



Effects of fine particulate matter (PM_{2.5}) on ovarian function and embryo quality in mice

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ARTICLE INFO

Handling Editor: Da Chen

Keywords:

PM_{2.5}
Ovary
Embryo
Oocyte
Mice

ABSTRACT

Fine particulate matter (PM_{2.5}) has an adverse effect on reproductive function, in particular causing reduced male reproductive function, but relatively few studies have directly targeted its effects on female reproduction. To investigate the effects of PM_{2.5} exposure on female reproduction, we exposed female mice to PM_{2.5} by intratracheal instillation for 28 days, and evaluated apoptosis of ovarian granulosa cells and oocytes and the quality embryos after insemination. Our results showed increased numbers of apoptotic granulosa cells and oocytes after exposure to elevated concentrations of PM_{2.5}, which had adverse effects on female fertility via compromising embryo development and quality. We conclude that PM_{2.5} induced apoptosis of ovarian granulosa cells and oocytes leading to disrupted embryo development and female fertility in mice.

1. Introduction

Particulate matter (PM) is one of the main air pollutants and there are increasing concerns regarding its harmful impact on human health due to its complex composition and wide distribution. The effects of PM on health depend on its chemical composition and size distribution (Chen et al., 2014). Compared with larger particles, airborne fine particulate matter (PM_{2.5}), whose aerodynamic particle diameter is less than 2.5 μm, can penetrate deep into the lung and pass through the air–blood barrier to enter circulating blood, and consequently have a serious effect on health. The PM_{2.5} comes from a wide range of sources, including motor vehicles, combustion of coal or oil, transformation products from nitrogen dioxide or sulfur dioxide, as well as organics, such as biogenic organics and dust. The major components of PM_{2.5} include the element carbon, organic carbon, sulfate, nitrate, ammonium, organic compounds (including polycyclic aromatic

hydrocarbons) and metals, many of which are noxious to the health (Srimuruganandam and Nagendra, 2012).

Epidemiologic and experimental studies support the concept that PM_{2.5} exposure has negative effects on multiple biological systems, such as the respiratory, cardiovascular, nervous and immune systems (Guaita et al., 2011; Halonen et al., 2009; Sram et al., 2017; Pearson et al., 2010). Exposure to PM_{2.5} can also affect both male and female reproductive systems (Carré et al., 2017). Studies on animals and humans found that certain air pollutants induced a decline in sperm quality (Najafi et al., 2015). Compared to many reports on the male reproductive system, there is less research into PM_{2.5} effects on females. In a study on humans, Slama et al. examined short-term PM_{2.5} exposure before conception in 1916 couples, and found that each incremental increase of 10 μg/m³ PM_{2.5} was associated with a 22% [95% confidence interval (CI), 6–35%] decrease in conception rate, to which both male and female may contribute (Slama et al., 2013). Nieuwenhuijsen et al.

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found a link between reduction in fertility rate and an increase in the ambient levels of PM_{2.5-10} (PM with an aerodynamic diameter within 2.5 and 10 µm) (Nieuwenhuijsen et al., 2014). In animal studies, PM_{2.5} caused ovarian injury through inflammatory and oxidative stress responses in mice (Gai et al., 2017). A recent study reported that secondary organic aerosol derived from diesel exhaust, mainly PM_{2.5} with the presence of benzoquinone as a toxic component, affected meiotic spindle formation and destabilized microtubules in mouse oocytes (Udagawa et al., 2018).

Given that animal studies of PM_{2.5} effects on female fertility were few and most of the human data came from retrospective studies, the influence of PM_{2.5} toxicity on female fertility and its underlying mechanisms need to be further explored. In the present study, we exposed female mice to PM_{2.5} by intratracheal instillation and investigated pathological and functional changes in the ovary and oocytes, along with embryonic development and fetal outcomes.

2. Materials and methods

2.1. Animals and ethical approval

Two hundred and ten four-week-old, non-parous and specific-pathogen-free Kunming female mice (18–22 g), together with forty-five six-week-old male mice (24–28 g) for copulation, were purchased from Shanghai Slack Laboratory Animal Company (Certificate No. SCXK2012-0002, Shanghai, China). Mice were housed in a temperature-controlled room at 24 ± 1 °C under a 12–12 h light-dark cycle with food and water *ad libitum*. Mice were randomly divided into five groups (42 per group): a baseline group (B) administered phosphate buffered solution (PBS), a control group (C) with PBS plus carboxymethylcellulose sodium (CMC), an atoxic water-soluble binder (De Giorgio et al., 2017), and three PM_{2.5} groups receiving 0.36 (L), 1.2 (M) or 2.4 (H) µg/µL, respectively. All animal experiments were conducted in accordance with the guidelines of the Animal Studies Committee of Xiamen University, China (approval ID: XMUMC 2011-10-08), and the study was approved by the Animal Care and Use Committee of Xiamen University.

2.2. PM_{2.5} sample collection and preparation of PM_{2.5} suspension

The PM_{2.5} were collected on Teflon filters with a diameter of 75 mm and pore diameter of 0.45 µm (PALL, USA) at a flow rate of 100 L/min using an eight-stage cascade impactor (MOUDI 131, MSP, USA) from 1st April to 1st June 2016. The sampler was set on the rooftop of Student Center, a three-story building about 10 m high located within the campus of Xiamen University in the Xiang'an District, where it was fairly open without surrounding heavy traffic but had a large brick factory 500 m southeast of the building. The MOUDI 131 sampler (Pipalatkhar et al., 2012, Sun et al., 2013) segregates airborne particle portions by the following size categories: ≤0.25, 0.25–0.44, 0.44–1.0, 1.0–1.4, 1.4–2.5, 2.5–10.0, 10.0–16.0 and > 16.0 µm. We chose filters from below 2.5 µm, which consisted of the entire particle components with an aerodynamic diameter of less than 2.5 µm. The PM_{2.5} samples were collected by repeatedly and gently scratching one way on the filter surface using a smooth blunt blade. Samples were stored at –80 °C. Before use, PM_{2.5} samples were re-suspended in PBS containing 0.2% CMC (Sigma, USA) to adjust PM_{2.5} suspensions at concentrations of 0.36, 1.2 or 2.4 µg/µL and then processed by ultrasonic concussion for 20 min.

The 24-hour PM_{2.5} deposition quantity for a mouse was calculated assuming an alveolar ventilation of about 24 mL/min per mouse and approximately 70% of the inhaled atmospheric particulates would be deposited in the deep airways and alveoli (Hougaard et al., 2008; Cassee et al., 2002). As the alveolar ventilation in mice is about 24 mL/min (Vorherr, 1982), the formula for 24-hour PM_{2.5} deposition in mice's alveolar was calculated as following: $N = 24 \text{ mL/min} \times A\mu\text{g/}$

Table 1

Experimental grouping and exposure profile.

Group	Exposure profile	PM _{2.5} samples (µg)	Relevant ambient PM _{2.5} concentration (µg/m ³)
Baseline	PBS	–	–
Control	PBS + CMC	–	–
L	PBS + CMC + 0.36 µg/µL PM _{2.5}	3.6	150
M	PBS + CMC + 1.2 µg/µL PM _{2.5}	12	500
H	PBS + CMC + 2.4 µg/µL PM _{2.5}	24	1000

Note: L, low dose group; M, medium dose group; H, high dose group.

$\text{m}^3 \times 60 \text{ min} \times 24 \text{ h} \times 70\% = 24 \times 10^{-3} \times 10^{-3} \text{ m}^3/\text{min} \times A\mu\text{g}/\text{m}^3 \times 60 \text{ min} \times 24 \text{ h} \times 70\% = 0.024A(\mu\text{g})$ (N: 24 h PM_{2.5} deposition; A: PM_{2.5} concentration). Table 1 shows the concentration of PM_{2.5} suspensions used and their roughly relevant ambient PM_{2.5} concentrations.

2.3. Intratracheal instillation of PM_{2.5} suspensions

The intratracheal instillation method was a combination and improvement of methods introduced by Gerlofs-Nijland (Gerlofs-Nijland et al., 2005) and Saber et al. (2012). Briefly, after inhalation of 0.1% isoflurane for about 15 sec, each mouse was positioned supinely with its forelegs secured on a table at a 60-degree angle, then the tongue was pressed towards the lower jaw using a small spatula. A 24-gauge BD Insyte catheter (Becton-Dickison, USA) was inserted into the trachea to a depth 1 cm above the bifurcation. A volume of 30 µL PBS, CMC, or PM_{2.5} suspension at 0.36, 1.2 or 2.4 µg/µL was injected into the trachea through the catheter connected to a pipette tip (Sigma, USA) between each breath within seconds. Immediately after the instillation, 150 µL of air was injected using a glass syringe to spread the instilled volume over the lung. Mice were administered 10.8, 36 or 72 µg PM_{2.5} every three days over a 28-day period of exposure. Mice were kept in isolated chambers where airborne particulates were filtered by high efficiency particulate air filters (HEPA) during the exposure period, except when PM_{2.5} administration was performed.

2.4. Animal assignment and specimen pretreatment

At day 28, after the last intratracheal instillation was performed, twenty-four female mice from each group were sacrificed by euthanasia via cervical dislocation. For each group, 8 mice were assigned for oocyte retrieval, annexin V staining and morphological observation, 8 mice for deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and caspase-3 staining of ovarian cells, and 8 mice for transmission electron microscopy of oocytes and granulosa cells. The ovaries were isolated and cultured in M2 medium for germinal vesicle (GV) oocyte collection, then fixed in 4% paraformaldehyde for TUNEL and caspase-3 staining, or were directly fixed with 0.25% glutaraldehyde for transmission electron microscopy. The another 8 mice were mated with male mice after superovulation to investigate blastocyst retrieval. The remaining 10 mice were mated without superovulation to observe insemination and their offspring.

2.5. GV oocyte morphology

Ovaries were obtained from each mouse and intact GV oocytes were collected under an inverted microscope (SMZ1000 Nikon Co., Tokyo, Japan) and incubated in M2 medium covered by paraffin oil in a 5% CO₂ and 95% air incubator. Oocytes that were blocked at GV stage were transferred and cultured in M2 medium plus 2.5 mM milrinone (Cayman, USA) for morphological observation after staining.

2.6. Annexin V staining for oocytes

To determine early apoptosis of oocytes, an annexin V probe kit (Beyotime, China) was used according to the manufacturer's instructions. Briefly, oocytes were cultured in M2 medium plus annexin V probe away from light at 5% CO₂, 95% air and 37 °C for 30 min. After washing, stained oocytes were fixed and co-stained with DAPI (Vector, Switzerland). Fluorescent signals were examined with a confocal laser scanning microscope (FV1000, Olympus Co., Japan).

2.7. Transmission electron microscopy of oocytes and granulosa cells

The fresh ovarian tissues ($n = 8$) were fixed with 0.25% glutaraldehyde overnight and then washed three times with PBS. Tissues were then fixed in 1% osmium tetroxide in cacodylate buffer for 1 h and then embedded in araldite. Ultrathin sections were stained in a saturated uranyl acetate solution in 50% ethanol. The cell ultrastructure, including cytoplasmic membrane, mitochondrial cristae, nuclear membrane and cytoplasmic organelles of oocytes and granulosa cells were observed by a transmission electron microscope (JEM-2100, Japan).

2.8. TUNEL and caspase-3 staining of ovarian cells

After an overnight incubation in 4% paraformaldehyde, the ovaries were dehydrated, embedded in paraffin, sectioned serially at 5 μm and slices attached to glass slides, which were then heated at 60 °C for 2 h. After a two-step xylene dewaxing, slides were washed in a series of graded ethanol/water solutions and treated for TUNEL and caspase-3 staining. Apoptosis analysis of ovarian cells was performed using TUNEL reaction mixture (Keygene Biotech, China), in which nuclei were stained with DAPI. TUNEL-positive and total cell nuclei were observed with a laser scanning microscope and ratios for the numbers of TUNEL-positive nuclei versus the total cell nuclei were calculated for eight different serial sections. The arithmetical means of the ratios were used for statistical analysis among the groups.

For caspase-3 staining, the slides were incubated in 0.01 M sodium citrate at 100 °C for 10 min, blocked with 1% bovine serum albumin (BSA)-supplemented PBS for 1 h, and incubated with 1:200 rabbit polyclonal anti-caspase-3 antibody (Cell Signaling Technology Inc., USA) at 4 °C overnight. After the incubation, slides were washed with PBS three times for 5 min each, and sections were labeled with fluorescence secondary antibody FITC-conjugated goat anti-rabbit IgG (1:200) at 37 °C for 30 min. Caspase-3-positive and total cell nuclei were observed with a confocal laser scanning microscope and ratios for the caspase-positive nuclei versus total cell nuclei were calculated in eight different serial sections.

2.9. Fertility trial

For embryo collection, 8 mice per group were superovulated to obtain more embryos via peritoneal injection of pregnant mare serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG) (Ningbo Sansheng Pharmaceutical Co. Ltd., China), both at 10 IU/0.1 mL per mouse with an interval of 48 h. The female mice were then mated with male mice separately at a ratio of 1:1 for copulation. On the next morning, successfully mated female mice detected by the presence of vaginal plugs were retrieved and designated as gestation day 0 (D0). At least 6 mice were successfully mated within 3 days for all the groups (Fig. 1A). At D3.5 of pregnancy, mice for embryo retrieval ($n = 6$) were sacrificed by euthanasia via cervical dislocation, then uteri were immediately flushed with KSOM to harvest the embryos. The remaining 10 mice were mated without superovulation. After spontaneous delivery, the number of mated and post-delivery mice and litter sizes were recorded (Fig. 1B).

For Oct4 and TUNEL staining, blastocysts were fixed with 4%

paraformaldehyde at room temperature for 30 min and then transferred to a permeabilization solution (0.5% Triton X-100) for 30 min. The blastocysts were incubated in 1% BSA-supplemented PBS for 1 h, followed by incubation with mouse anti-Oct4 monoclonal antibody at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA., US) at 4 °C overnight. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 5 min each, the blastocysts were labeled with fluorescence secondary antibody Alexa Fluor 546-conjugated donkey anti-mouse IgG (1:100) at 37 °C for 1 h. TUNEL staining in blastocysts was performed following instructions of the kits. The blastocysts were then co-stained with DAPI, mounted on glass slides with mounting medium, and examined using a confocal laser scanning microscope.

2.10. Statistical analysis

Data were expressed as the mean \pm standard error of the mean and were analyzed by ANOVA using SPSS 19.0 statistical software (IBM Corp, USA) followed by Fisher's protected least significant difference test. The P value < 0.05 was considered statistically significant.

3. Results

3.1. Litter sizes of post-delivery and mated mice

After PM_{2.5} treatment for 28 days, the litter sizes of mice from L group were similar to B and C groups. Compared with the other groups, H group showed a significantly reduced average litter size from post-delivery ($P < 0.05$; Fig. 2B) and mated ($P < 0.05$; Fig. 2C) mice. The H group, but not L group, showed a significantly reduced average litter size from mated mice compared with the baseline and control groups ($P < 0.05$; Fig. 2C).

3.2. Apoptosis in granulosa cells and oocytes

The detection of apoptotic granulosa cells surrounding the growing follicles is shown in Fig. 3A, and the ratio of apoptotic granulosa cells was significantly elevated in M and H groups compared with B, C or L group ($P < 0.05$; Fig. 3B). The percentage of caspase-3 positive staining in total granulosa cells was increased in H group compared with the other groups ($P < 0.05$; Fig. 3D). The annexin V positive signal, which was visualized as green fluorescence, was found in the membrane and zona pellucida of early apoptotic oocytes (Fig. 3E), and the ratios of annexin V positive cells to total cells in M and H groups were markedly higher than those in other groups ($P < 0.05$; Fig. 3F).

3.3. Changes in ovarian ultrastructure

As shown in Fig. 4, morphologically normal nuclei with homogeneous chromatin, clear nucleoli and double-layer nuclear membranes, and normal mitochondrial and endoplasmic reticulum structure were observed in the oocytes and granulosa cells from B and C groups. In the M and H groups, but not in the L group, obvious morphological abnormalities were found in the cytoplasmic membrane, mitochondrial cristae, nuclear membrane and cytoplasmic organelles of oocytes and granulosa cells. Nuclear membrane collapse, chromatin margination and apoptotic bodies were found in some granulosa cells from the H group (Fig. 4B). Increased mitochondrial and cytoplasmic density and decreased mitochondrial volume in granulosa cells, and mitochondrial swelling and cristae breakage in the oocytes were also observed in the M and H groups (Fig. 4C).

3.4. Embryo quality

Mice exposed to PM_{2.5} at different doses produced similar numbers of oocytes but exhibited decreased blastocyst formation rates and increased the percentages of lysed embryos compared with mice not

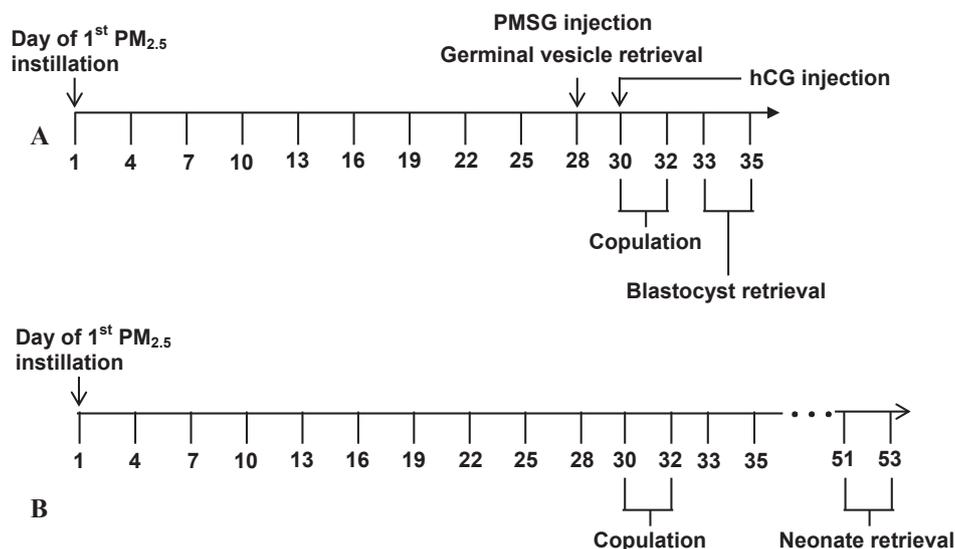


Fig. 1. Exposure profile and the days of euthanasia and oocyte retrieval. Copulation and successful mating occurred at day 30, 31 or 32, blastocyst retrieval was conducted 3.5 days after pregnant (A), and neonate retrieval was performed when spontaneous delivery occurred (B).

exposed to PM_{2.5} (Fig. 5; Table 2). Ratios for the number of Oct4 positively stained cells to total cells per blastocyst were significantly reduced (Fig. 6B), and percentages of apoptotic cells in total cells per blastocyst were significantly elevated (Fig. 6C) in the M and H groups as compared with the B, C or L group ($P < 0.05$).

4. Discussion

In this study, we investigated the effect of PM_{2.5} on ovaries, follicle, oocytes and embryos in a murine model via intratracheal instillation of PM_{2.5} *in vivo*. Our results suggested that PM_{2.5} decreased reproductive function in female mice, augmented apoptosis in ovarian granulosa cells and oocytes, and compromised the quality of embryos.

In the present study, the expression of caspase-3 in ovarian granulosa cells was dose-dependently increased by PM_{2.5} exposure. The caspase family plays an important role in the reactive oxygen species (ROS)-mediated mitochondrial apoptotic signaling pathway, of which caspase-3 is one of the most important proteins. Caspase-3 activation

indicates that apoptosis has entered an irreversible stage. The ROS-mediated apoptotic signaling pathway involves the release of mitochondrial cytochrome C into the cytoplasm to activate caspase-9, triggering downstream caspase cascades, and ultimately activating caspase-3 located at the end of the apoptotic cascades (Tang et al., 2009). In the ovary, excessive ROS induced apoptosis of granulosa cells via the involvement of the ROS-mediated mitochondria apoptotic pathway (Wu et al., 2011). The main toxic components in PM_{2.5}, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals, stimulate ROS overproduction, and excessive ROS causes cell apoptosis by inducing oxidative damage to DNA, proteins, lipids and other macromolecules (Agarwal et al., 2012). Our results suggested that PM_{2.5} induced apoptosis of granulosa cells, also indicated by TUNEL positive staining in the ovary. Granulosa cells physiologically protect oocytes against oxidative stress, and granulosa cell apoptosis increased oxidant levels, reduced meiotic competency, and increased levels of pro-apoptotic as well as apoptotic factors leading to oocyte apoptosis (Tiwari et al., 2015). Oocyte quality may be evaluated by early cell apoptosis,

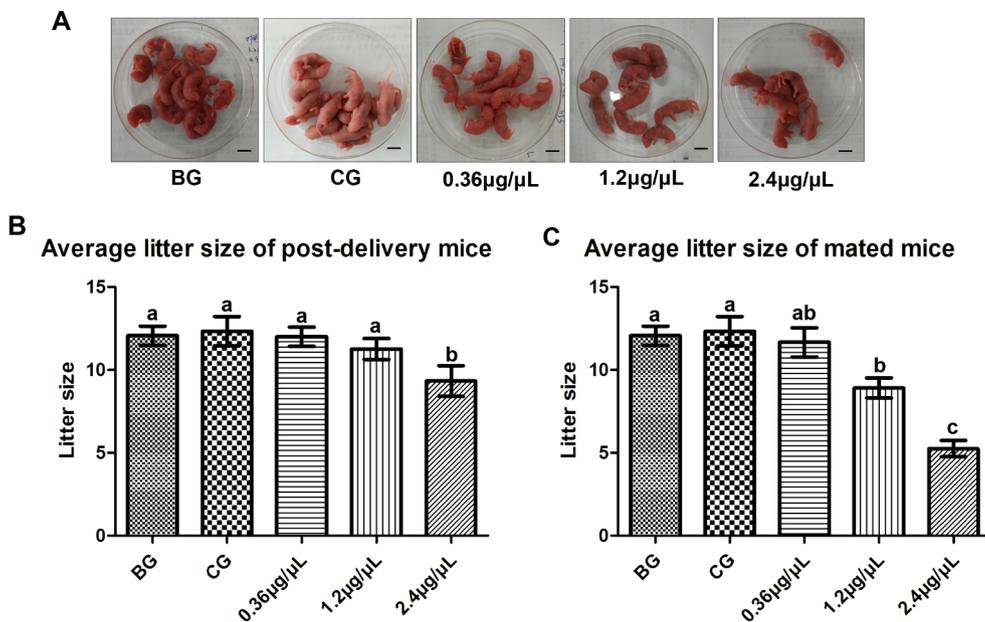


Fig. 2. Litter size of mice after administration of fine particulate matter (PM_{2.5}) for 28 days. Representative litter sizes from different groups were photographed (A). Histograms refer to average litter size of post-delivery mice (B) and mated mice (C) groups. a vs. b, $P < 0.05$; a, ab or b vs. c, $P < 0.05$. The bars represent mean \pm SEM.

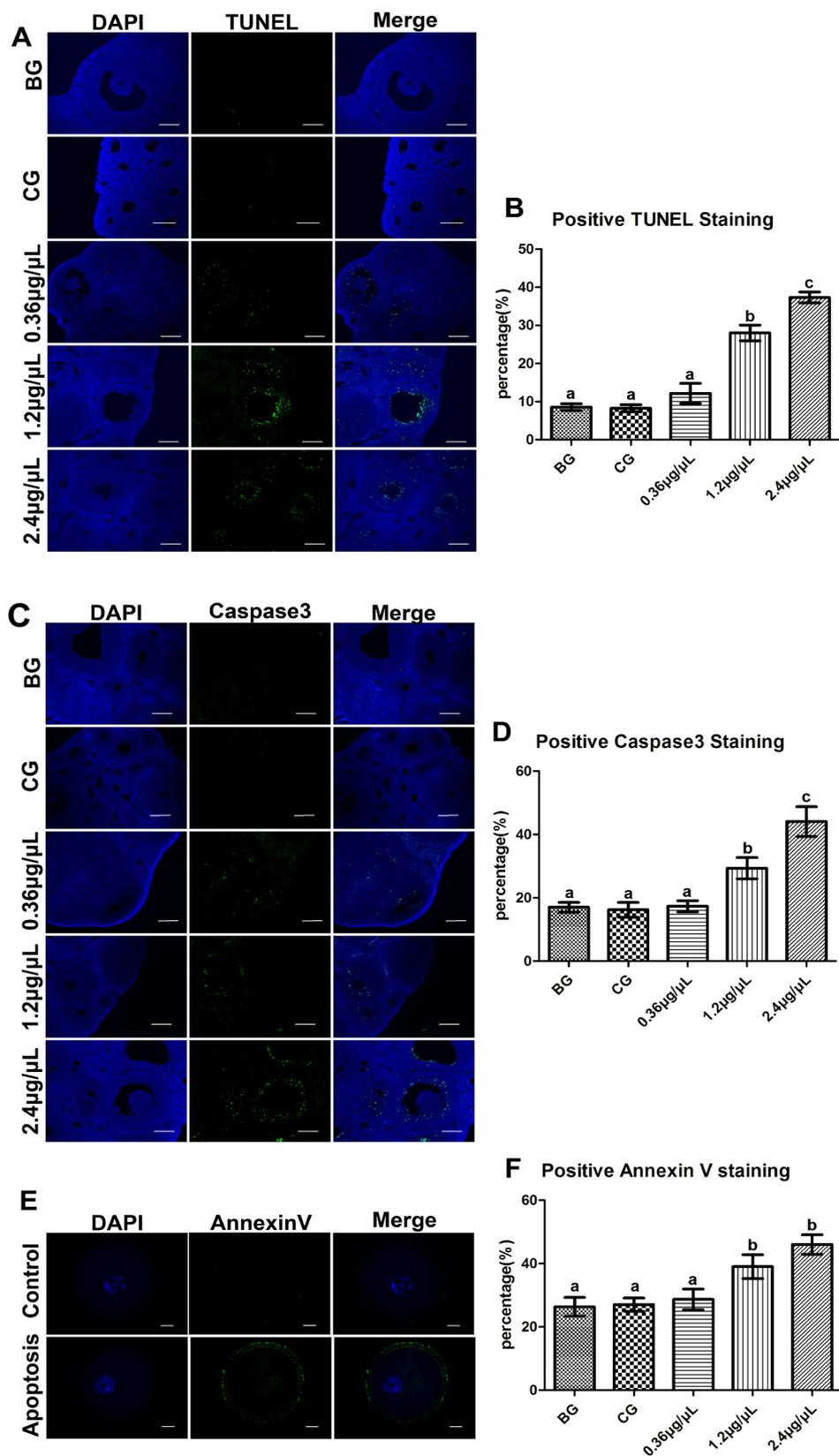


Fig. 3. Immunofluorescence images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (A) and caspase-3 (C) staining in ovaries, and annexin V (E) staining in oocytes, in which positive staining is displayed in green. The intact oocytes show that fluorescence is restricted to the zona pellucida, whereas early apoptotic oocytes have fluorescence in both zona pellucida and membranes (E). DNA stained with DAPI was blue. Scale bar = 100 µm. The corresponding histograms delineate the percentages of follicles with positive TUNEL (B), caspase-3 (D) or annexin V (F) staining and comparisons among the groups. Scale bar = 20 µm. a vs. b or c, $P < 0.05$. The bars show mean \pm SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detected by annexin V staining of oocytes. Our results indicated that the higher concentrations of PM_{2.5} resulted in a greater number of early apoptotic oocytes. We propose that granulosa cell apoptosis affected the quality of oocytes.

Mitochondria provide the main generation center of ATP in cells,

but also play a vital role in the process of apoptosis. In this study, we found that mitochondrial swelling and cristae breakage in oocytes from 1.2 and 2.4 µg/µl PM_{2.5}-treated animals, which suggested disturbance of mitochondrial function. Three kinds of apoptotic pathways are caused by mitochondria alterations: release of proteins that stimulate

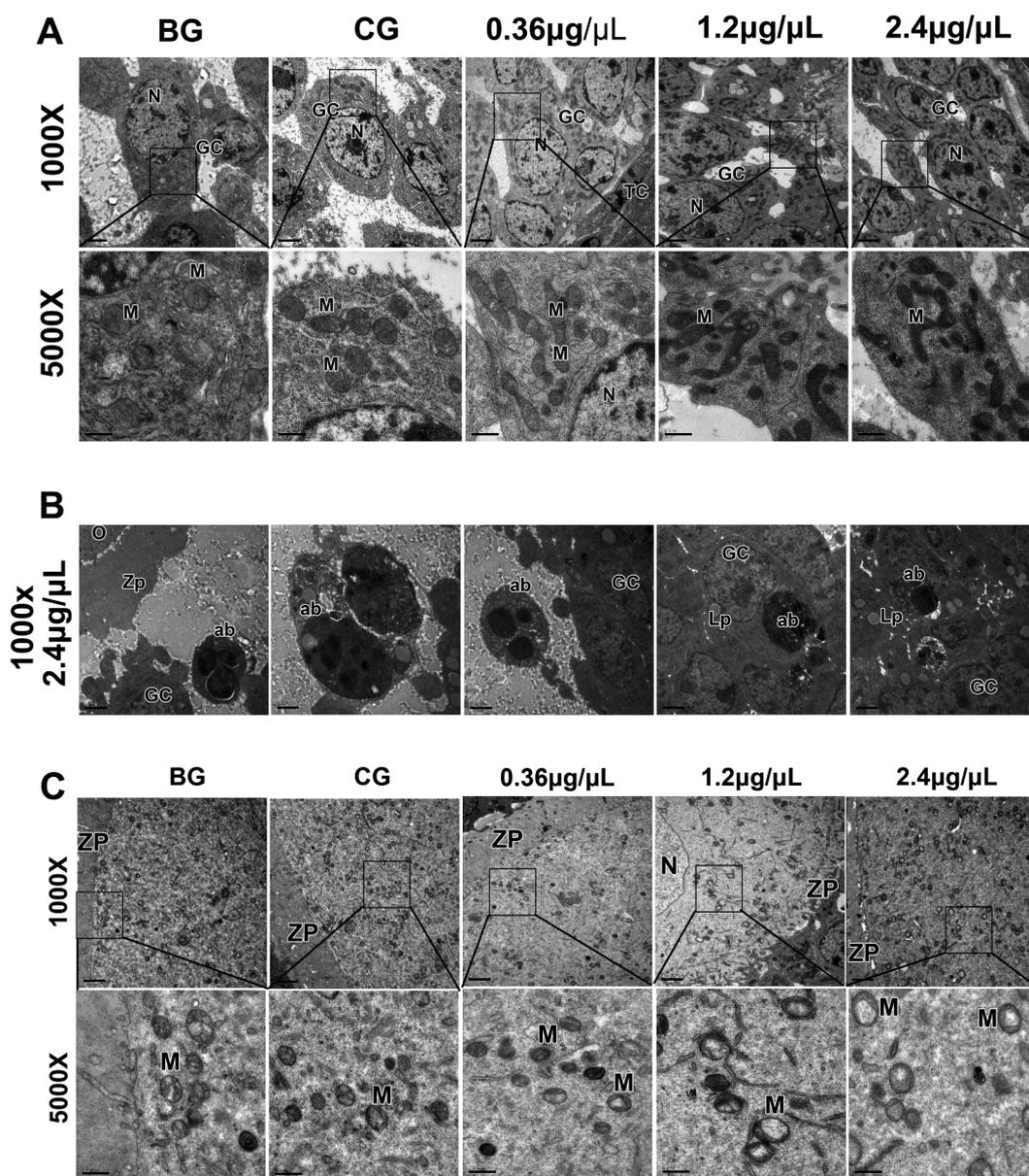


Fig. 4. The ovarian ultrastructure in different groups. Ovary tissues were processed by fixation in osmium tetroxide, then embedded in araldite for ultrathin sections. The cell ultrastructure was observed by transmission electron microscopy after staining with saturated uranyl acetate solution. A: electron micrographs of granulosa cells from different groups; B: apoptotic body from high dose ($2.4 \mu\text{g}/\mu\text{L}$) $\text{PM}_{2.5}$ -treated group; C: electron micrographs of mitochondria from different groups. GC: granulosa cell; M: mitochondria; N: nucleus; TC: theca cell; ab: apoptotic bodies; Lp: lipid; O: oocyte; Zp: zona pellucidum. Bars represent $2 \mu\text{m}$ in $1000\times$ and $0.4 \mu\text{m}$ in $5000\times$ pictures.

the caspase family such as cytochrome C, destruction of electron transport, oxidative phosphorylation and ATP production, and change of cellular redox potential (Matsuda et al., 2012). Therefore, $\text{PM}_{2.5}$ may activate the apoptotic pathway and cause apoptosis of granulosa cells and oocytes via mitochondrial dysfunction. This may be one of the important mechanisms by which $\text{PM}_{2.5}$ affects the reproductive system in female mice.

High-quality oocytes are vital to successful fertilization and embryo development. Previous research demonstrated an increased percentage of embryonic apoptosis when murine embryos were exposed to high levels of diesel exhaust particles *in vitro*, and blastocyst distribution and inner cell mass (ICM) morphology were altered (Januário et al., 2010). Another study reported that there were no significant differences in ovarian response and blastocyst numbers between long-term $\text{PM}_{2.5}$ -exposed and non-exposed mice by *in vitro* fertilization. However, the ratio of inner cell mass/trophectoderm (ICM/TE), which is clinically

associated with embryo chromosome ploidy and reflects blastocyst pluripotency, was decreased by 25%, in the $\text{PM}_{2.5}$ -exposed group compared with the non-exposed group (Maluf et al., 2009). In the present study, we found that $\text{PM}_{2.5}$ exposure decreased the expression of Oct4 and increased cell apoptosis in blastocysts, mainly in the ICM. As an important indicator of blastocyst quality, Oct4 is mainly expressed in the ICM before embryonic implantation and plays a key role in ICM formation, which is essential for embryonic development (Liu et al., 2004). If an embryo lacks Oct4, the blastocysts will not form an ICM and die shortly after implantation (Palmieri et al., 1994). Our current results, showing $\text{PM}_{2.5}$ -induced down-regulation of Oct4 expression, suggested $\text{PM}_{2.5}$ exposure compromised embryo quality. In general, ICM is more sensitive to toxicants than the trophectoderm. This could explain why the apoptosis mainly occurred in the ICM in our study. We propose that $\text{PM}_{2.5}$ may affect the quality of blastocyst by decreasing Oct4 expression and increasing ICM apoptosis. Decreased

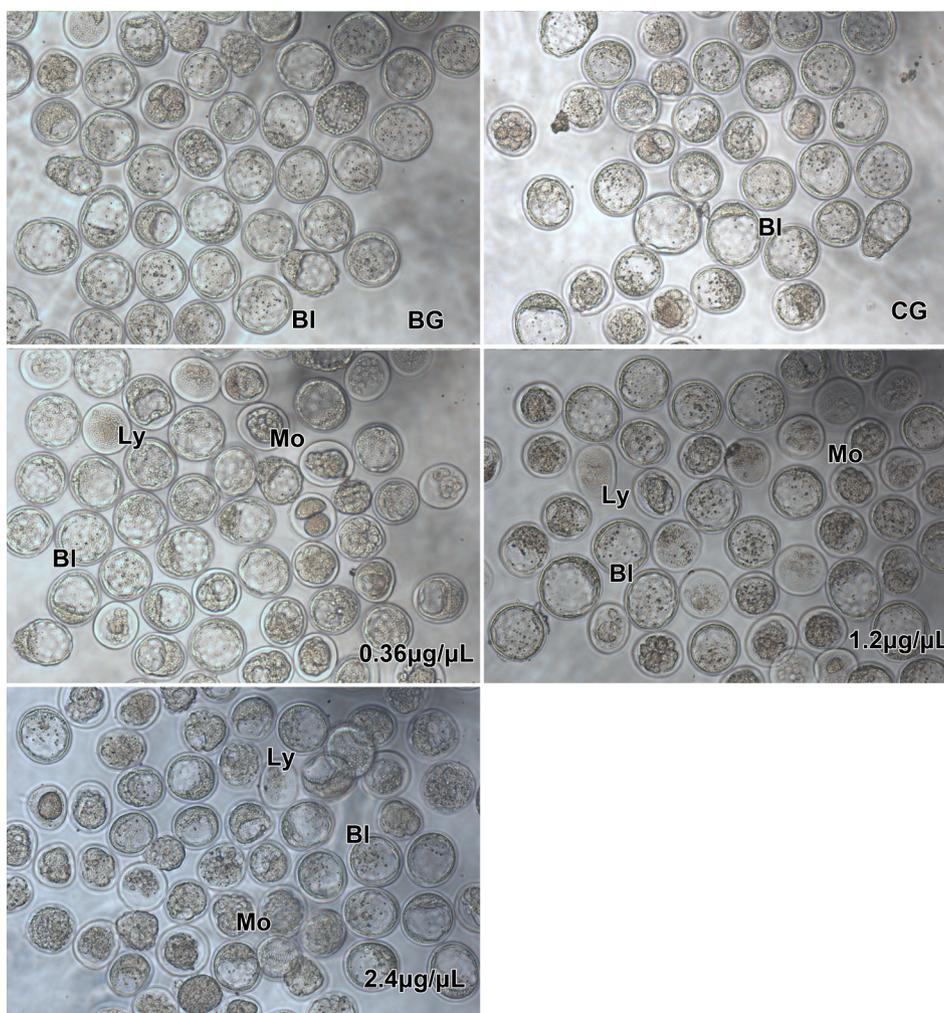


Fig. 5. Fine particulate matter ($PM_{2.5}$) affects embryo development *in vivo*. (A) Morphology of different stages of embryos in different groups. BI: Blastocyst; Mo: Morula; Ly: Lysed.

Table 2
Groups with morula, blastocyst and lysed of embryos.

Treatment	Total embryos, n	Embryos/mouse, n	Morula, n(%)	Blastocyst, n(%)	Lysed, n(%)	Others*
Baseline	185	31	34(18.4) ^a	135(73.0) ^c	11 (5.9) ^f	5
Control	220	37	40(18.2) ^a	155(70.5) ^c	21 (9.5) ^f	4
0.36 µg/µL	170	28	29(17.1) ^a	110(64.7) ^c	21(12.4) ^f	10
1.2 µg/µL	151	25	39(25.8) ^b	78(51.7) ^d	19(12.6) ^f	15
2.4 µg/µL	159	27	54(34.0) ^b	63(39.6) ^e	23(14.5) ^g	19

Note: a vs. b: $P < 0.01$; c vs. d: $P < 0.05$; c vs. e: $P < 0.01$; f vs. g: $P < 0.05$.

* Arrested before morula.

blastocyst quality affected subsequent embryo implantation and development, which may be associated with early miscarriage.

In the present study, litter size decreased after $PM_{2.5}$ exposure. In the 1.2 µg/µL and 2.4 µg/µL $PM_{2.5}$ treatment groups, we found that the average litter size from mated female group was different from that of the post-delivery female group, which may be attributable to miscarriage in the mated group, as few post-delivery mice after detection of vaginal plugs failed to deliver any offspring. We speculated that $PM_{2.5}$ exposure increased the risk of miscarriage. In a clinical investigation, Perin et al. found that 531 pregnant women from either spontaneous pregnancy or *in vitro* fertilization had a 2.6-fold increase in early miscarriage rate when exposed to high concentrations of PM in their follicular phase; they also found that short-term PM exposure resulted in an increased risk of early miscarriage with no obvious changes in

biological and ovarian stimulation parameters (Perin et al., 2010a, 2010b). Our results provide further the experimental evidence to support an unfavorable impact of $PM_{2.5}$ upon the probability of miscarriage.

According to China's Ambient Air Quality Standards revised in 2012, China's current annual and daily standard criteria for average $PM_{2.5}$ concentrations are 75 and 35 µg/m³, respectively. These standards are approximately three times higher than the current suggested levels of $PM_{2.5}$ proposed by the World Health Organization (WHO), in which the annual level of $PM_{2.5}$ is recommended below 10 µg/m³ and daily level of $PM_{2.5}$ below 25 µg/m³. However, many cities in China have not yet met these standards (Lowson and Conway, 2016). It is imperative to increase awareness among the population and public authorities to protect the environment as much as possible, and to make

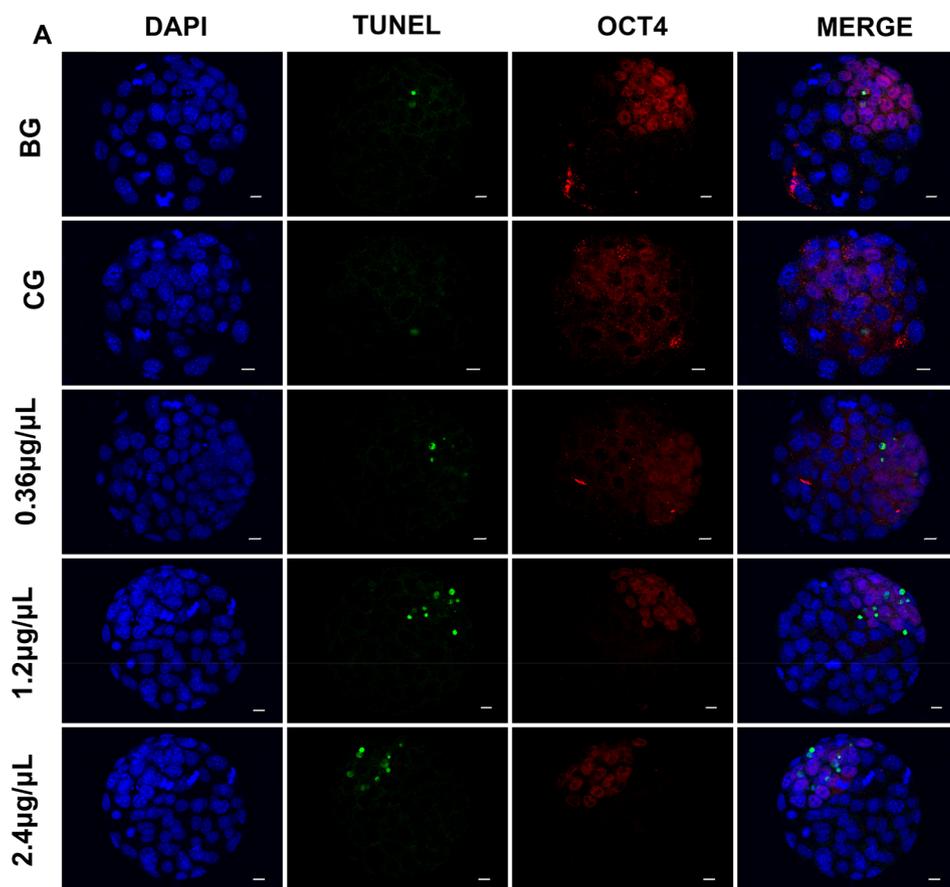


Fig. 6. Immunofluorescence images for Oct4 positive staining (red) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive staining (green) in embryos with DAPI-stained nuclei in blue (A). The number of Oct4-positive cells indicates the cell number of the inner cell mass in an embryo (B). The number of TUNEL-positive cells was counted and the total cell number was estimated by counting the nuclei for each embryo, then the overall percentage of apoptotic cells was calculated per embryo (C). a vs. b or c, $P < 0.05$. Bars represent the mean \pm SEM in the corresponding histograms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

steady progress to meet the Chinese standards to promote a healthy life for current and future generations.

There are some limitations in our study. We instilled PM_{2.5} with doses of 1.2 and 2.4 $\mu\text{g}/\mu\text{L}$ in mice to simulate the ambient PM_{2.5} concentrations at 500 and 1000 $\mu\text{g}/\text{m}^3$, respectively, which is practically absent or rarely encountered in the actual living environment. We used such high doses because we predicted that exposure to PM_{2.5} at high levels would elicit obvious effects on the female reproductive system, making it experimentally more practical to exploring its underlying mechanisms of reproductive toxicity. Our current investigation into the effects of PM_{2.5} on ovary, follicle, oocyte and embryo development in female mice, and its possible mechanisms, adds more information to the field of environmental toxicology and reproductive health.

Statement of author contributions

Hai-Long WANG and Xin-Ru HONG designed the study and applied for Research Ethics Board approval. Bao-Qiong LIAO, Shu-Juan XIE, and Chao-Bin LIU collected and analyzed the data and prepared the manuscript. Yu LIU and Ya-Bin DENG assisted with animal experiments.

Shu-Wen HE, Xian-Pei FU, Bin-Bin FU, and Ya-Long WANG assisted with analysis tools of data. Ming-Huang CHEN, Yan-Hong LIN, Fei-Ping LI and Xi XIE discussed the results and manuscript. All authors approved the final manuscript.

Declaration of Competing Interest

There are no conflicts of interest to declare.

Acknowledgements

This study was supported by grants from The National Key Research and Development Program of China under Grant No. 2017YFD0501905. This work was also supported by research grants from the National Natural Science Foundation of China (81172677, 81773448, 81973051 and 31672248) and the Natural Science Fund of Fujian Province (2015J01493), China. Prof. C. Liu was supported by a research grant from Fujian Hygiene, Science and Education Fund of Key Clinical Specialty Discipline Construction Program (2012149).

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