Ovarian Damages Produced by Aerosolized Fine Particulate Matter (PM$_{2.5}$) Pollution in Mice: Possible Protective Medications and Mechanisms

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Abstract

Background: Ambient aerosol fine particulate matter (PM$_{2.5}$) is associated with male reproductive toxicity in experiments and may have adverse effects in the female. However, studies evaluating the protective effects and precise mechanisms of aspirin, Vitamin C, Vitamin E, or ozone against toxic effects of PM$_{2.5}$ are sparse. This study was conducted to investigate the possible protective effects and mechanisms of aspirin, Vitamin C, Vitamin E, or ozone on fertility in female mice treated with PM$_{2.5}$.

Methods: Eighty-four ICR mice were divided into six groups: control group, PM$_{2.5}$ group, PM$_{2.5}$ + aspirin group, PM$_{2.5}$ + Vitamin C group, PM$_{2.5}$ + Vitamin E group, and PM$_{2.5}$ + ozone group. PM$_{2.5}$ was given by intratracheal instillation every 2 days for 3 weeks. Aspirin, Vitamin C, and Vitamin E were given once a day by oral gavage for 3 weeks, and ozone was administered by intraperitoneal injection once a day for 3 weeks. The levels of anti-Müllerian hormone (AMH), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were measured using enzyme-linked immunosorbent assay. Western blotting analysis was used to analyze the expressions of Bcl-2, Bax, and caspase-3 in ovaries. Changes in histological structure were examined by light microscope and electron microscopy was used to detect ultramicrostructure.

Results: The results demonstrated that PM$_{2.5}$ decreased AMH levels ($P < 0.001$); however, aspirin ($P < 0.001$), Vitamin C ($P < 0.001$), Vitamin E ($P = 0.001$), and ozone ($P = 0.002$) alleviated the decrease. Changes of IL-6, TNF-α, 8-OHdG, Bax/Bcl-2, and caspase-3 in PM$_{2.5}$ group were increased compared to control group ($P < 0.001$), while in PM$_{2.5}$ + aspirin, PM$_{2.5}$ + Vitamin C, PM$_{2.5}$ + Vitamin E, and PM$_{2.5}$ + ozone groups, they were statistically decreased compared to PM$_{2.5}$ group ($P < 0.001$ or $P < 0.05$).

Conclusions: PM$_{2.5}$ cause the damage of ovaries, and aspirin, Vitamin C, Vitamin E, and ozone antagonizes the damage. The protective mechanism is probably due to its ability to blunt the inflammatory and oxidative stress caused by PM$_{2.5}$, which subsequently suppressing the expression of apoptotic regulatory protein and reducing the incidence of ovary apoptosis.

Key words: Aspirin; Fine Particulate Matter; Ozone; Reproductive Toxicity; Vitamin C; Vitamin E

Introduction

Ambient aerosol fine particulate matter (PM$_{2.5}$) is ubiquitous and persistent in real life. Numerous epidemiological studies have documented that PM$_{2.5}$ has potential adverse effects on humans and animals including cardiovascular events,[1,2] lung cancer,[3] and chronic obstructive pulmonary disease.[4] In addition to the adverse effects of PM$_{2.5}$ on respiratory

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and cardiovascular systems, recent studies have shown that PM exposure is a risk factor for degraded semen quality in males.[5,6] However, a few studies have investigated the influence of PM-induced reproductive toxicity in females.

$PM_{2.5}$-induced inflammatory and oxidative stress responses have been considered as an important part in the triggering of cellular pathological process.[7,8] Aspirin is a classic nonsteroidal anti-inflammatory drug and has obvious anti-inflammatory effects as well as antioxidant effects demonstrated experimentally in both animals and humans.[9,10] Vitamin C (ascorbic acid) is an essential water-soluble vitamin in the human body, which is not only an effective antioxidant[11] but also can both prevent histamine release and increase the detoxification of histamine,[12] thus essentially acting as an anti-inflammatory agent. Vitamin E is an essential lipid-soluble vitamin in the human body, which is an effective antioxidant.[13] In addition, Vitamin E has been shown to be cardioprotective against peroxidative damage induced by tobacco smoke.[14,15] Ozone is a very reactive gas and is well known as a potent oxidant.[16] However, recent studies have clearly shown that a small and precisely ozone dose can induce upregulation of antioxidant enzymes.[17,18] Further, other results have confirmed that neither structural nor enzymatic cell damage occurs if an appropriate ozone therapeutic window is used.[19] Finally, ozone therapy has proven successful in treating a variety of illnesses.[18,20]

Despite these previous studies, little information is available about the effects of aspirin, Vitamin C, Vitamin E, and ozone on female reproductive endocrine function and ovarian structure in $PM_{2.5}$-treated mice.

The aim of the present study was to explore the effects of aspirin, Vitamin C, Vitamin E, and ozone on female reproductive performance and ovarian structure in $PM_{2.5}$-treated mice. The results of this investigation could provide an approach for preventing or alleviating the toxicity produced by aerosolized $PM_{2.5}$ pollution.

METHODS

Animals
Eighty-four, 4-week-old, specific-pathogen-free, ICR female mice, weighing 18–22 g, were purchased from Beijing Weitonglihua Laboratory Animal Co., Ltd., (Beijing, China). The mice were acclimatized to the laboratory environment for 7 days before starting the experiment. They were housed in plastic cages and allowed free access to standard mice chow and tap water. Animals were maintained under standard conditions with a 12 h light-dark cycle at 24 ± 2°C and 50 ± 5% humidity. All protocols used in this experiment were approved by the Animal Care and Use Committee (Beijing, China).

Fine particulate matter suspension preparation
All of the particulate matter (PM) used in this study had a diameter of <2.5 μm. It was provided by the Chinese Research Academy of Environmental Science in Beijing, China. The atmospheric $PM_{2.5}$ sampling location was on the roof of the academy, during March 2016. The daily $PM_{2.5}$ samples were collected on membrane filters for 24 h/day using a $PM_{2.5}$ air sampler (Tianhong, China). After sampling, the membrane filters were packed in clean aluminum foil and stored at −20°C until use.

Each time before use, the $PM_{2.5}$ membrane filters were weighed, and then the weighed membrane filter was placed in normal saline (NS). A dose of 10 mg of particles was suspended in 1.5 ml of NS and sonicated for 30 min to scatter the particles. After 30 min of ultrasonic oscillation, the supernatant fluid was obtained. The instillation method was a nonsurgical intratracheal instillation adapted from Bai *et al.*'s method.[21] Specific procedures are as follows: using the left hand to catch mice under conditions of no anesthesia, make the head of mice tilt 15°, with the right hand to hold Eppendorf and absorb the $PM_{2.5}$ particles suspension that has been prepared, dripped into the nasal cavity of mice dropwise, and then waiting for the automatic suction. In addition, the process of drip should be soft, and observe the response of mice, and meanwhile, the velocity should not be too fast. The exposure groups were treated every 2 days for up to 11 times.

Drug preparation
Aspirin and Vitamin C were dissolved in 0.9% NS. Vitamin E was dissolved in corn oil. Aspirin was prepared as a solution of 1.5 mg/ml, Vitamin C as a solution of 4.5 mg/ml, and Vitamin E as a solution of 40 mg/ml; each was administered by oral gavage depending on randomization. All drugs were prepared freshly immediately before use. The ozone was obtained from an ozone therapeutic apparatus (Kastner, Germany), with the concentration of the ozone at 30 μg/ml, and administered by intraperitoneal injection.

Animal experiments
Mice were divided randomly into six equal groups of 14 animals each: (1) control group, (2) $PM_{2.5}$ group, (3) $PM_{2.5}$ + aspirin group, (4) $PM_{2.5}$ + Vitamin C group, (5) $PM_{2.5}$ + Vitamin E group, and (6) $PM_{2.5}$ + ozone group.

After one week of acclimation, each mouse in the five exposed groups was instilled with a $PM_{2.5}$ suspension at the same concentration, and the exposed concentration used in this study was 10 mg/kg. Each $PM_{2.5}$ solution was freshly prepared with NS. For the control group, each mouse was instilled with the suspension using the same method and dosage as that used for the other five exposure groups.

Mice in $PM_{2.5}$ + aspirin group ($n = 14$), $PM_{2.5}$ + Vitamin C group ($n = 14$), and $PM_{2.5}$ + Vitamin E group ($n = 14$) were each administered either aspirin (15 mg/kg), or Vitamin C (45 mg/kg), or Vitamin E (100 mg/kg) once a day by oral gavage according to their respective group assignment. Mice in $PM_{2.5}$ + ozone group ($n = 14$) were administered ozone (0.4 mg/kg) once a day by intraperitoneal injection. The mice in the control group ($n = 14$) and $PM_{2.5}$ group ($n = 14$) were administered NS once a day by oral gavage using the same method and dosage as that used for other intervention groups. The interventions were repeated daily for 22 days.

Serum sampling and processing

After finishing the last administration, all mice were sacrificed one day later. Blood samples were collected from the hearts of all mice and then centrifuged at 3000 rpm for 30 min to obtain the serum. The serum samples were quickly removed and kept at −80°C for further analysis of the anti-Müllerian hormone (AMH) levels.

Measurement of anti-Müllerian hormone in the serum of female mice

The levels of AMH were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Jiamay Biotech Co., Ltd., Beijing, China), as per the manufacturer's protocols.

Ovarian inflammatory cytokines measurement

The levels of the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) in mice ovaries were assayed using a TNF-α and IL-6 ELISA kit (Jiamay Biotech Co., Ltd., Beijing, China), as per the manufacturer's instructions.

Ovarian oxidative stress measurement

The ovarian concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were performed through ELISA with the use of 8-OHdG kits (Jiamay Biotech Co., Ltd., Beijing, China). All procedures followed the manufacturers' recommendation.

Protein extraction and Western blotting

The ovarian tissues were lysed with ice-cold Pro-PREPTM buffer (Jiamay Biotech Co., Ltd., Beijing, China). Then, the tissue lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C and the supernatants were used for the assays. Concentrations of the proteins were determined using a BCA Protein Assay Kit (Thermo Pierce, Rockford, IL, USA). The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 1 h at 37°C by the use of 5% nonfat milk and incubated overnight at 4°C with the rabbit polyclonal anti-Bcl-2 antibody (Jiamay Biotech Co., Ltd., Beijing, China), rabbit polyclonal anti-Bax antibody (Jiamay Biotech Co., Ltd., Beijing, China), rabbit polyclonalanti-caspase-3 antibody (Jiamay Biotech Co., Ltd., Beijing, China), and rabbit polyclonal anti-β-actin antibody (Jiamay Biotech Co., Ltd., Beijing, China) in the Primary Antibody Dilution Buffer and subsequently with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C. Immunoreactive bands were detected using the Enhanced chemiluminescence system (Jiamay Biotech Co., Ltd., Beijing, China). After washing four times with TBS-T, the band intensity was quantified by densitometry.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the *in situ* Cell Death Detection Kit (Jiamay Biotech Co., Ltd., Beijing, China), to identify ovarian apoptosis in the 5 μm-thick paraffined sections. TUNEL assay was performed as per the manufacturer's instructions. Briefly, the ovarian paraffin sections were deparaffinized by heating at 60°C for 4 min, then washed twice in xylene and rehydrated through a graded series of ethanol and double-distilled water. The sections were incubated with 20 μg/ml of proteinase K for 15 min at room temperature and then washed four times with phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 2% H_2O_2 in PBS for 5 min at room temperature and washing twice in PBS for a total of 10 min. Then, 50 μl terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the tissue, and the sections were incubated in a humidified atmosphere for 1 h at 37°C in the dark. Meanwhile, for negative control, sections were incubated with 50 μl reaction buffer without TdT treatment. All sections were then incubated with stop/wash buffer for 30 min, and then added with anti-digoxigenin conjugate for 30 min at room temperature, after which the sections were stained with 4',6-diamidino-2-phenylindole. TUNEL images were obtained under a light microscope.

Histopathological examination

Mice were deeply anesthetized and then euthanized 24 h after the last treatment. The ovaries were removed and then fixed in 10% formalin at room temperature. Ovaries were dehydrated and embedded in paraffin. Next, tissue samples were sectioned serially every 5 μm and the sections then stained with hematoxylin and eosin and examined under a light microscope.

Electron microscopy

Two mice were taken from each group for the examination of electron microscope (EM) 24 h after the last treatment. Animals were anesthetized and perfused through the aorta with warm saline followed by a mixed solution of 4% paraformaldehyde and 2% glutaraldehyde (Sigma, St. Louis, MO, USA). The ovaries were removed after 1 h at 4°C. The tissues were fixed in 3% glutaraldehyde overnight and then rinsed with 0.1 M phosphate buffer (PB) three times. After postfixed with 1% osmium tetroxide (Sigma, St. Louis, MO, USA) in sodium PB for 2 h at 4°C, tissues were dehydrated with a graded series of ethanol washes, and then the dehydrated specimens were embedded in araldite for 24 h. Ultrathin sections (1 μm) were cut from the embedded tissue and then stained by 3% uranyl acetate-lead citrate. Ovarian ultrastructures were observed using an EM.

Statistical analysis

All values were analyzed by the statistical software package SPSS version 22.0 (IBM Co., Ltd., New York, USA). Data were expressed as mean ± standard deviation (SD). Comparisons between groups were analyzed using single-factor analysis of variance, followed by the least significant difference test. Moreover, differences were considered statistically significant if the $P < 0.05$.

RESULTS

Anti-Müllerian hormone levels in the serum of female mice

Concentrations of AMH in the serum of mice are presented in Figure 1. AMH levels in $PM_{2.5}$ group were statistically decreased

when compared to the levels in the control group ($P < 0.001$). The concentrations of AMH in $PM_{2.5}$ + aspirin ($P < 0.001$), $PM_{2.5}$ + Vitamin C ($P < 0.001$), $PM_{2.5}$ + Vitamin E ($P = 0.001$), and $PM_{2.5}$ + ozone ($P = 0.002$) groups were significantly increased compared to the levels in $PM_{2.5}$ group. Moreover, interestingly, the changes of AMH concentrations in $PM_{2.5}$ + aspirin ($P = 0.407$) and $PM_{2.5}$ + Vitamin C ($P = 0.051$) groups were not significant relative to the control, whereas statistically significant differences were identified in $PM_{2.5}$ + Vitamin E ($P = 0.002$) and $PM_{2.5}$ + ozone ($P = 0.002$) groups when compared to the control group.

Changes in inflammatory cytokines in ovarian tissue

To observe the changes in ovarian inflammation, we measured IL-6 and TNF-α. As shown in Figure 2, we found significant increases in IL-6 and TNF-α levels in the $PM_{2.5}$ group when compared to the levels in the control group ($P < 0.001$). The IL-6 and TNF-α levels in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups were statistically decreased when compared to the levels in $PM_{2.5}$ group ($P < 0.001$ or $P < 0.05$). In addition, there were no statistically significant differences of IL-6 and TNF-α concentrations between $PM_{2.5}$ + aspirin group and control group ($P = 0.086$ and $P = 0.061$, respectively). However, significant differences were observed in $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups when compared to the control group ($P < 0.001$ or $P < 0.05$).

Changes of oxidative stress in ovarian tissue

The concentrations of 8-OHdG, a marker of oxidative stress, were determined to observe the changes of oxidative stress in ovarian. 8-OHdG concentrations are presented in Figure 3. As shown in Figure 3, 8-OHdG concentrations were significantly higher in $PM_{2.5}$ group than that of the

Figure 1: Effects of different medications and ozone on the levels of serum AMH in $PM_{2.5}$-treated female mice. Values are mean ± SD (n = 6/group). Using one-way ANOVA, followed by LSD multiple range test, comparing with control group, significant difference is indicated by *P < 0.001 and §P < 0.01. Comparing with $PM_{2.5}$ group, significant difference is indicated by †P < 0.001 and ‡P < 0.01. $PM_{2.5}$: Fine particulate matter; SD: Standard deviation; ANOVA: Analysis of variance; AMH: Anti-Müllerian hormone; LSD: Least significant difference.

control group ($P < 0.001$). In addition, the 8-OHdG levels in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups were statistically lower as compared to the levels in $PM_{2.5}$ group ($P < 0.001$). In addition, compared to the control group, significant differences were observed in $PM_{2.5}$ + Vitamin E ($P = 0.001$) and $PM_{2.5}$ + ozone ($P < 0.001$) groups, while no statistically significant differences were observed in $PM_{2.5}$ + aspirin ($P = 0.087$) and $PM_{2.5}$ + Vitamin C ($P = 0.058$) groups.

Changes of apoptotic regulatory protein expression in ovaries

Western blotting analysis was used to analyze the expressions of antiapoptotic protein Bcl-2 and apoptosis-promoting protein Bax and caspase-3 in ovarian tissues of both groups. As the results shown in Figure 4, proapoptotic Bax protein increased, and antiapoptotic Bcl-2 protein decreased significantly in $PM_{2.5}$ group compared to control group while the increases of Bax protein and the decreases of Bcl-2 in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups were slightly [Figure 4a]. The Bax/Bcl-2 ratio showed an significant increase in $PM_{2.5}$ group ($P < 0.001$) as compared to control group and the statistically significance decreases were observed in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups compared to $PM_{2.5}$ group ($P < 0.001$). Moreover, the ratios of Bax/Bcl-2 in $PM_{2.5}$ + aspirin ($P = 0.256$) and $PM_{2.5}$ + Vitamin C ($P = 0.242$) groups were not significant relative to the control, while statistically significant differences were identified in $PM_{2.5}$ + Vitamin E ($P = 0.004$) and $PM_{2.5}$ + ozone ($P = 0.016$) groups when compared to the control group [Figure 4b]. In addition, as shown in Figure 4c and 4d, the expression of apoptosis-promoting protein caspase-3 in $PM_{2.5}$ group showed a significant increase as compared to control group ($P < 0.001$), and comparing with $PM_{2.5}$ group, significant decreases were identified in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups ($P < 0.001$). Nevertheless, no significant differences were found between $PM_{2.5}$ + aspirin ($P = 0.24$), $PM_{2.5}$ + Vitamin C ($P = 0.069$), and control group.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Apoptosis in ovarian follicles was assessed in histological sections by TUNEL assay. TUNEL-positive staining was observed in the ovarian tissue of both groups. As shown in Figure 5, ovarian tissue in groups of control, PM2.5 + aspirin, PM2.5 + Vitamin C, PM2.5 + Vitamin E and PM2.5 + ozone were stained with light brown color. However, a deeper brown color appeared in $PM_{2.5}$ group, which suggested that the number of apoptotic cells in ovarian tissue from the $PM_{2.5}$ group was higher than that of other groups.

Changes of ovarian histopathologic and follicle counting

To understand the effects of aspirin, Vitamin C, Vitamin E, and ozone after exposure to $PM_{2.5}$, changes in the histological structure of the ovary were examined. As shown in Figure 6, ovarian histological structures were damaged after exposure
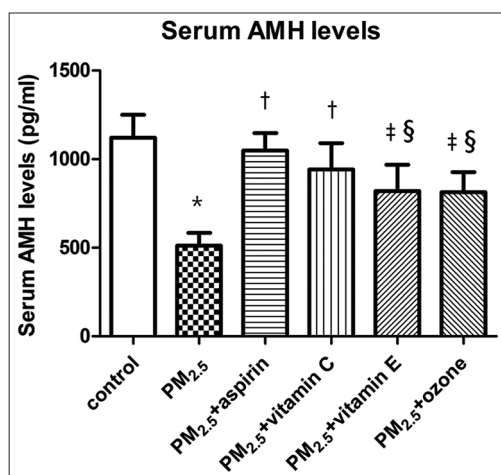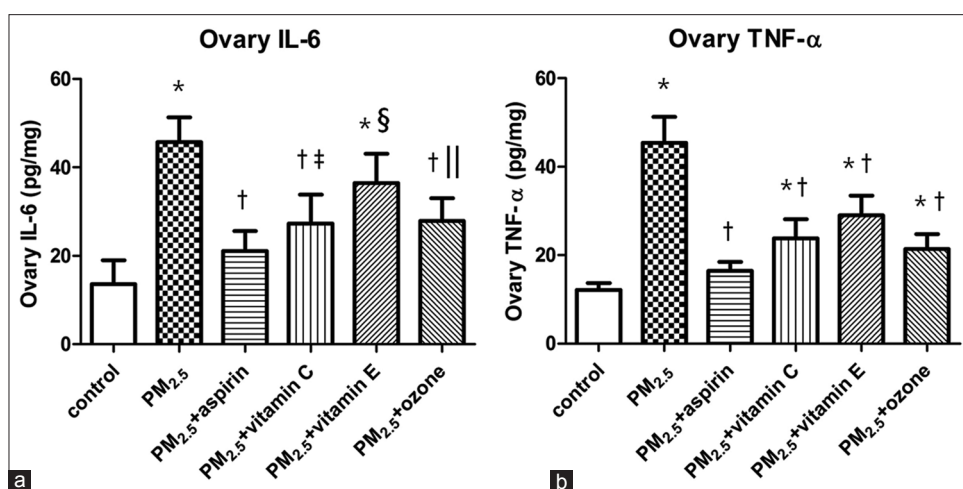
Figure 2: Effects of different medications and ozone on the levels of ovarian inflammation in PM$_{2.5}$-treated mice. (a) The concentrations of IL-6 in ovarian tissue of female mice. (b) The concentrations of TNF-α in ovarian tissue of female mice. Values are mean ± SD (n = 6/group). Using one-way ANOVA, followed by LSD multiple range test, comparing with control group, significant difference is indicated by *P < 0.001, †P < 0.01 and ‡P < 0.05. Comparing with PM$_{2.5}$ group, significant difference is indicated by †P < 0.001 and §P < 0.05. SD: Standard deviation; ANOVA: Analysis of variance; PM$_{2.5}$: Fine particulate matter; TNF-α: Tumor necrosis factor-α; IL: Interleukin; LSD: Least significant difference.
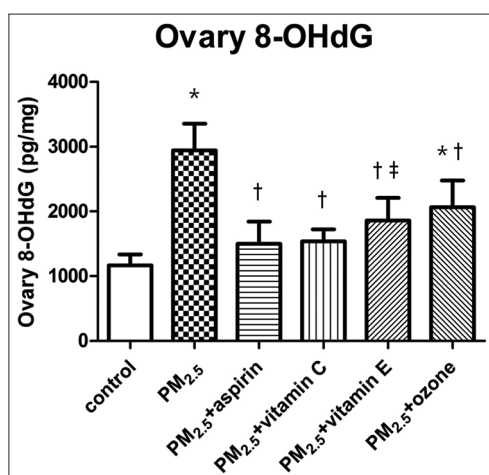

Figure 3: Effects of different medications and ozone on the levels of ovarian 8-OHdG in PM$_{2.5}$-treated mice. Values are mean ± SD (n = 6/group). Using one-way ANOVA, followed by LSD multiple range test, comparing with control group, significant difference is indicated by *P < 0.001 and ‡P < 0.01. Comparing with PM$_{2.5}$ group, significant difference is indicated by †P < 0.001. SD: Standard deviation; ANOVA: Analysis of variance; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; PM$_{2.5}$: Fine particulate matter; LSD: Least significant difference.

to PM$_{2.5}$ when compared to the control group that can be seen as hemorrhage and vascular congestion in ovarian structures. However, after treatment with aspirin, Vitamin C, Vitamin E, and ozone, the damage in ovarian histological structures was statistically decreased.

Changes in ovarian ultrastructure

In all groups, EM was used to detect the ovarian tissue ultramicrostructure to observe the damage to mitochondria in the ovarian tissue. Comparisons of the ultrastructural changes of mitochondria in mice ovarian tissue in different groups are depicted in Figure 7. Control groups displayed normal ultrastructural features of mitochondria. As shown in Figure 7

(control), the individual mitochondria morphology was intact and the outer membrane was smooth, and the mitochondrial cristae were arranged regularly. In the PM$_{2.5}$ group, the mitochondria swelled, mitochondrial membrane appeared impaired, mitochondrial cristae were arranged in a chaotic manner and occasionally disappeared, and mitochondria showed vacuolization. However, after treatment with aspirin, Vitamin C, Vitamin E, or ozone, the mitochondrial membranes maintained their integrity, mitochondrial cristae could still be seen and demonstrated relatively regular arrangement, although the mitochondria exhibited slight swelling. And meanwhile, the level of vacuolization in the mitochondria was less than that in PM2.5 group.

Discussion

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian inflammation in mice treated with fine particulate matter

Previous studies reported that PM$_{2.5}$ could induce acute systemic inflammatory responses.[22] Aspirin is a classical anti-inflammatory drug, and previous studies have shown that ozone has obvious anti-inflammatory effects.[23] Furthermore, a study by Dhalla *et al.*[24] reported that oxidative stress could initiate the inflammatory process to cause tissue damage. Since both Vitamins C and E exhibit antioxidant properties, we speculated that they might blunt the inflammation caused by PM$_{2.5}$ through their antioxidant abilities. To observe the protective effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian inflammation after the exposure of PM$_{2.5}$, we measured inflammatory cytokines IL-6 and TNF-α in ovarian tissue. IL-6 might stimulate the production of neutrophils and the proliferation of B-lymphocytes, while TNF-α is a multifunctional cytokine that might alter the normal barrier function of the endothelium and thereby increase the permeability of the same.[25]
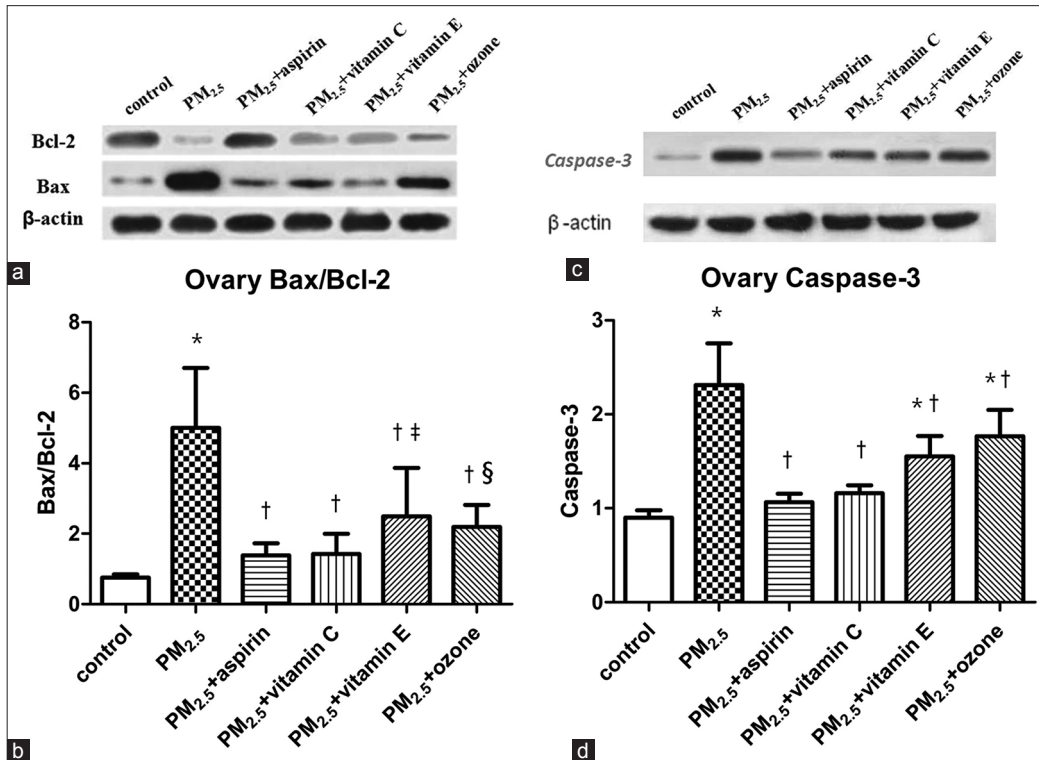
Figure 4: Effects of medications and ozone on the expression of ovarian apoptotic regulatory proteins in $PM_{2.5}$-treated mice. (a) Images of Western blot for Bcl-2 and Bax. (b) Comparisons of Bax/Bcl-2 ratio. (c) Images of Western blot for caspase-3. (d) Comparisons of the expression of caspase-3. Values are mean ± SD. Using one-way ANOVA, followed by LSD multiple range test, comparing with control group, significant difference is indicated by *$P < 0.001$, ‡$P < 0.01$ and §$P < 0.05$. Comparing with $PM_{2.5}$ group, significant difference is indicated by †$P < 0.001$. SD: Standard deviation; ANOVA: Analysis of variance; LSD: Least significant difference; $PM_{2.5}$: Fine particulate matter.
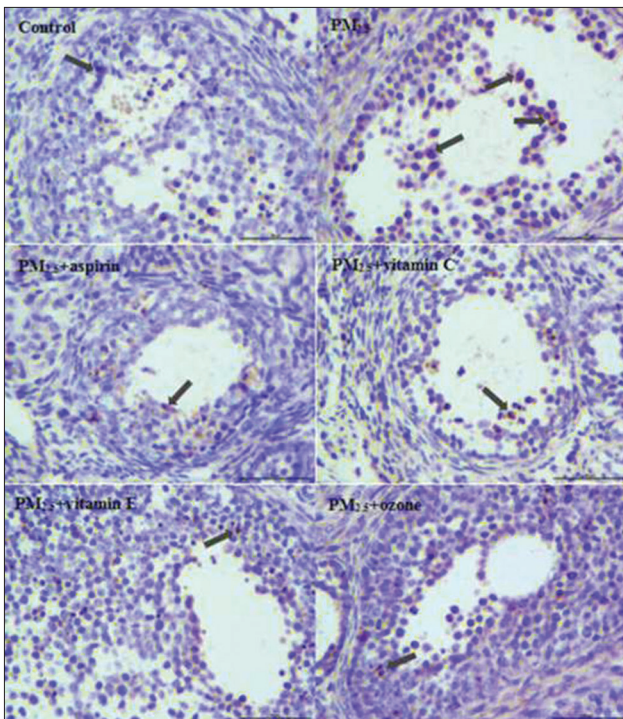

Figure 5: Apoptotic cells were determined by TUNEL staining in the ovarian tissues (original magnification, ×400). TUNEL-positive cells were found in the granulosa cells (black arrows). TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
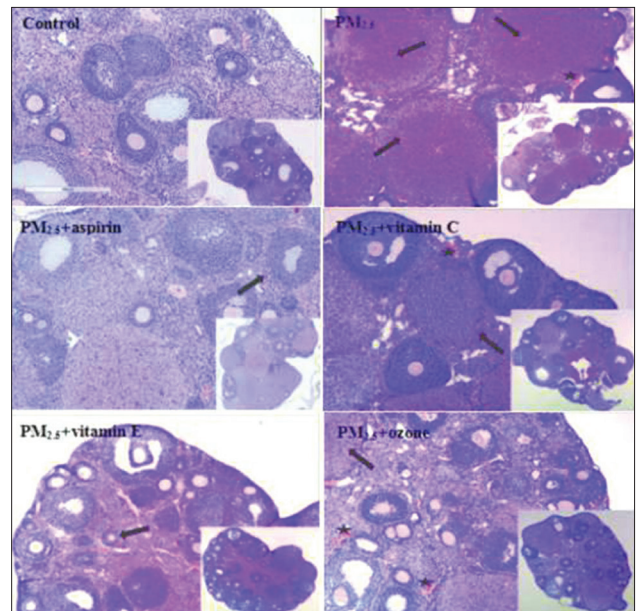

Figure 6: Comparison of the ovarian appearance changes in mice of different groups under light microscopy (hematoxylin and eosin staining, original magnification, ×100). The normal ovarian histological structures were observed in control group. Hemorrhage (thin arrow) and vascular congestion (star) were found in $PM_{2.5}$ group. However, in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups, the changes in hemorrhage (thin arrow) and vascular congestion (star) were decreased compared to the $PM_{2.5}$ group. $PM_{2.5}$: Fine particulate matter.
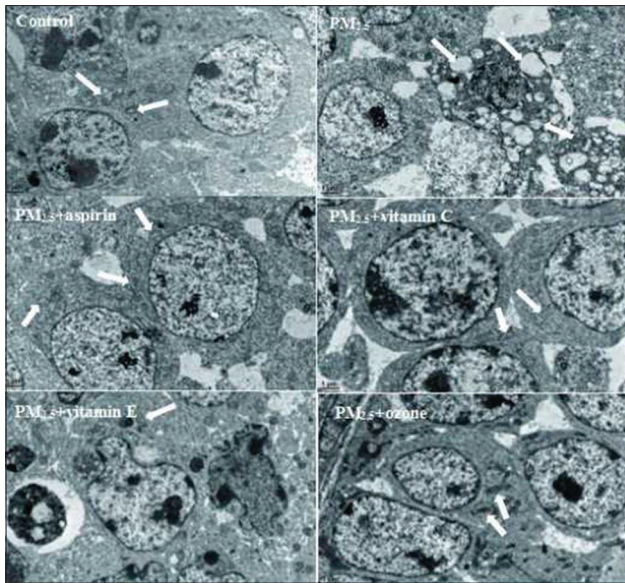
Figure 7: Comparison of the ultrastructural alterations in mitochondria in ovarian tissues in mice of different groups (original magnification ×10,000). The normal ovarian ultramicrostructures were observed in control group. In $PM_{2.5}$ group, arrows indicate the abnormal mitochondria with swollen mitochondria, absence of cristae and vacuolization. In addition, in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups, although mild mitochondrial swelling and irregularly arrayed cristae were still observed, the alterations were decreased compared to the $PM_{2.5}$ group. $PM_{2.5}$: Fine particulate matter.

The results of the present experiments showed that $PM_{2.5}$ exposure increased the levels of IL-6 and TNF-α in ovarian tissue in mice, and aspirin, Vitamin C, Vitamin E, and ozone all alleviated the inflammatory response. In addition, the morphological changes caused by inflammation, in the $PM_{2.5}$ group, we observed hemorrhage, vascular congestion, and mitochondrial structural changes, such as swelling, membrane impairment, disordered arrangement of cristae, and vacuolization. However, after the administration of any one of aspirin, Vitamin C, Vitamin E, or ozone, the pathological changes were ameliorated. All results demonstrated that the significant ovarian inflammation caused by $PM_{2.5}$ was reduced through the use of experimental anti-inflammatory agents.

This suggests that exposure to $PM_{2.5}$ results in an inflammatory response that is related to ovarian injury and appears to be the most likely mechanism for ovarian injury. Meanwhile, the anti-inflammatory effects of aspirin, Vitamin C, Vitamin E, or ozone may be involved in the protection from this exposure.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian oxidative stress in mice treated with fine particulate matter

Dhalla *et al*.[24] reported that oxidative stress may play important roles in $PM_{2.5}$-induced injures. Exposure to $PM_{2.5}$ leads to an imbalance of prooxidants and antioxidants in the cellular environment, which in turn causes an increase in the generation of reactive oxygen species. These oxygen free radicals initiate the lipid peroxidation process by attacking membranes resulting in cellular injury, including apoptosis and increased membrane permeability in cells and mitochondria.[26] Therefore, interventions that enhance antioxidative properties may contribute to the protection of ovarian tissue, by reducing ovarian apoptosis and thereby improving the preservation of ovarian reserve.

Vitamins C and E are widely used as antioxidative agents and are known to have minimal side effects. The protective effects of Vitamins C and E on oxidative damages have been reported in a previous study.[27] Similarly, aspirin and ozone also have antioxidant properties in addition to their anti-inflammatory effects.[10,23,28] However, their protective function against $PM_{2.5}$-induced damages has never been clarified. Moreover, aspirin, Vitamin C, Vitamin E, and ozone are attractive from the clinical point of view because of their low cost and minimal side effects.

Reactive oxygen species give rise to the formation of DNA-protein crosslinks, strand breakage, and alteration of the purine and pyrimidine bases.[29] 8-OHdG is an excision repair product of oxidative DNA damage and is one of the most common biomarkers of oxidative damage in DNA.[30] Therefore, ovarian tissue 8-OHdG concentrations were measured as an indicator of oxidative stress in the present study.

Our data showed that aspirin, Vitamin C, Vitamin E, and ozone were able to restrict the oxidative stress caused by $PM_{2.5}$. This study suggested that increased 8-OHdG levels were consistently relieved by aspirin, Vitamin C, Vitamin E, and ozone treatment. In addition, after treatment with aspirin and Vitamin C, the levels of 8-OHdG reach the same levels as the control, which indicates that the antioxidant effect of aspirin and Vitamin C is better than Vitamin E and ozone in $PM_{2.5}$-induced oxidative damage. Meanwhile, the number of apoptotic cells in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, and $PM_{2.5}$ + ozone groups decreased significantly compared to $PM_{2.5}$ group, which revealed that Vitamin C, Vitamin E, aspirin, and ozone were able to reduce the apoptosis of ovarian cells presumably by restricting the oxidative stress following exposure to $PM_{2.5}$. Moreover, we demonstrated that antioxidative protection translates to better preservation in ovarian reserve, measured as smaller decreases in AMH concentrations.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on serum anti-Müllerian hormone in mice treated with fine particulate matter

AMH is a dimeric glycoprotein of the transforming growth factor-superfamily and exclusively produced by granulosa cells of small follicles in the ovary.[31] Several studies have validated AMH as an early, reliable, and clinically useful biomarker of ovarian reserve.[32-34] Compared to other endocrine markers of ovarian reserve such as serum concentrations of follicle-stimulating hormone (FSH), inhibin B, and estradiol, the serum AMH concentration seems to be a more accurate predictor as it offers several advantages;[35] it is the only marker that was thought

to be stable throughout the phases of the menstrual cycle and it is relatively unaffected following hormonal therapy;[36-38] however, FSH, inhibin B, and estradiol are more dependent on cycle stage. In addition, AMH is detectable in the peripheral circulation;[39] therefore, it is considered a noninvasive method of evaluating ovarian reserve. Thus, in the present study, serum AMH concentrations are used as a hormonal indicator of ovarian reserve.

The results of our study showed that serum concentrations of AMH in the $PM_{2.5}$ group were significantly lower than control group. However, after treatment with aspirin, Vitamin C, Vitamin E, or ozone, the levels of AMH significantly increased compared to the $PM_{2.5}$ group, with no significant differences found between $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, and the control group. These results indicated that $PM_{2.5}$ can cause a decrease in ovarian reserve, and that treatment with aspirin, Vitamin C, Vitamin E, or ozone could ameliorate this damage. As reported previously, smoking is associated with a more rapid decline in AMH levels compared to nonsmokers.[40,41] These results indicate that aspirin, Vitamin C, Vitamin E, and ozone are able to improve the ovarian reserve to different degrees. Further, the mechanism may be related to either or both the anti-inflammatory and antioxidant effects of these agents.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian apoptosis in mice treated with fine particulate matter

Apoptosis is a biologically functional model of cell suicide essential for both development and maintenance of tissue homeostasis, and that is regulated by the dynamic balance between proapoptotic factors and antiapoptotic factors. To investigate the protective mechanism of these interventions on $PM_{2.5}$-induced lesions, we stained the ovarian cells by TUNEL to measure apoptosis. The results showed that TUNEL-positive cell numbers in $PM_{2.5}$ treatment groups were higher than that in the control group and $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, or $PM_{2.5}$ + ozone groups.

In addition, these findings correlated well with the morphological changes observed under the microscope. Mice treated with aspirin, Vitamin C, Vitamin E, or ozone, when exposed to $PM_{2.5}$, had reduced numbers of apoptotic cells compared to the mice exposed to $PM_{2.5}$. This suggests that apoptosis might contribute to the lesion in ovaries following exposure to $PM_{2.5}$ and antiapoptosis might be the protective mechanism displayed by aspirin, Vitamin C, Vitamin E, or ozone. Moreover, previous studies have demonstrated that oxidative stress can induce apoptosis by a mitochondrial pathway.[42] Therefore, the apoptosis of ovarian cells observed in the $PM_{2.5}$ group in this study may be related to the oxidative damage.

It is well known that caspases are a family of intracellular cysteine proteases that act as proapoptotic factors that play an important role in the initiation and execution of apoptosis in mammalian cells. Among the caspases, caspase-3 is

ultimately responsible for most apoptotic processes.[43] To examine whether caspase-3 is involved in $PM_{2.5}$-induced apoptosis, the expression of caspase-3 in ovarian cells was measured. The present results showed that $PM_{2.5}$ markedly increased the expression of caspase-3 in a manner that might be relevant to $PM_{2.5}$-induced apoptosis in ovarian cells. And interestingly, we have also observed that the expression of caspase-3 in $PM_{2.5}$ + aspirin, $PM_{2.5}$ + Vitamin C, $PM_{2.5}$ + Vitamin E, or $PM_{2.5}$ + ozone group was significantly decreased compared to the $PM_{2.5}$ group, which indicates that aspirin, Vitamin C, Vitamin E, or ozone might have protective effects on $PM_{2.5}$-induced apoptosis in ovarian cells. These results also suggest that $PM_{2.5}$-induced apoptosis was dependent on caspase-3 activation, and aspirin, Vitamin C, Vitamin E, or ozone can decrease $PM_{2.5}$-induced apoptosis of ovarian cells by reducing the activity of caspase-3.

Many studies have demonstrated that the mitochondrial pathway is the most common mechanism of apoptosis and constitutes the core part of the apoptotic pathway in mammalian cells.[44] However, the integrity of the mitochondrial membranes is regulated by the Bcl-2 family of proteins.[45] The Bcl-2 family of proteins can be separated into antiapoptotic and proapoptotic members, including the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax. In this study, we found that $PM_{2.5}$ enhanced the expression of the proapoptotic protein Bax and significantly decreased that of the antiapoptotic Bcl-2, resulting in a marked increase of the Bax/Bcl-2 ratio. Moreover, after treatment with aspirin, Vitamin C, Vitamin E, or ozone, the expression levels of Bax were reduced when compared to the $PM_{2.5}$ group, while levels of Bcl-2 were increased which resulted in a decrease in the ratio of Bax/Bcl-2. The imbalance of Bax and Bcl-2 expressions and increased Bax/Bcl-2 ratios observed in these experiments are consistent with enhanced apoptosis in the $PM_{2.5}$ group. In addition, these results suggest that aspirin, Vitamin C, Vitamin E, or ozone can reduce the apoptosis of ovarian cells by decreasing the ratio of Bax/Bcl-2. Obviously, a reduced apoptosis rate of ovarian cells could decrease the reproductive toxicity caused by $PM_{2.5}$.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian histopathology in mice treated with fine particulate matter

The protective effects of aspirin, Vitamin C, Vitamin E, and ozone on $PM_{2.5}$ injury to the ovary were also demonstrated by histopathology. Our histological findings support our biochemical and molecular findings. Ovarian damage following exposure to $PM_{2.5}$ was demonstrated by hemorrhage, vascular congestion, and follicular degeneration. These results provided a strong pathological basis for the lower concentration of serum AMH in the $PM_{2.5}$ group. However, the administration of aspirin, Vitamin C, Vitamin E, or ozone protected ovaries from injuries induced by $PM_{2.5}$, as shown in our histopathological results. The comparison of the protective effects of aspirin, Vitamin C, Vitamin E, or ozone demonstrated that the administration of aspirin and

Vitamin C provided better protective effects and resulted in lesser histopathological damage to the ovary.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian ultrastructure in mice treated with fine particulate matter

Results of the present study showed that some abnormal ultrastructural alterations in the ovarian mitochondria such as swelling, cristae disorder, and vacuolation occurred after exposure of $PM_{2.5}$. However, the ovarian mitochondria of the mice treated with aspirin, Vitamin C, Vitamin E, or ozone appeared to be normal. This protective effect was demonstrated by the mitochondrial cristae reappearing and the internal structure of the microtubules maintaining their integrity, indicating that treatment with aspirin, Vitamin C, Vitamin E, or ozone ameliorated the damages of $PM_{2.5}$. These ultrastructural damage results hint that $PM_{2.5}$ exposure induces strong ultrastructural changes in mitochondria, which subsequently leads to mitochondrial dysfunction. As it is reported in the literature, PM exposure could induce significant decreases in mitochondrial membrane potential and impaired oxidative phosphorylation.[46] Further, this also suggests that $PM_{2.5}$ exposure causes mitochondrial damage linked to the mitochondrial dysfunction and the abnormal alterations in mitochondrial morphology. Aspirin, Vitamin C, Vitamin E, and ozone protected the structural integrity of mitochondria so that they could maintain their normal function and ultimately preventing reproductive toxicity caused by $PM_{2.5}$.

Effects of aspirin, Vitamin C, Vitamin E, and ozone on ovarian function in mice treated with fine particulate matter

In animals, $PM_{2.5}$ exposure caused ovarian tissues inflammation and oxidative stress reactions while the protective effects of aspirin, Vitamin C, Vitamin E, and ozone are clear; however, whether the results of the present study can be applied to humans is not clear. Furthermore, our experiment is the only study at the animal level, and therefore, there is a need to strengthen the epidemiological investigations to confirm our findings.

In this experimental study, compared to Vitamin C, Vitamin E, and ozone, the protective effect of aspirin is better than other interventional measures. Reasons may be that aspirin is not only a very good anti-inflammatory agent but also has a strong antioxidant effect – the two main mechanisms for damage caused by $PM_{2.5}$. In addition, aspirin is a drug with a history of more than two thousand years and has proved to have anti-inflammatory, anticancer,[47] anticoagulation,[48] and antirheumatic[49] effects. This study represents the first demonstration that aspirin can significantly reduce ovarian tissue damages caused by $PM_{2.5}$.

Vitamins C and E are two of the essential vitamins for humans, and Vitamin E is often used in the protection of the reproductive system. However, in this experiment, its protective effect on reproductive toxicity caused by $PM_{2.5}$ was inferior to aspirin. The reason may be that Vitamin E appears to only work through the mechanism of resistance to oxidative stress.

Ozone as a new treatment method has several studies that confirmed its obvious anti-inflammatory and antioxidant effects, but in this study, the protective effects in $PM_{2.5}$ + aspirin group and $PM_{2.5}$ + Vitamin C group were significantly better. This may indicate that we did not use the optimum concentration of ozone to protect from$PM_{2.5}$-induced injuries; and of course, there is a need for further research.

One limitation of this study was the exposure mode of $PM_{2.5}$. In the present study, we used intratracheal instillation to perform the $PM_{2.5}$ exposure, which is clearly not a physiological route of delivery for humans. However, intratracheal instillation can get more accurate exposure dose of $PM_{2.5}$. Another limitation of this study was the short exposure durations and large particle concentrations, while in real life in chronic exposure, there would be lower concentrations for a longer period. This likely does not represent the spatial and temporal variability of the process during daily human contact. Furthermore, we also did related research of $PM_{2.5}$ impact on reproductive axis, but unsuccessful, of course further researches are needed. In addition, in the present study, we only examined the mechanisms of inflammation and oxidative stress, and there may be alternative existing mechanisms yet to be elucidated. Despite these limitations, our study included a carefully standardized experimental condition and the instilled dose of particles in our study was strictly calculated per previous studies.

In conclusion, evidence from this study provides strong biochemical and histopathological support that $PM_{2.5}$ could cause the damage of ovaries, and aspirin, Vitamin C, Vitamin E, and ozone reduced the damage and ameliorated the changes in ovarian structure caused by $PM_{2.5}$ and may be useful in protecting the ovaries from $PM_{2.5}$-induced damage in humans. Moreover, aspirin and Vitamin C have shown to be more effective in reducing tissue damage compared to treatment with Vitamin E and ozone. The protective effect of aspirin, Vitamin C, Vitamin E, and ozone is probably due to its ability to blunt the inflammatory and oxidative stress caused by ambient $PM_{2.5}$, which subsequently suppressing the expression of apoptotic regulatory protein in ovary and reduce the incidence of ovary apoptosis. These results indicate that moderate intake of aspirin, Vitamin C, Vitamin E, or ozone is warranted when exposure to $PM_{2.5}$ is unavoidable.

Conflicts of interest
There are no conflicts of interest.

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