Polystyrene microplastics induced female reproductive toxicity in mice

Zhiqiang Liu\textsuperscript{a,}, Qingrui Zhu\textsuperscript{a,}, Luyao Zhang\textsuperscript{a,}, Lin Meng\textsuperscript{a,}, Xiangwei Fu\textsuperscript{b,}, Yunpeng Hou\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} State Key Laboratories of Agrobiotechnology, College of Biological Sciences, China Agricultural University, No.2 Yuanmingyuan Xilu, Haidian District, Beijing 100193, PR China
\textsuperscript{b} Key Laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, China Agricultural University, No.2 Yuanmingyuan Xilu, Haidian District, Beijing 100193, PR China

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\textbf{A B S T R A C T}

Plastics have caused serious environmental pollution. In recent years, microplastics (MPs) have caused widespread concern about their potential toxicity on animals and humans, especially on organ and tissue deposition. However, there is little known about the reproductive toxic effects of MPs in female mammals. In this study, the reproductive toxicity of polystyrene MPs (PS-MPs) in female mice was evaluated after continued exposure for 35 days. Results showed that PS-MPs could accumulate in heart, liver, spleen, kidney, brain, large intestine, small intestine, uterus, ovary and blood of exposed mice. Moreover, PS-MPs exposure increased the IL-6 level and decreased malondialdehyde (MDA) level in mouse ovaries. The results also showed that PS-MPs exposure decreased the first polar body extrusion rate and the survival rate of superovulated oocytes. Meanwhile, PS-MPs reduced the level of glutathione (GSH), mitochondrial membrane potential (MMP), endoplasmic reticulum calcium ([Ca\textsuperscript{2+}]\textsubscript{ER}) and increased reactive oxygen species (ROS) in oocytes. In conclusion, our study illustrated that PS-MPs exposure induced the inflammation of ovaries and reduced the quality of oocytes in mice, which provided a basis for studying the reproductive toxic mechanism of PS-MPs in female mammals.

1. Introduction

The production and use of plastic substances have grown rapidly since the 1940s, because of its physicochemical stability, degradation-resistant and low processing cost (Schirinzi et al., 2017; Jambeck et al., 2015; Al-Salem et al., 2010). The wide application of plastics in industrial production and daily life has brought great benefits and convenience to people, but a lot of plastics are also being released into the environment by humans (Andrady and Neal, 2009). Plastics are difficult to degrade and have resulted in a large accumulation in terrestrial and aquatic ecosystems (Browne et al., 2011; Ziajahromi et al., 2017; Cole et al., 2011; Moore, 2008). Plastic pollution has aroused extensive concern in the world, especially microplastics (MPs). MPs are pieces of plastic less than 5 millimeters in diameter. MPs can be classified as primary and secondary MPs. Primary MPs are manufactured to microsize for specific commercial uses and secondary MPs are produced by large plastics under various environmental effects (Barnes et al., 2009; Thompson et al., 2004; Ryan et al., 2009; Lin et al., 2018). MPs are pervasive and have been detected in a variety of environments around the world such as inland rivers, soil and air, even in the Antarctic and Arctic regions (Ding et al., 2020; Lahive et al., 2019; Almeda et al., 2021; Anderson et al., 2016; Horton et al., 2017; Bessa et al., 2019; Nor and Obbard, 2014; Ivar do Sul and Costa, 2014).

Due to MPs being tiny and biorefractory, they are easily absorbed by animals and plants and could accumulate continuously (Della Torre et al., 2014; Li et al., 2020b; Cole et al., 2013; Khalid et al., 2020; Lusher et al., 2013; Liu et al., 2021). And they can be delivered from the lower trophic levels enriched to the higher trophic levels by food chain (Miranda and de Carvalho-Souza, 2016; Setala et al., 2014; da Costa Araujo et al., 2020; Farrell and Nelson, 2013). MPs can enter the human body via consumed and inhaled. MPs have been found in tap water, bottled water, sugar, salt, beer, honey and personal care products such as cosmetics, even in the air. Air and food are the main source of human MPs uptake (Rist et al., 2018; Cox et al., 2019; Dris et al., 2016).

Recent studies showed that MPs were detected in human hands and facial skin, hair, saliva, placenta and stool (Schwabl et al., 2019; Abbasi and Turner, 2021; Ragusa et al., 2021). It has reported that humans may ingest 0.1–5 g of MPs per week from a variety of exposure routes on globally average (Senathirajah et al., 2021). There is widespread concern that MPs may affect human health in addition to the environmental problems caused by MPs.

Previous studies have shown that MPs could accumulate in
organisms which induced a lot of adverse effects. Some studies showed that MPs could induce hepatic inflammation, lipid metabolic disorders and oxidative stress in fish, and affect the survival, growth, feeding and reproduction in Daphnia and result in damage in early embryonal development of sea urchin (Eltemsah and Bohn, 2019; Zhang et al., 2020a; Rist et al., 2017; Cong et al., 2019; Trifuoggi et al., 2019; Jin et al., 2018; Liu et al., 2019; Jaikumar et al., 2019; Lu et al., 2016; Zitouni et al., 2021). Some studies have revealed that MPs were detected in the guts of mice, and then induced intestinal dysbacteriosis, barrier dysfunction, inflammation and hepatic lipid disorder in mice (Jin et al., 2019; Li et al., 2020a; Lu et al., 2018). Other studies also found that MPs exposure interfered with spermatogenesis and GnRH level in male rats, and induced metabolic disorder and dysplasia in the next generation of mice (Amereh et al., 2020; Luo et al., 2019a, 2019b; Park et al., 2020). Polystyrene (PS) is widely used in food and makeup packaging such as polyfoam, and it is easier to be mixed into food and absorbed by human body. PS-MPs could induce testicular inflammation, reduce the number of viable epididymis sperm and increase the rate of sperm deformity in male mice after exposure to PS-MPs for 35 days (Hou et al., 2021a). In addition, Hou et al. found that PS-MPs could induce pyroptosis and apoptosis of ovarian granulosa cells via the NLRP3/Caspase-1 signaling pathway maybe triggered by oxidative stress in rats (Hou et al., 2021b). These results suggested that PS-MPs treatment could induce the dysfunction of reproductive system. Moreover, MPs can induce more severe adverse effects with absorbed pollutants on their surface. It had shown that environmental contaminants co-exposure with MPs induced more toxicity than environmental contaminants and virgin MPs (Zhang et al., 2020b; Deng et al., 2018; Tang et al., 2020; Yang et al., 2020; Santos et al., 2020).

These studies confirmed that MPs could induce reproductive dysfunctions. However, most studies focus on aquatic organisms, and the effect of MPs on reproductive systems in mammals is still ambiguous, especially in females. The aim of this study was using mice as an animal model to explore the effect of PS-MPs on female reproductive system in mammals, including the ovarian organ index, follicle number, the quality and developmental competence of oocytes. These results could provide a basis for further studies on the effect of MPs on female reproductive health, specifically further assessment of the roles of PS-MPs in the female mammals.

2. Material and methods

2.1. Materials

All chemicals and culture media components were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) unless otherwise stated. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were obtained from the Ningbo Second Hormone Factory (Zhejiang, China). Opti-num cutting temperature compound (OCT) was purchased from Sakura Finetek (USA). Paraformaldehyde solution (4%) was obtained from Solarbio Life Science (Beijing, China). Polyfluorine labelled PS-MPs (G-PS-MPs) and non-fluorescent (plain) PS-MPs suspensions (1.0% w/v, 10 ml) were purchased from Tianjin Baseline ChromTech Research Centre (Tianjin, China) and stored at 4 °C. G-PS-MPs were used for the accumulation test, and PS-MPs were used for the toxicity test. The morphology and size of PS-MPs and G-PS-MPs were examined by a scanning electron microscope (Focus-Beam Technology, Beijing, China; Fig. 1). Both PS-MPs and G-PS-MPs were spherical morphology in uniform size, and the sizes were around 0.7918 ± 0.00273 and 0.7939 ± 0.00282 μm in diameter, respectively.

Five-week-old specific pathogen free (SPF) CD-1® (Institute of Cancer Research, ICR) female mice were purchased from the Beijing Vital River Experimental Animals Centre (Beijing, China). Upon arrival, mice were left to acclimatize for one week under standard conditions of temperature (22–25 °C) and light (12-h light/12-h dark cycles), with ad libitum access to rodent food and water. All animal experiments were carried out in accordance with the principles and guidelines for the use of laboratory animals and approved by the Institutional Animal Care and Use Committee of China Agricultural University.

2.2. Experimental design

In this experiment, mice were used as experimental animals to explore the effect of PS-MPs on the female reproductive system. The mice were given ultrapure water with or without PS-MPs/G-PS-MPs via oral gavage and were randomly assigned to three groups as follows: (1) The control group was given 30 mg/kg body weight ultrapure water; (2) The PS-MPs treatment group were exposed to 30 mg/kg body weight PS-MPs; (3) The G-PS-MPs treatment group were exposed to 30 mg/kg body weight G-PS-MPS for 35 days. The concentration of PS-MPs (30 mg/kg body weight) was determined based on reproductive toxicity study in male mice (Jin et al., 2021).

Experiment 1. The accumulation of PS-MPs in various tissues and organs of female mice by G-PS-MPs treatment. Heart, liver, spleen, lung, kidney, brain, large intestine, small intestine, uterus, ovary and blood were collected from both control and G-PS-MPs-treated mice.
Experiment 2. The basic physiological effects of PS-MPs in mice. Body-weight, consumption of food and water were measured in both control and PS-MPs-treated mice on Day 0, 7, 14, 21, 28, and 35. Organ indexes and serum hormone levels were detected in both control and PS-MPs-treated mice at Day 35. Experiment 3. The effects of PS-MPs on mouse ovaries. The relative expression levels of ovarian inflammation-related genes, oxidative stress-related genes and apoptosis-related genes were tested in both control and PS-MPs-treated group. The levels of ovarian inflammation-related proteins and oxidative stress-related proteins were examined in both control and PS-MPs-treated group. Experiment 4. The effects of PS-MPs on the quality and developmental competence of mouse oocytes. The rates of germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE), quality of mitochondria and parthenogenetic activation (PA) were evaluated in both control and PS-MPs-treated group.

2.3. Fluorescence measurements of blood and organs

To observe the existence of PS-MPs in the ovary of mice, after exposed to G-PS-MPs for 35 days, the ovary immersed in the OCT were frozen in liquid nitrogen, then cut into 5 µm-thick sections using freezing microtome (Leica, Berlin, Germany). The fluorescence of G-PS-MPs in the ovary of the mice were observed under a confocal laser scanning microscope (Nikon A1R, Tokyo, Japan).

In order to quantify the amounts of MPs accumulated in various organs in the mice, fluorescence spectroscopy was used to detect the concentration of MPs accumulated in the fresh tissues, to avoid the damage of the fluorescence of G-PS-MPs by freezing. Twenty-four hours after the last treatment with G-PS-MPs (Day 36), the mice were sacrificed, and the organs and blood samples were collected and detected the accumulation of PS-MPs in mice according to previous studies with modification (Walczak et al., 2015; Loeschner et al., 2013). Briefly, all the organs were carefully weighed, cut into pieces and digested in digestion buffer at a weight ratio of 1:20, except for ovaries which digested at a ratio of 1:100. Digestion buffer contains 50 mM NH4HCO3 (pH = 7.4) with 1 g/l proteinase K (Sigma-Aldrich, St. Louis, MP, USA) and 5 g/l SDS. The samples were thoroughly vortexed and incubated at 60 °C under continuous stirring on a magnetic stirrer for 4 h. The blood samples were mixed with digestion buffer at a volume ratio of 1:4. Fluorescence of G-PS-MPs in the samples was measured using a FLx800 microplate reader (BioTek, US) at excitation/emission wavelengths of 488/518 nm. The concentration of PS-MPs was determined based on previously prepared standard calibration curves in each organ separately, obtained by spiking blank organ homogenates (prepared as described above) with serial dilutions of PS-MPs ranging from 0 to 40 µg/ml (for organs) and 0–80 µg/ml (for blood). The amounts of MPs per gram tissue were obtained in all tested organs, including heart, liver, spleen, lung, kidney, brain, large intestine, small intestine, uterus, ovary and blood.

2.4. ELISA analyses of serum reproductive hormones, inflammatory cytokines and oxidative stress-related proteins

The levels of follicle stimulating hormone (FSH), luteinising hormone (LH), estradiol (E2) and progesterone (P) in serum and the levels of inflammatory cytokines interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-a (TNF-a), as well as the levels of oxidative stress-related proteins superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) in ovaries were measured by ELISA kits (Jingmei Bio-technology, Yancheng, China), according to manufacturer’s instructions.

2.5. Ovarian follicle classification and counting

After PS-MPs exposure, ovarian follicles were classified and counted as previous study (Liu et al., 2019a). In brief, after fixation in 4% paraformaldehyde solution, the ovaries were dehydrated in ethanol and xylene, and embedded in paraffin. The paraffin-embedded ovaries were serially sectioned at 5 µm thickness by a microtome (RM2255, Leica, Berlin, Germany). Then, the ovarian sections were dewaxed in xylene, rehydrated in ethanol and stained using hematoxylin and eosin (HE). The number of follicles in each section were counted under a light microscope (DMS500, Leica, Berlin, Germany).

2.6. Oocyte maturation and superovulation

Germinal vesicle (GV) oocytes were obtained from the ovaries of female mice. Briefly, the mice were euthanized by cervical dislocation 44–46 h after they were primed with 10 IU PMSG. Ovaries were immediately transferred to M199 medium with 4 mM hypoxantheme (HX-M199 media), and GV oocytes were carefully isolated from the ovaries under a microscope (SZ61, Olympus, Tokyo, Japan) using a pair of sterilized needles in HX-M199 media. Cumulus cells surrounding the oocyte were removed using a narrow-pore glass pipette. After being washed three times with M16 medium (M7167, Sigma), denuded oocytes were cultured in M16 medium covered with mineral oil and kept at 37 °C incubator with a humidified atmosphere of 5% CO2. The rates of germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE) were evaluated after 2 h and 12 h incubation, respectively.

For superovulation, PS-MPs-treated or control mice were intraperitoneal (IP) injected with PMSG, followed by IP injection with 10 IU hCG 48 h later. The cumulus-oocyte complexes (COCs) of ovulation were collected from the oviducts 14 h after injection of hCG. The cumulus cells were denuded by using Dulbecco’s phosphate buffered saline (DPBS, Gibco, Grand Island, New York) containing 0.1% (w/v) hyaluronidase. The numbers of ovulated and morphologically normal (live) oocytes were counted using a light microscope (SZ61, Olympus, Tokyo, Japan).

2.7. Measurement of intracellular ROS and glutathione (GSH), and oocytes apoptosis

The level of intracellular ROS in the oocytes was measured using CellROX™ Green Reagent (C10444, 1:500, Invitrogen, Eugene, OR, USA). In brief, metaphase II (MII) stage oocytes were incubated in CellROX reagent for 30 min at 37 °C, and then were washed three times using PBS. In like manner, the level of intracellular GSH was measured using a 20 µM ThiolTracker Violet (T10096, Invitrogen). Photographs were taken using fixed microscopic parameters by Cellence Entry (Olympus, Tokyo, Japan) and the intensity of fluorescence from each oocyte was measured by a fluorescence microscope (Olympus IX73, Tokyo, Japan) and quantified using EZ-C1 Free-Viewer (Nikon, Tokyo, Japan).

To detect the apoptosis in oocytes, matured oocytes were stained with an Annexin-V staining kit (Vazyme) according to the manufacturer’s instructions. The oocytes were stained with 100 µl of binding buffer containing 5 µl of Annexin-V-FITC for 12 min in the dark at 37 °C. After being washed three times with DPBS, the oocytes were transferred onto a glass slide and covered with a coverslip. Green fluorescence apoptosis signals were observed by a fluorescence microscope (Olympus IX73). The green fluorescence occurring at the zona pellucida were considered as an indication of a nonapoptotic cell, whereas green fluorescence found in the membrane and zona pellucida were deemed to indicate apoptosis had occurred.

2.8. Mitochondrial distribution and the measurement of Mitochondrial Membrane Potential (MMP) and ATP level

The mitochondrion is an important organelle in oocyte maturation and its dysfunction could compromise oocyte meiosis and fertilization. Therefore, mitochondrial distribution and function were investigated in mouse III oocytes using superovulation. Three mitochondrial
Mitochondrial membrane potential (MMP) is an indicator of mitochondrial function. Oocytes were incubated with JC-1, a fluorescent probe and a MMP assay kit (Beyotime Institute of Biotechnology, China), according to manufacturer’s instructions. In brief, mouse MII oocytes were incubated to 10 µM JC-1 in 100 µl working solution at 37.0 °C in 5% CO₂ for 30 min. They were washed three times with PBS and observed under a fluorescence microscope (Nikon A1R, Tokyo, Japan). MMP was calculated as the ratio of red fluorescence (corresponding to activated mitochondria (J-aggregates)) to the green fluorescence (corresponding to less activated mitochondria (J-monomers)). The average ATP level in each oocyte was determined by using an Enhanced ATP Assay Kit, S0027 (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. In brief, serial dilutions of ATP standard were prepared before examining, ranging from 0 to 32 pmol ATP. Ten denuded MII oocytes were mixed with 50 µl of lysis buffer in a 0.2 ml centrifuge tube on ice, and then homogenized by vortex until lysis occurred. All procedures were operated on ice before measurement. The ATP assay buffer of 50 µl was added to 96-well plates and equilibrated for 3–5 min at room temperature. Then, standard solutions and ATP detection diluent were added into each well. Subsequently, samples were also added into each well and luminescence activity was measured immediately using a FLx800 microplate reader (BioTek, US). ATP level of samples was determined from the standard curve. The total amount of ATP was divided by the number of oocytes in each sample to obtain the mean level per oocyte (p mol/oocyte).

2.9. Determination of Ca²⁺ levels in oocytes

To measure the Ca²⁺ levels in the intracellular, mitochondrial and endoplasmic reticulum, the MII oocytes were incubated in HX-M199 medium containing with 5 µM Fluo-3 AM or Rhod-2 AM or Fluo-4 AM at 37.0 °C for 30 min, and then washed three times and recovered in HX-M199 medium for 20 min. The fluorescence of oocyte was observed using a laser scanning confocal microscope (Nikon A1) after washing with DPBS for three times. The fluorescence intensity was assessed using NIS-Elements AR.

2.10. Parthenogenic activation (PA) of oocytes and embryo culture

The PA was performed as previously described (Liu et al., 2019b). MII oocytes were collected from mice with or without PS-MPs treatment, and then washed three times and incubated in activation medium (calcium (Ca²⁺)-free human tubal fluid (HTF)) contains 10 mM strontium chloride (SrCl₂) and 5 µg/ml cytochalasin B) for 2.5 h at 37 °C in a humidified atmosphere with 5% CO₂. Subsequently, the oocytes were transfected into regular HTF without SrCl₂ for 3.5 h under the same incubation conditions, prior to culture in KSOM plus (+) amino acids (KSOM/AA) medium (EmbryoMax®, KSOM + AA with D-Glucose and Phenol Red, EMD Millipore, Bilerica, MA, USA). The day on which PA was performed was considered as Day 0. The two-cell embryo and blastocyst stages were assessed on Day 1 and 4, respectively.

2.11. RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was isolated from ovaries using Trizol reagent (Invitrogen). Then RNA from each group was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was conducted using an ABI 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: 94 °C for 30 s; 42 cycles at 94 °C for 5 s; and 60 °C for 34 s. The 2^−ΔΔCt method was used to determine relative transcriptional levels of target genes with β-actin for normalization. The relative expression levels of ovarian inflammation-related genes Il-6, Il-10 and Tnf-α, oxidative stress-related genes Nrxox1, Sod1 and Gpx1 and apoptosis-related genes Caspase3, Bax and Bcl-2 were measured. The sequences of all primers used are listed in Table S1.

2.12. Statistical analysis

Each experiment was biologically repeated at least three times and data expressed as means ± standard error of means (SEM). All analyses were performed using SPSS 22.0 Software (SPSS Inc., Chicago, USA). Analysis of independent sample t-tests were used for the comparison between PS-MPs-treated and control groups. The level of statistical significance was set at P < 0.05.

3. Results

3.1. Accumulation of G-PS-MPs in mouse organs

As shown in Fig. 2, the fluorescence of G-PS-MPs were observed in the treated-ovarian frozen section after G-PS-MPs exposure, but not in the control group. The accumulations of PS-MPs were quantified by fluorescence spectroscopy using external standard calibration curves (Fig. S1) and the results are shown in Table 1.

3.2. Effects of PS-MPs on the body weight, consumption of food and water, and organ indexes

There were no significant changes in body weights and consumption of food and water between PS-MPs-treated group and control group mice (Fig. 3A, C and D). Moreover, there were no significant differences in organ indexes of mice after treatment PS-MPs, including the heart, liver, spleen, lung, kidney, brain, hypothalamus, uterus and ovary (Fig. 3B).

3.3. Effects of PS-MPs on the number of ovarian follicles, the levels of serum hormone, ovarian inflammation, oxidative stress and apoptosis in mice

The 5 µm ovarian sections continuous were examined after intra-gastric administration by HE staining (Fig. 4A). The numbers of primordial follicles, primary follicles and antral follicles were counted (Fig. 4B). Compared to control, there was no significant difference in the number of primordial follicles, primary follicles and secondary follicles in the PS-MPs-treated ovaries. However, the number of antral follicles was significantly reduced in the PS-MPs-treated ovaries, compared to that in the control. These results indicated that PS-MPs may affect ovarian follicle development in mice. Then, levels of FSH, LH, E2 and P in serum were not different between the PS-MPs-treated group and the control group (Fig. 4C, D and E).

Following exposure to PS-MPs, there were no statistically significant differences in mRNA levels of inflammation-related genes Il-6, Il-10 and Tnf-α in ovaries between the PS-MPs-treated and the control groups (Fig. 5A). Moreover, the levels of IL-6, IL-10 and Tnf-α were detected in the ovary supernatants, compared with the control group, the IL-6 significantly enriched in the ovaries of mice with PS-MPs treatment (Fig. 5B). In this study, the expressions of glutathione peroxidase (gpx1), peroxidase dismutase 1(sod1) and NADPH oxidase organizer 1 (noxo1) were not significantly different in the PS-MPs-treated ovaries compared to the control group (Fig. 5C). In addition, the levels of GSH, SOD and...
As shown in Fig. 6 B, there were no statistically significant differences in MII oocytes, the oocytes of superovulation at MII were collected (Fig. 6 A and C). To determine the effect of PS-MPs on maturation of oocytes in vivo and in vitro, GV oocytes were cultured in the M16 medium for oocytes in vitro maturation and the female mice were stimulated with hormones and in vitro, GV oocytes were cultured in the M16 medium for oocytes in vitro maturation and the female mice were stimulated with hormones.

**3.4. Effects of PS-MPs on the quality and developmental competence of mouse oocytes**

To determine the effect of PS-MPs on maturation of oocytes in vivo and in vitro, GV oocytes were cultured in the M16 medium for oocytes in vitro maturation and the female mice were stimulated with hormones and the oocytes of superovulation at MII were collected (Fig. 6A and C). As shown in Fig. 6B, there were no statistically significant differences in GVBD rates between PS-MPs-treated and control groups, but the proportion of PBE was significantly decreased in PS-MPs-treated group. The numbers of superovulated oocytes were not significantly different between PS-MPs-treated and control mice. However, the survival rate of oocytes was significantly decreased in the PS-MPs-treated group than that in control group (Fig. 6D). These results indicated that PS-MPs may affect the maturation of oocytes.

After exposure to PS-MPs, ROS and GSH generation were assessed in the control group, the level of MDA was significantly decreased in PS-MPs-treated group compared to the control group, respectively (Fig. 5D). One of the most important functions of mitochondria is the regulation of ROS. The expression of apoptosis-related genes Caspase-3, Bax and Bcl-2 were also examined. The result showed that Caspase-3 and the ratio of Bax/Bcl-2 was not significantly different between the PS-MPs treatment group and the control group (Fig. 5E). Collectively, these results indicated that PS-MPs induced ovarian inflammation, but did not induce apoptosis.

**Table 1**

Organ accumulation of PS-MPs in different tissues of mice after 24 h of the last treatment with G-PS-MPs expressed as µg PS-MPs/g (ml) tissue (at least three biological replicates each group).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration of PS-MPs (µg PS-MPs/ g (ml) tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>45.35 ± 21.63</td>
</tr>
<tr>
<td>Liver</td>
<td>69.86 ± 25.31</td>
</tr>
<tr>
<td>Spleen</td>
<td>106.31 ± 18.02</td>
</tr>
<tr>
<td>Lung</td>
<td>103.70 ± 14.41</td>
</tr>
<tr>
<td>Kidney</td>
<td>81.56 ± 19.36</td>
</tr>
<tr>
<td>Brain</td>
<td>27.78 ± 5.01</td>
</tr>
<tr>
<td>Large intestine</td>
<td>9.95 ± 5.74</td>
</tr>
<tr>
<td>Small intestine</td>
<td>53.44 ± 11.13</td>
</tr>
<tr>
<td>Uterus</td>
<td>32.79 ± 5.82</td>
</tr>
<tr>
<td>Ovary</td>
<td>62.60 ± 7.82</td>
</tr>
<tr>
<td>Blood</td>
<td>135.86 ± 7.23</td>
</tr>
</tbody>
</table>

Data represent means ± SEM.

MDA were detected in the mice ovary supernatants. Compared to the control group, the level of MDA was significantly decreased in PS-MPs-treated ovaries, whereas no differences were observed in the levels of SOD and GSH (Fig. 5D). The expression of apoptosis-related genes Caspase-3, Bax and Bcl-2 were also examined. The result showed that Caspase-3 and the ratio of Bax/Bcl-2 was not significantly different between the PS-MPs treatment group and the control group (Fig. 5E). Collectively, these results indicated that PS-MPs induced ovarian inflammation, but did not induce apoptosis.

**Fig. 2.** Freezing microtome section images showing the presence of G-PS-MPs in mice ovary. Black and grey arrows indicate G-PS-MPs (scale bar = 50 µm).

**4. Discussion**

Recent studies have shown that the effects of MPs on mammals and humans are getting more and more attention (Prata et al., 2020; Wright and Kelly, 2017). To our knowledge, this study is the first to systematic explore the effects of PS-MPs on ovary function and developmental competence of oocytes in female mice.
Fig. 3. Effects of PS-MPs on mice bodyweight, organ index and the consumption of food and water of mice after exposure to PS MPs for consecutive 35 days. (A) Bodyweight was measured once a week (n = 84). (B) Organ index was calculated after PS MPs exposure (organ weight (mg) / body weight (g)%, n = 10). (C) Consumption of food was measured once a week during the exposure period (n = 13). (D) Consumption of water was measured once a week during the exposure period (n = 13). The data was expressed as the mean ± SEM (independent sample t-tests).

Fig. 4. Effects of PS-MPs on ovarian follicle numbers and serum hormone levels. (A) Representative images of HE-stained ovarian tissue Section (5 µm) demonstrating representative classification of ovarian follicles (scale bar = 20 µm). Primordial follicle, insert: scale bar = 20 µm. (B) Average number of primordial follicles, primary follicles, secondary follicles and antral follicles per (n = 5). The levels of LH (C), FSH (D), E2 and P (E) in serum were measured by ELISA. The data was expressed as the mean ± SEM. *P < 0.05 vs. control (independent sample t-tests).
Fig. 5. Effects of PS-MPs exposure on ovarian inflammation, oxidative stress and apoptosis in mice. (A) The relative mRNA levels of Il-6, Il-10 and Tnf-α in ovaries were examined with q-PCR by normalizing to β-actin (n = 5). (B) The levels of IL-6, IL-10 and TNF-α in ovaries were measured by ELISA (n = 6). (C) The relative mRNA levels of Noxo1, Sod1 and Gpx1 in ovaries were examined with q-PCR by normalizing to β-actin (n = 7). (D) The levels of SOD, GSH and MDA in ovaries were measured by ELISA (n = 6). (E) The relative mRNA levels of Caspase3 and the ratio of Bax/Bcl-2 in ovaries were examined with q-PCR by normalizing to β-actin (n = 8). The data was expressed as the mean ± SEM. *P < 0.05 vs. control, **P < 0.01 vs. control (independent sample t-tests).

Fig. 6. PS-MPs treatment impairs the maturation of oocytes in vitro and in vivo. (A) Representative images of the GVBD and PBE mouse oocytes (scale bar = 200 µm). (B) GVBD and PBE rates of oocytes in vitro maturation (n = 3). (C) Representative images of ovulatory oocytes (scale bar = 200 µm). (D) The ovulation number and the survival number of oocytes (n = 5). The data was expressed as the mean ± SEM. *P < 0.05 vs. control (independent sample t-tests).
MPs can be absorbed by lymphocytes in the gastrointestinal tract, enter the circulatory system in the mussel, *Mytilus edulis* (L.) (Browne et al., 2008; Hussain et al., 2001). But there is little known about the accumulation and distribution of MPs in different tissues of mice, especially the reproduction system. Walczak et al. found that poly styrene nanoparticles (PS-NPs) could accumulate in the heart, liver, spleen, lung, kidney, brain, testis, large intestine and small intestine (Walczak et al., 2015). Besides the organs mentioned above, we also found that PS-MPs could accumulate in the uterus, ovary and blood of mice after being exposed to the G-PS-MPs for persistent 35 days. The result showed that spleen had the highest concentration of PS-MPs and large intestine had the lowest concentrations of PS-MPs. One possible reason may be that spleen is the “blood bank” of the animal body. We may not be able to clear blood from the spleen which resulted in higher concentration of Ps-MPs than that in other organs. Another explanation could be that spleen is the largest lymphoid organ in mammals and contains a large number of macrophages and lymphocytes, which can absorb and phagocytic foreign bodies in the blood. On the other hand, there is very little blood in the large intestine. And the main function of large intestine is to absorb water from food scraps as a digestive organ, so it absorbed almost no microplastics. It suggests that blood residue may affect the amounts of PS-MPs that accumulate in organs.

Previous study showed that the survival, growth and reproduction of medaka larvae were all significantly affected after a 14 days pre-exposure with the 10 µm non-fluorescent PS-MPs (Cong et al., 2019). Jin et al. found that decreases of food consumption and body weights of mice were observed in the groups of exposure PS-MPs of 4 µm and 10 µm, but no significant changes in 0.5 µm group. Moreover, there were no significant changes on consumption of water or paired testis/body weight after treatment with various sizes of PS-MPs (Jin et al., 2021). In our study, PS-MPs treatment did not affect mice body weights, organ indexes and consumption of food and water. These suggested that the effects of PS-MPs on body weight and food consumption of mice might be size dependent, and PS-MPs treatment with the size of 1 µm had no adverse effect on organ indexes and water consumption of mice, compared to the control group. Further studies are needed to investigate the effect of long-term treatment of PS-MPs in mice.

Ovary is an important reproductive and endocrine organ of the female, and its main function is to produce oocytes and secrete steroid hormones. PS-MPs may induce ovarian inflammation, oxidative stress and even apoptosis, because PS-MPs enter the ovaries as foreign objects. A recent study found that PS-MPs induced fibrosis via Wnt/β-Catenin signaling pathway activation and granulosa cells apoptosis of ovary through oxidative stress in rats, ultimately resulted in decrease of ovarian reserve capacity (An et al., 2021). It would result in reproduction dysfunction in females, once ovarian function is impaired (Wang et al., 2017; Hummitzsch et al., 2013). The follicle is the basic structural unit of the ovary and follicle development can be divided into gonadotropin-independent stage (preantral follicles) and gonadotropin-dependent stages (antral follicles). In the study, although the levels of FSH, LH, E2 and P in serum and the apoptosis level of ovary were not affected by the PS-MPs treatment, we did observe decreased number of ovarian antral follicles in the PS-MPs-treated group compared to that in the control. The possible explanation could be that PS-MPs could induce metabolic disturbances in mice, and nutrient metabolism is also critical for follicular development in the ovary (Jin et al., 2019; Dupont et al., 2014). In addition, our results showed that the level of IL-6 significantly increased and MDA significantly decreased in mice treated with PS-MPs compared to the control group, respectively. IL-6 is a proinflammatory factor that plays an important role in acute
Fig. 8. Effects of PS-MPs on mitochondria of mouse oocytes. (A) Representative images for the mitochondrial distribution pattern in mouse oocytes by staining with Mito Tracker (scale bar = 50 µm). (B) The percentage of mitochondria with three distribution patterns (n = 3). (C) Representative images of MMP in mouse oocytes stained with JC-1 (scale bar = 50 µm). (D) MMP levels (red/green fluorescence intensity) were detected in mouse oocytes. (E) The levels of ATP were evaluated in mouse oocytes. The data was expressed as the mean ± SEM. ***P < 0.001 vs. control (independent sample t-tests).

Fig. 9. Effects of PS-MPs on calcium homeostasis in mouse oocytes. (A) Representative images of Fluo-3 AM fluorescence (green), Rhod-2 AM fluorescence (red) and Fluo-4 AM fluorescence (green) in mouse oocytes (scale bar = 50 µm). (B) Quantification of the relative levels of [Ca^{2+}]_c, [Ca^{2+}]_mt and [Ca^{2+}]_ER in mouse oocytes. The data was expressed as the mean ± SEM. ***P < 0.001 vs. control (independent sample t-tests).
inflammatory responses. The increase of IL-6 levels in the ovary of mice indicated the occurrence of ovarian inflammation after PS-MPs treatment for 35 days. Jin et al. also found that PS-MPs induced testicular inflammation and testosterone level of mice to decline (Jin et al., 2021). MDA is the end-product of lipid peroxidation. The level of MDA is an important parameter reflecting the antioxidant potential of the body, which can reflect the lipid peroxidation rate and strength of the body, and also indirectly reflect the degree of tissue peroxidation damage. However, in our study, the level of MDA significantly decreased in PS-MPs-treated group compared to control group. Previous studies showed that PS-MPs exposure could induce lipid disorder in mice and their offspring (Luo et al., 2019a, 2019b; Deng et al., 2017). Lu et al. found that the hepatic triglyceride (TG) and total cholesterol (TC) levels decreased in PS-MPs-treated group mice compared to that in control group mice. And the relative mRNA levels of some key genes related to lipogenesis and TG synthesis decreased in the liver and epididymal fat (Lu et al., 2018). The decreased level of MDA might be caused by the decline of fatty acids in PS-MPs-treated group in the study. These results might be the reason for the decline in the number of ovarian antral follicles (Wang et al., 2012a, 2012b; Devine et al., 2012). But the reasons for the decline in MDA level and the number of ovarian antral follicles need to be further explored.

MPs could reduce oocyte numbers, sperm velocity and the development of offspring in oysters after being exposed to 2 µm and 6 µm PS-MPs for 2 months (Sussarellu et al., 2016). In our study, the result also showed that the quality and maturation of oocytes were adversely affected after mice were treated with PS-MPs compared to the control group, such as the significant increase of ROS and MMP in oocytes, and the significant decrease of GSH, [Ca\(^{2+}\)\(_{ER}\)], the proportion of first polar body extrusion and the survival number of oocytes. And these results suggested the decline of oocyte quality and maturation. Even though we did not find differences in vitro development of PA embryos between PS-MPs-treated and control groups, decreased oocyte quality may still affect the in vivo development. Collectively, our study provides a new sight for studying the reproductive toxicity of PS-MPs to female mammals.

5. Conclusion

In the study, our results indicated that PS-MPs could lead to the decline of antral follicles number and the quality of oocytes in mice. It suggested that PS-MPs exposure could induce reproductive toxicity in female mice. Our study provides a basis for further exploring the molecular mechanism of PS-MPs exposure induced reproductive dysfunction in female mammals.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127629.

References


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Table 2

Effects of PS-MPs on subsequent development of mouse oocytes following parthenogenetic activation (n = 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of oocytes</th>
<th>No. of Cleavage (%)</th>
<th>No. of Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82</td>
<td>82(100 ± 0.00)</td>
<td>68(84.23 ± 5.48)</td>
</tr>
<tr>
<td>PS-MPs</td>
<td>108</td>
<td>103(92.65 ± 7.35)</td>
<td>81(73.91 ± 7.49)</td>
</tr>
</tbody>
</table>

The data was expressed as the mean ± SEM (independent sample t-tests).


