay, and it has been seen as an indicator of cellular pyroptosis. Inflammatory reaction in testis might be one of the reasons in the PM2.5 associated spermatogenesis damage. PM2.5 exerted systemic impact as evidenced by increased leucocytes in blood (Ferguson et al., 2016). Previous study and our study found obvious inflammatory effects in lung tissues of mice exposed to PM2.5 which contributed to the pulmonary toxicity (Jin et al., 2017; Sun et al., 2017). Systemic inflammation or pulmonary inflammation could cause renal illnesses (Aztatzi-Aguilar et al., 2016), central nervous system injury (Calderon-Garciduenas et al., 2008), hepatic inflammation (Li et al., 2018) or testicular dysfunction (Elhija et al., 2005). Therefore, we speculate the inflammatory reaction in testis observed in the present study might be a consequence of systemic inflammation triggered by PM2.5, at least partially. Previous study demonstrated that 1.75 μm carboxylate microspheres could travel into testis through oropharyngeal aspiration for 6 weeks in mice (Li et al., 2017a,2017b). Therefore, the further study is required to explain the direct damages of PM2.5 in testis.

Spermatogenesis was dominated by numerous factors including protein kinases and phosphatases, or the transcriptional and translational processes (Ellis and Standfield, 2014). FOXO1 is a member of the forkhead transcription factor family that has a variety of functions, such as control of cellular growth, regulation of glucose metabolism and stem cell homeostasis (Tothova and Gilliland, 2007). The FOXO1 was indicated a requirement in spermatogenesis, from long-term spermatogonial stem cells (SSCs) self-renewal to the initiation of spermatogenesis and meiosis, or germ cell survival (Tothova and Gilliland, 2007). Altered expression of FOXO1 would result in spermatogenetic failure and FOXO1 functions were highly-context-dependent (Goertz et al., 2011; Salih and Brunet, 2008). FOXO1 was a potential target for miR-183/96/182, a microRNA cluster similar in sequence and usually function synergistically. The miR-183/96/182 cluster could directly repress expression of FOXO1 (Ichiyama et al., 2016). Our data showed a significantly decrease in this miR-183/96/182 cluster, demonstrating a possibility that FOXO1 increase may be led by the down-expressed microRNA cluster.

The present study strongly suggested that PM2.5 exposure was correlated with sperm quality decline and testicular damage depend on the NALP3 pathway. Furthermore, altered FOXO1 expression regulated by miR-183/96/182 cluster might somehow explain the impairment. Although we just provide preliminary information in this study, it has a number of strengths: A whole-body exposure mouse model was used to mimic the real world exposure of humans, thus achieving a more compelling outcome. We set the filtered air group, unfiltered air group and concentrated air group to achieve analogous doses treatment and different time courses in purpose to observe a time dependency. Time-dependency changes were found in testosterone levels, pro-caspase1 expressions and miR96 expressions. Almost all changes of parameters we measured were more obvious in the concentrated air group than that of the unfiltered air group. Additionally, to our knowledge, this is the first study revealing the NALP3 pathway involved in the PM2.5 induced testicular damage. There are also some limitations: We calculated the PM2.5 concentration due to its content in the chamber. The inner dose deposited in target organs of mice was unknown. Sperm formation was a complex process not only including spermatogenesis in the testis but also sperm maturation in the epididymis. So, sperm quality decline after PM2.5 exposure may not just be attributed to the testicular impairment.

5. Conclusion

Overall, in the present study we established a real time whole-body PM2.5 mouse model to evaluate the male reproductive disorders. Our results demonstrated that PM2.5 exposure was associated with reduced sperm quality in a time-depended and dose-depended manner. The decreases of sperm quality might be partly explained by the inflammatory reaction in testis which was as a consequence of systemic inflammation triggered by PM2.5. The mechanism of testicular damage might be through the NALP3 inflammasome pathway and spermatogenetic failure depended on miR-183/96/182 cluster targeting to FOXO1.

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Conflicts of interest

The author declares no conflict of interest.

Novelty statement

In the present study we established a real time whole-body PM2.5 mouse model to evaluate the male reproductive disorders. Our results demonstrated that PM2.5 exposure was associated with reduced sperm quality depending on concentration of PM2.5 and exposure time. The down-regulated sperm quality might be explained by the testicular damage through the NALP3 inflammasome pathway and spermatogenetic failure dependent on miR-183/96/182 cluster targeting to FOXO1. This study will raise more public concern over the detrimental effects of PM2.5 on male reproductive health as well as increase our understanding of the underlying mechanism. Furthermore, it will allow us to focus on improving environment and optimizing male reproductive potential.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.10.108.

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