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Research Paper

Reproductive Toxicity of Inhaled Polyethylene Microplastics: Oxidative Stress and Caspase-3–Mediated Apoptosis in the Ovaries of Female Wistar Rats

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ABSTRACT

Background: The increasing global production of plastics has led to widespread environmental contamination, including airborne microplastics capable of entering the respiratory tract, reaching systemic circulation, and inducing oxidative stress. This study aimed to assess the reproductive toxicity of sub-acute inhalation exposure to polyethylene (PE) microplastics on ovarian folliculogenesis, apoptosis, and oxidative stress in female Wistar rats (*Rattus norvegicus*).

Methods: Twelve adult female Wistar rats were randomly divided into control and experimental groups (n=6 each). The experimental group was exposed to aerosolized PE microplastics in a chamber for 4 h/day over 28 days. Ovarian tissues were examined for follicle counts (primary, secondary, antral, and atretic), caspase-3 expression in antral follicle granulosa cells, and oxidative stress markers by measuring the malondialdehyde to superoxide dismutase (MDA/SOD) ratio.

Results: The PE microplastic exposure significantly reduced the numbers of primary ($p=0.0137$), secondary ($p=0.0053$), and antral follicles ($p=0.0033$), while increasing atretic follicles ($p=0.0062$). Caspase-3 expression in antral follicle granulosa cells was markedly upregulated ($p=0.0173$). Additionally, the MDA/SOD ratio was significantly elevated ($p=0.0043$), indicating oxidative stress induction. These findings suggest that PE microplastic inhalation disrupts folliculogenesis through oxidative stress and apoptosis pathways.

Conclusion: Sub-acute inhalation of polyethylene microplastics induces oxidative stress and caspase-3–mediated apoptosis in ovarian tissue, leading to follicular depletion and potential reproductive toxicity.

Keywords: Microplastics, Oxidative stress, Ovarian follicles, Polyethylene, Toxicity

Introduction

Plastic materials are widely used in modern life due to their durability, flexibility, and low cost [1,2]. However, their extensive use and improper disposal have led to global plastic pollution, resulting in the generation of microplastics (<5 mm) and nanoplastics (<1 μm) through physical, chemical, and biological degradation processes. These small polymer particles are now ubiquitous in the environment, including soil, water, and air [3,4]. Airborne microplastics have emerged as an important route of human exposure, primarily through inhalation, allowing their entry into the respiratory tract and systemic circulation [5].

Recent studies have reported that inhaled microplastics can trigger inflammation, oxidative stress, and organ dysfunction in various animal models. Microplastics can cross biological barriers, accumulate in tissues, and generate reactive oxygen species (ROS), leading to lipid peroxidation and cellular injury [6 ,7]. Among plastic polymers, polyethylene (PE) is one of the most abundantly produced and persistent forms, commonly detected in airborne particulate matter and indoor dust. Despite increasing concern, information on the reproductive effects of inhaled PE microplastics remains scarce [8].

Ovarian function relies on the integrity of folliculogenesis, a process highly sensitive to oxidative imbalance and apoptotic signaling [9]. Excessive ROS production can damage granulosa cells, disrupt steroidogenesis, and impair oocyte maturation. The oxidative stress level can be assessed by the ratio of malondialdehyde (MDA), a marker of lipid peroxidation, to superoxide dismutase (SOD), an endogenous antioxidant enzyme [10,11]. Concurrently, activation of caspase-3 serves as a key indicator of apoptosis, mediating follicular atresia and loss of ovarian reserve [12].

Given the lack of data on reproductive toxicity following microplastic inhalation, this study aimed to evaluate the effects of sub-acute exposure to polyethylene microplastics on ovarian folliculogenesis, caspase-3 expression, and oxidative stress status (MDA/SOD ratio) in female Wistar rats (*Rattus norvegicus*). Clarifying these mechanisms is essential to understanding how environmental airborne microplastics may contribute to reproductive dysfunction and to inform future toxicological risk assessments.

Materials and Methods

Experimental Animals and Ethical Approval

This experimental laboratory study employed a posttest-only control group design and was performed on 12 adult female Wistar rats (*Rattus norvegicus*), weighing 150–200 g. Animals were housed under controlled environmental conditions at 25 ± 2°C, relative humidity of 40–70%, and a 12-hour light/dark cycle. Standard pellet diet and water were provided *ad libitum*. Rats were randomly assigned to two groups (n=6 each), namely a control group (unexposed) and an experimental group (exposed to PE microplastic). The exposure group received 15 mg/m³ of PE microplastics via inhalation for 28 consecutive days, while the control group received no treatment (Figure 1). The exposure concentration of 15 mg/m³ was selected based on recommendations from the Occupational Safety and Health Administration. The exposure route and duration were conducted in accordance with Organisation for Economic Co-operation and Development Test Guideline 412 for subacute inhalation toxicity studies [13,14]. All animals were sacrificed during the proestrus phase. Experimental protocols were reviewed and approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya (Approval No. 254/EC/KEPK/08/2023). Schematic illustration was created using BioRender.com

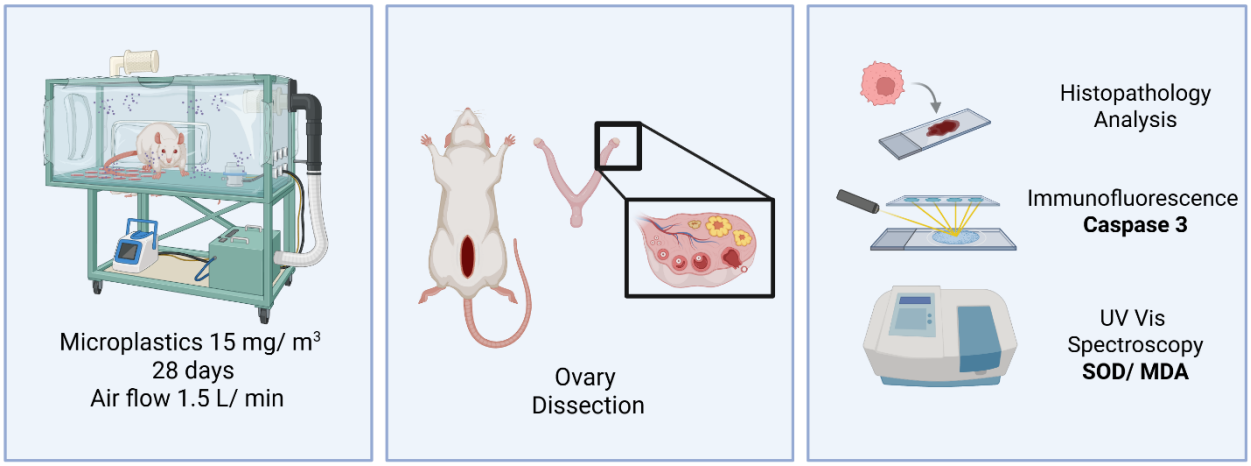

Figure 1. Schematic diagram of the experimental design.
Overview of the experimental workflow illustrating polyethylene microplastic inhalation exposure, duration, sampling, and subsequent analyses, including histological, immunofluorescence, and biochemical assessments.

Microplastic Exposure

Industrial-grade PE microplastics were obtained from a certified supplier (CV. Subur Kimia Jaya, Indonesia). Characterization of microplastic size and shape was conducted by our research group and reported previously (14). Exposure was conducted in a 60 × 60 × 60 cm acrylic chamber equipped with a regulated airflow (1.5–2 L/min) and a blower system to ensure uniform aerosol distribution, simulating an environmental airstream [15]. The whole-body inhalation method was used, with rats exposed to aerosolized PE microplastics for 4 h/day at the same time each day for 28 consecutive days [13].

Vaginal Cytology for Estrous Cycle Determination

Estrous cycle stages were identified via vaginal cytology to confirm synchronization at the proestrus phase before sacrifice [16]. Vaginal epithelial cells were collected using a sterile cotton swab moistened with 0.9% NaCl solution and gently inserted 1–2 cm into the vaginal canal. The collected sample was smeared on a glass slide, fixed in 70% ethanol for 5 min, air-dried, and stained with methylene blue for 15 min. Slides were rinsed with distilled water, dried, and examined under a light microscope for cytological evaluation of the estrous phase.

Assessment of Oxidative Stress Markers (superoxide dismutase and malondialdehyde levels)

Following 28 days of exposure, animals were euthanized during the proestrus phase, and ovarian tissues were excised, rinsed in phosphate-buffered saline (PBS), and homogenized. Tissue homogenates were prepared in 1.17% Tris-KCl buffer (pH 7.6), followed by centrifugation at 4 °C, 4,000 rpm for 15 min. Supernatants were used for biochemical assays. For SOD activity, each 500 µL sample was mixed with 200 µL EDTA (100 mM), 100 µL NBT (25 mM), 100 µL xanthine (25 mM), and

100 µL xanthine oxidase (1 unit). The mixture was vortexed and incubated at 37 °C for 30 min, followed by centrifugation at 4,000 rpm for 5 min. Absorbance was read at 580 nm using a spectrophotometer. The MDA levels were quantified via the thiobarbituric acid reactive substances method, and the MDA/SOD ratio was calculated as an index of oxidative stress [14,17].

Histological Examination and Follicle Counting

Ovarian tissues were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 5 µm thickness. Sections were stained with hematoxylin and eosin (H&E) and examined under an Olympus BX53 light microscope. Subsequently, whole-slide bright-field images were acquired at 20× magnification using an Aperio scanner, followed by follicle identification across serial histological sections [18]. Two independent, trained investigators, blinded to slide identity, evaluated the follicles. In the event of differing opinions, an independent third expert was consulted to reach a consensus. Follicles were classified and counted as primary, secondary, antral, or atretic based on established morphological criteria [18].

Immunofluorescence Detection of Caspase-3

Tissue sections were deparaffinized in xylene (2×2 min) and rehydrated in graded ethanol (100%, 90%, 80%, and 70%) followed by PBS washes. Non-specific binding was blocked with 1% bovine serum albumin for 30 min at room temperature. Slides were incubated overnight at 4 °C with primary antibody anti-caspase-3 fluorescein Isothiocyanate (Santa Cruz Biotechnology, Cat. No. sc-7272). After washing with PBS, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000) for 5 min. Fluorescence was visualized using an Olympus IX71 fluorescence microscope, and caspase-3 expression was semi-quantitatively analyzed in granulosa cells of antral follicles. Quantitative analysis of fluorescence intensity was conducted using ImageJ (NIH, USA) [17].

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using the Mann- Whitney U test. Analyses were conducted in SPSS software (version 26.0, IBM Corp., USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Histological examination was performed to evaluate the effect of PE microplastic inhalation on folliculogenesis and ovarian morphology. The analysis revealed distinct morphological differences between the control and experimental groups (Figure 2). The control group exhibited normal ovarian architecture with well-organized follicles at various developmental stages, whereas the PE-exposed group displayed a marked reduction in healthy follicles and an increase in follicular atresia. Quantitative analysis demonstrated significant decreases in follicle numbers across developmental stages in the experimental group, compared to the control group, including primary follicles (7.0±1.826 vs. 12.4±3.007, p=0.0137), secondary follicles (2.3±0.471 vs. 6.2±2.482, p=0.0053), and antral follicles (1.7±0.471 vs. 4.4±0.800, p=0.0033). Conversely, the number of atretic follicles was significantly elevated in the PE-exposed group (4.2±1.067 vs. 1.4±1.019, p=0.0062), indicating disrupted folliculogenesis and accelerated follicular degeneration.

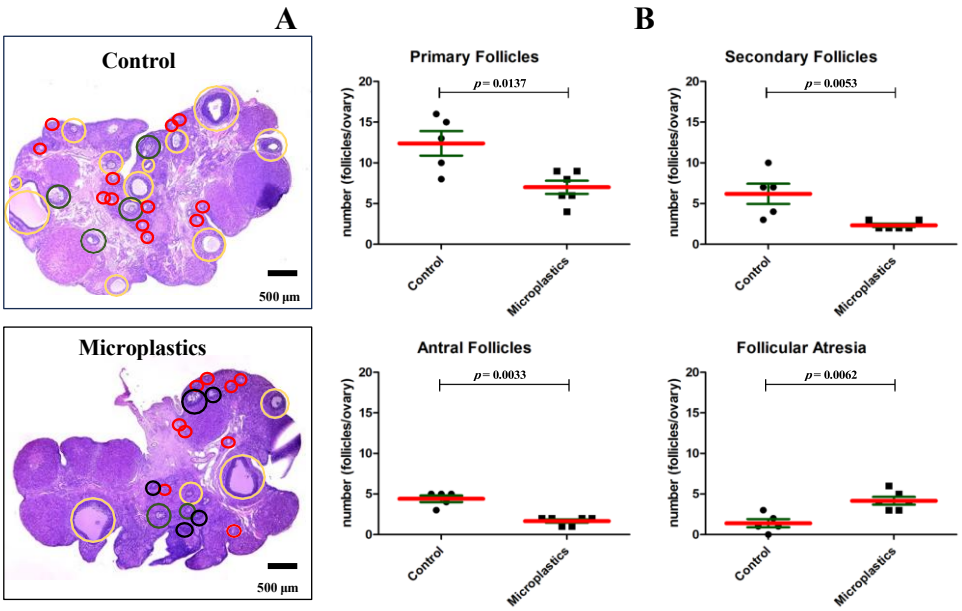

Figure 2. Histopathological analysis of ovarian follicles following polyethylene (PE) microplastic exposure.
A) Representative histological images of ovarian tissue from the control (upper panel) and PE-exposed (lower panel) groups. Red, yellow, green, and black circles indicate primary, secondary, antral, and atretic follicles, respectively. Scale bar = 500 µm.
B) Quantitative analysis of follicle counts (primary, secondary, antral, and atretic). Data are presented as mean (red line) ± SD (green line).

Moreover, immunofluorescence analysis was conducted to assess whether microplastic exposure induces apoptosis in granulosa cells through caspase-3 activation. Caspase-3 expression was predominantly localized within the cytoplasm of granulosa cells in antral follicles (Figure 3). Antral follicles were selected for caspase-3 assessment since they represent a critical developmental stage that is still viable yet susceptible to apoptotic signaling, allowing for a more accurate evaluation of regulated apoptosis, compared to atretic follicles, in which apoptosis is already advanced and extensive (19,20). However, the result does not fully represent apoptosis in advanced-stage atretic follicles. The fluorescence intensity and distribution were markedly higher in the PE-exposed group (14.1±7.095 IU), compared to the control group (5.0±2.848 IU). Quantitative evaluation confirmed a significant increase in caspase-3 expression following PE microplastic exposure (p=0.0173), suggesting activation of apoptotic pathways within ovarian tissue.

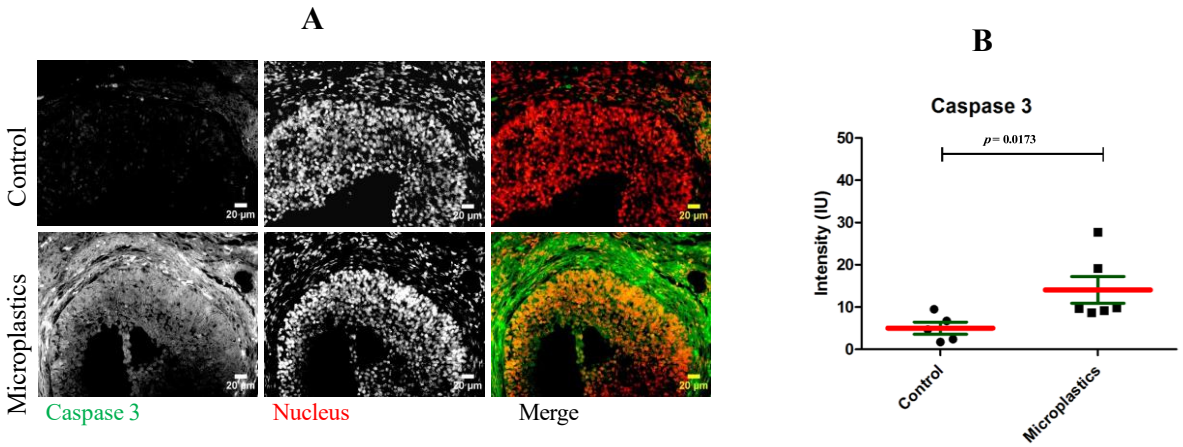

Figure 3. Apoptosis analysis in granulosa cells of antral follicles.
A) Immunofluorescence staining of antral follicles showing caspase-3 expression (green) and nuclear counterstaining with 4′,6-diamidino-2-phenylindole (red). Scale bar = 20 µm.
B) Quantification of caspase-3 fluorescence intensity in granulosa cells. Data are presented as mean (red line) ± SD (green line).

Lastly, oxidative stress in ovarian tissue was evaluated through biochemical analysis of key markers. Previously, it was demonstrated that inhaled microplastics disrupt oxidative balance, reflected by altered individual marker levels [21]. In the present study, a significantly higher MDA/SOD ratio was observed in the PE-exposed group (1.7±0.564), compared to the controls (0.5±0.415; p=0.0043) (Figure 4), indicating increased lipid peroxidation and reduced antioxidant capacity. This elevated oxidative stress aligns with heightened caspase-3 expression and follicular atresia, supporting a mechanistic link among oxidative injury, apoptosis, and impaired folliculogenesis. Notably, in this study, the MDA/SOD ratio was assessed in the whole ovarian tissue, which precluded the identification of specific differences between atretic and antral follicles.

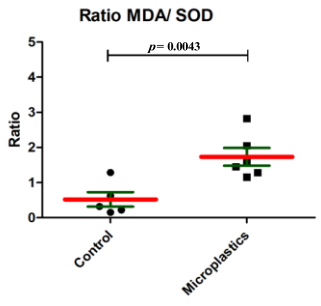

Figure 4. Oxidative stress analysis in ovarian tissue.
Biochemical quantification of oxidative stress represented by the malondialdehyde to superoxide dismutase ratio in ovarian homogenates. Data are presented as mean (red line) ± SD (green line).

Discussion

This study demonstrated that subacute inhalation of PE microplastics leads to structural and functional impairment of the ovary in female Wistar rats. The observed reduction in primary, secondary, and antral follicles, along with an increased number of atretic follicles, indicates that microplastic exposure disrupts normal folliculogenesis. These morphological alterations were accompanied by a rise in oxidative stress markers (MDA/SOD ratio) and elevated caspase-3 expression in granulosa cells, suggesting that oxidative stress and apoptosis are key mechanisms of ovarian damage.

The increase in atretic follicles accompanied by a reduction in antral follicles reflects enhanced follicular atresia during the gonadotropin-dependent antral stage [22]. Antral follicles that fail to receive adequate survival signals, such as sufficient follicle-stimulating hormone stimulation and anti-apoptotic support, undergo granulosa cell apoptosis and transition into atretic follicles [23,24]. This inverse relationship indicates a shift in ovarian follicular dynamics from maturation toward degeneration.

The ovary is highly sensitive to oxidative imbalance, as ROS play a dual role in both normal signaling and cellular injury [25, 26]. In the present study, the elevated MDA/SOD ratio in the PE-exposed group reflected enhanced lipid peroxidation and a weakened antioxidant defense system. Such redox imbalance can impair

granulosa cell function, alter steroidogenesis, and accelerate follicular atresia [27,28]. Granulosa cells are crucial for supporting oocyte maturation and hormone synthesis; therefore, their dysfunction or apoptosis leads to follicular loss and reproductive decline [22,29].

Caspase-3 activation further supports that apoptosis contributes to the observed ovarian damage. This enzyme acts as a final effector in the intrinsic apoptotic pathway, commonly triggered by mitochondrial dysfunction and oxidative stress [30-32]. The strong cytoplasmic localization of caspase-3 in granulosa cells of PE-exposed rats suggested mitochondrial-mediated apoptosis. Excessive ROS can damage mitochondrial membranes, promote cytochrome c release, and activate caspase cascades, leading to controlled but irreversible cell death [33,34].

These findings are consistent with prior research showing that particulate pollutants and endocrine disruptors promote ovarian oxidative stress and apoptosis [35). Studies involving fine particulate matter (PM2.5) and bisphenol A exposure have demonstrated similar increases in ROS and caspase-3 activity, resulting in follicular atresia [36,37]. Results of the current study extend this understanding by providing experimental evidence that inhaled PE microplastics, commonly present in the environment, elicit comparable reproductive toxicity through oxidative apoptotic mechanisms.

Importantly, the inhalation route represents a relevant yet understudied pathway for microplastic exposure [38]. Airborne particles can penetrate the lower respiratory tract, enter circulation, and accumulate in distant organs, including the ovary [39,40]. The findings from this study underscore that even short-term exposure to PE microplastics can impair ovarian health.

In conclusion, PE microplastic inhalation induces oxidative stress–mediated apoptosis in granulosa cells, leading to disrupted folliculogenesis and increased follicular atresia. These findings highlight microplastic exposure as a potential environmental risk factor for female reproductive dysfunction and warrant further investigation into long-term and low-dose effects.

Conclusions

Inhalation of PE microplastics induces oxidative stress and caspase-3–mediated apoptosis in ovarian granulosa cells, resulting in impaired folliculogenesis and increased follicular atresia in female Wistar rats. The elevated MDA/SOD ratio and enhanced caspase-3 expression suggest that oxidative injury is a primary mechanism underlying microplastic-induced ovarian toxicity. These findings provide experimental evidence that airborne microplastics may pose a reproductive health risk. Further studies are needed to evaluate chronic and low-dose exposures, elucidate systemic distribution, and clarify potential implications for human fertility and endocrine function.

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Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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