RESEARCH ARTICLE

Polystyrene microplastics of different particle sizes cause reproductive disorders in male mice

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Microplastics, an emerging pollutant, have attracted widespread attention for their potential toxicity. To date, there are few studies on the impact of microplastics on the mammalian reproductive system. This study aimed to investigate the effects of microplastics of different particle sizes and doses on reproductive damage in male Kunming mice. Polystyrene microplastics (PS-MPs) with different particle sizes of 5 μm and 60 nm and doses of 10, 100, and 1,000 μg/d were administered by gavage to experimental animals for 35 consecutive days. Changes in body weight and testicular weight, sperm count, and HE staining sections of testicular tissue were examined. The results showed that the body weight of mice in the groups exposed to different particle sizes and doses of microplastics demonstrated a gradual increasing trend, but the increase was significantly lower in the microplastic groups than that in the control group. There were no significant differences in testicular weight of the mice between the microplastic-treated groups and the control group. In addition, HE stained tissue sections and sperm counting revealed that the sperm count and motility of mice in the microplastic-treated groups were significantly reduced compared to the control group, and this reduction was dose-dependent, suggesting that microplastics could cause damage to mammalian male reproduction. This study provided a theoretical basis for further research on the effects of microplastics on male reproduction in mammals.

Keywords: polystyrene microplastics; mammalian reproduction; impairment; mouse.

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Introduction

While infertility is a complex clinical phenomenon, its incidence is on the rise globally. Studies have shown that infertility caused by male factors mainly includes impaired spermatogenesis and reduced sperm quality [1, 2]. This condition is caused by a variety of factors including but not limited to reproductive system abnormalities [3, 4], hormonal imbalances due to endocrine dysfunction [5], genetic factors [6], infectious diseases, poor lifestyle habits, and environmental factors [7, 8]. In recent years, microplastic pollution has become a global environmental issue, and its potential impact on ecosystems and human health has attracted widespread attention. As a new type of pollutant, microplastics are widely distributed in the environment and have potential bioaccumulation, making their impact on the reproductive system a hot topic of current research.

Microplastics are plastic particles with a diameter of less than 5 mm, which are tiny plastic particles produced by human activities [9-11]. These

plastic particles come from the breakdown of microplastics of origin or large pieces of microplastic waste through physical, chemical, or biological degradation [9, 12]. They are found mainly in oceans, urban beaches, sediments, and rivers [13, 14]. Currently, polyethylene, polypropylene, and polystyrene are the three most common polymers in marine microplastics [15]. These tiny microplastic particles are difficult to degrade, easily absorbed by organisms, and accumulated in the body, and have gradually evolved into a global environmental pollution issue [16]. With the increasing use of plastic products, microplastics are causing more and more widespread environmental pollution, and its impact is becoming increasingly severe. The possibility of human ingestion of microplastics through the food chain has raised health concerns [17-19]. Although the long-term effects of microplastics on human health have not been adequately studied, it has been shown that microplastics can enter the human body through respiration and food intake while accumulating in the body [19]. It has also been demonstrated that, as the trophic level of the food chain goes up, the content of microplastics accumulated in organisms increases. The intake of microplastics has a serious impact on human life safety. Past studies showed that microplastics could accumulate in the gastrointestinal tracts, livers, kidneys, and brains of aquatic organisms and mammals [20-22]. A previous study revealed that, while microplastics affected the reproduction of female oysters, sperm velocity was reduced in oysters after exposure to 2 μm or 6 μm polystyrene microplastics (PS-MPs) [23]. Subsequent studies found that exposure of marine medaka *Oryzias melastigma* to 2 μg/L of 10 μm polystyrene microplastics led to increased interstitial tissue and disorganized seminiferous tubules, and these testicular abnormalities were induced in a concentration-dependent manner [24]. Moreover, polyethylene microplastics can trigger a significant inflammatory response in the intestines of mice [25]. Taken together, microplastics have been shown to exert adverse effects on aquatic organisms and mammals including physical condition, oxidative damage, intestinal barrier function, inflammation, and neurotoxicity. Notably, the male reproductive system in rodents can also be impaired by microplastics. Studies have shown that 4 μm or 10 μm polystyrene microplastics can decrease sperm quantity and quality, and even lead to disruption of the blood-testis barrier [26, 27]. These studies suggest that microplastics may have toxic effects on the mammalian reproductive system.

While existing studies have shown that microplastics can cause damage to the reproductive system of aquatic organisms, there is still very little research on whether microplastics cause reproductive damage in male mammals. The present study aimed to investigate the effects of polystyrene microplastics of different sizes (5 μm and 60 nm) and doses (10 μg/d, 100 μg/d, and 1,000 μg/d) on the reproductive system of male Kunming mice to determine whether polystyrene microplastics induced reproductive disorders by measuring several indicators including changes in mouse body weight and testicular tissue weight, sperm quality, and testicular tissue structure. This study would reveal the impairment effects of polystyrene microplastics on the male reproductive system and provide new insights into the health risks of mammals imposed by polystyrene microplastics.

Materials and methods

Experimental animals

Fifty-six (56) 5-week-old male Kunming mice were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, Henan, China). All animals were fed with regular feed, had free access to water, and were reared at 25°C with 50 - 60% humidity under natural light conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Huanghuai University (Zhumadian, Henan, China) in accordance with the Institutional Animal Ethics guidelines.

Administration of PS-MPs

After 2 days acclimation, all animals were randomly divided into 7 groups with 8 mice in each group including (1) ultrapure water group, (2) 5 μm PS-MPs (Tianjin Baseline Chrom Tech Research Centre, Tianjin, China) 10 μg/d, (3) 5 μm PS-MPs 100 μg/d, (4) 5 μm PS-MPs 1,000 μg/d, (5) 60 nm PS-MPs 10 μg/d, (6) 60 nm PS-MPs 100 μg/d, and (7) 60 nm PS-MPs 1,000 μg/d. The experimental period was 35 days. The mice in group 1 were gavaged with 200 μL of ultrapure water every day, while the other groups were gavaged with 200 μL of microplastics of different doses every day. Since PS-MPs were insoluble in ultrapure water, PS-MPs were prepared by mixing them thoroughly in an oscillator before gavage.

Body and testis weight measurement

The body weights of mice were recorded weekly throughout the experiment period. Testes weights of mice were recorded at the end of the experiment, where the animals were euthanized, and the testicular tissue from both sides was dissected and weighed.

Sperm counting and morphological observations

Mice in each group were sacrificed by cervical dislocation. The epididymis and testes on both sides of each mouse were removed and placed into 1 mL of 37℃ physiological saline (PBS) to prepare a sperm suspension. The number of sperms in each group were counted using ML-608JZ-II sperm counter (MaiLang, Nanning, Guangxi, China). The sperm counting and morphological observations were performed as described previously [28].

HE histopathological examination

The ipsilateral testes were taken from each group of mice and fixed in Bouin's solution (Servicebio, Wuhan, Hubei, China). The samples were removed from the fixative after 12 hours, rinsed with distilled water, dehydrated in a gradient of 70%, 80%, 95%, and 100% ethanol for 10 min at each concentration, treated with xylene for 30 min, and then embedded in liquefied paraffin. After the paraffin solidified, tissues were sliced with a thickness of 5 μm using RM2016 microtome (Leica Instrument, Shanghai, China). The intact slices were baked in an oven at 55°C, dewaxed with xylene, and then stained using hematoxylin solution (Servicebio, Wuhan, Hubei, China) and eosin dye. The slices were imaged under Eclipse E100 optical microscope equipped with DS-U3 camera (Nikon Precision Co., Ltd., Shanghai, China).

Scanning electron microscopy (SEM)

Fresh testicular tissues with an area of no more than 3 mm^2 were collected while caution was made to minimize mechanical damage such as pulling, contusion, and squeezing. The samples were gently rinsed with PBS to remove blood stains and hairs on the surface. The tissue surface to be scanned was well-preserved and labelled. The samples were immediately soaked in electron microscope fixative at room temperature for 2 h and then transferred to 4°C for storage. The fixed samples were rinsed three times with 0.1 M phosphate buffer (pH 7.4) for 15 min each time. The tissues were fixed with 1% osmium in 0.1 M phosphate buffer (pH 7.4) at room temperature in the dark for 1-2 h. After fixation, they were rinsed three times with 0.1 M phosphate buffer (pH 7.4) for 15 min each time and gradually dehydrated in a gradient of 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 15 min at each concentration and in isoamyl acetate for 15 min. The samples were dried in a critical point dryer, placed on conductive carbon double-sided tape, and sprayed with gold for about 30 s on the sample stage of the ion sputtering apparatus. Images were collected using Regulus 8100 scanning electron microscope (Hitachi High-Tech, Co., Ltd., Shanghai, China).

Statistical analysis

Prism8 (GraphPad, Boston, MA, USA) was employed for statistical analysis of this study. The one-way ANOVA was used for results comparison between the experimental groups. The *P* value less than 0.05 was defined as the significant difference.

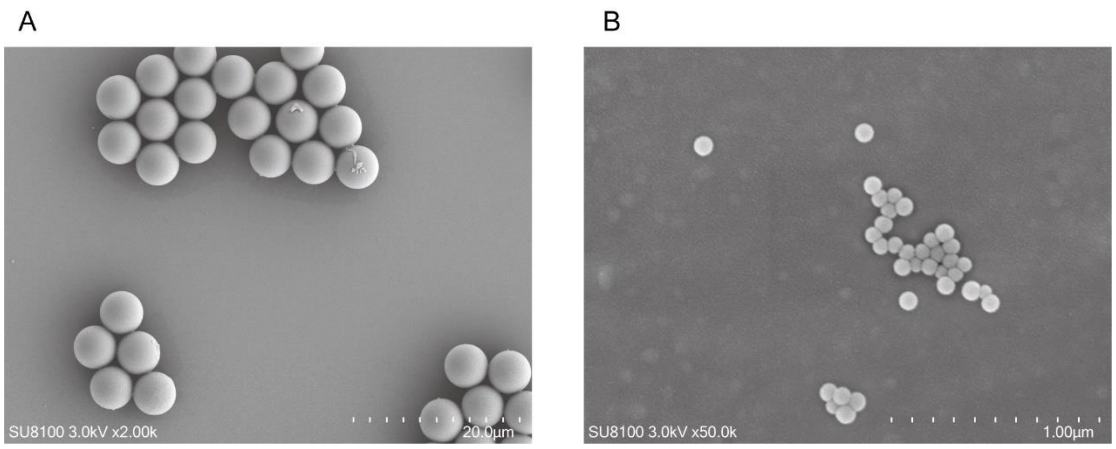

Figure 1. Characteristics of different sizes of polystyrene microplastics (PS-MPs). Scanning electron microscope (SEM) photomicrographs showing various sizes of PS-MPs. **A.** PS-MPs of 5 µm. **B.** PS-MPs of 60 nm.

Results

Characteristics of PS-MPs with different particle sizes

The microscopic characteristics of microplastics including their surface texture and shape were observed under a SEM. The results demonstrated that both 5 µm and 60 nm PS-MPs were spherical, uniform in size, and dispersedly distributed (Figure 1)

The effects of PS-MPs with different particle sizes and doses on the body weight and testis weight of mice

Within five weeks of continuous administration, mice in each group were weighed every week. The results showed that the body weight of mice in all experimental groups displayed an upward trend. Notably, mice in groups with different doses and particle sizes of microplastics showed a slower increase in body weight compared with those in the control group. At the end of the experiment, the body weights of mice in all microplastic-treated groups were lower than that in the control group (Figure 2A). Given that all mice were housed in the same environment, these results indicated that treatment with different particle sizes and doses of microplastics affected the weight growth of mice. After the experiment, the testis weight of mice was statistically analyzed. There were no significant differences in testis weight of mice between groups treated with PS-MPs of different particle sizes (5 µm or 60 nm) and doses (10, 100, and 1,000 µg /d) and the control group (Figure 2B), implying that these microplastics had no significant damage to the testicular tissue of mice.

The effect of PS-MPs with different particle sizes and doses on mouse sperm quality

The sperm counts of mice treated with PS-MPs of different particle sizes (5 µm or 60 nm) and doses (10, 100, and 1,000 µg/d) were compared with that of the control group. The results revealed that the sperm count in each group of 60 nm MPS 10, 100, and 1,000 µg/d was significantly lower than that in the control group ($P < 0.001$). Moreover, the decrease in the sperm count of the treatment groups displayed a dose-dependent trend. Specifically, the sperm count showed a gradually decreasing trend as the dose of microplastics increased. Likewise, the sperm count in each group of 5 µm PS-MPs 10, 100, and 1,000 µg/d was significantly lower than that in the control group and showed a gradually decreasing trend with the increase of doses of microplastics ($P < 0.001$) (Figure 3). Collectively, these results indicated that different doses of 5 µm and 60 nm microplastics had damaging effects on sperm count and spermatogenesis in mice.

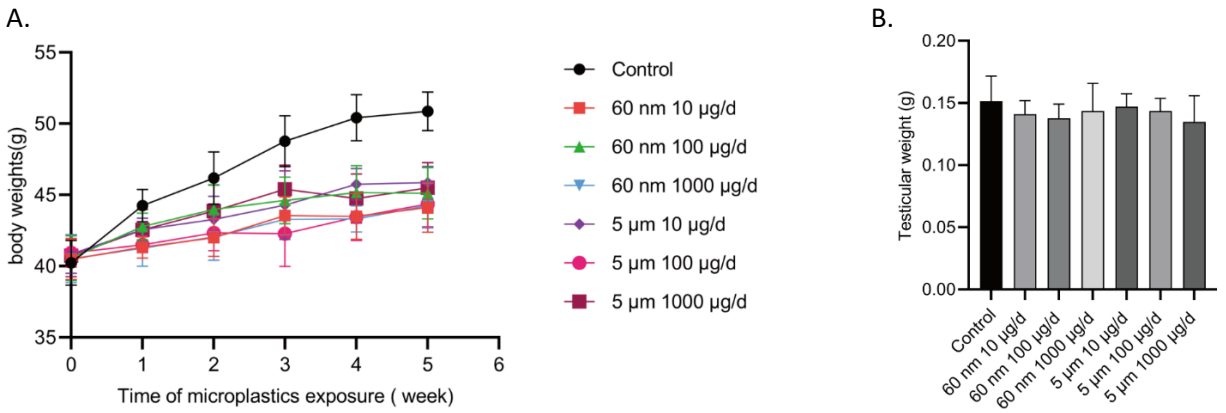

Figure 2. The body weight and testicular weight of mice in control and PS-MPs exposed groups. **A.** The body weight in each group of mice was weighed weekly. **B.** Testicular weights of mice in different groups weighed on the 35th day.

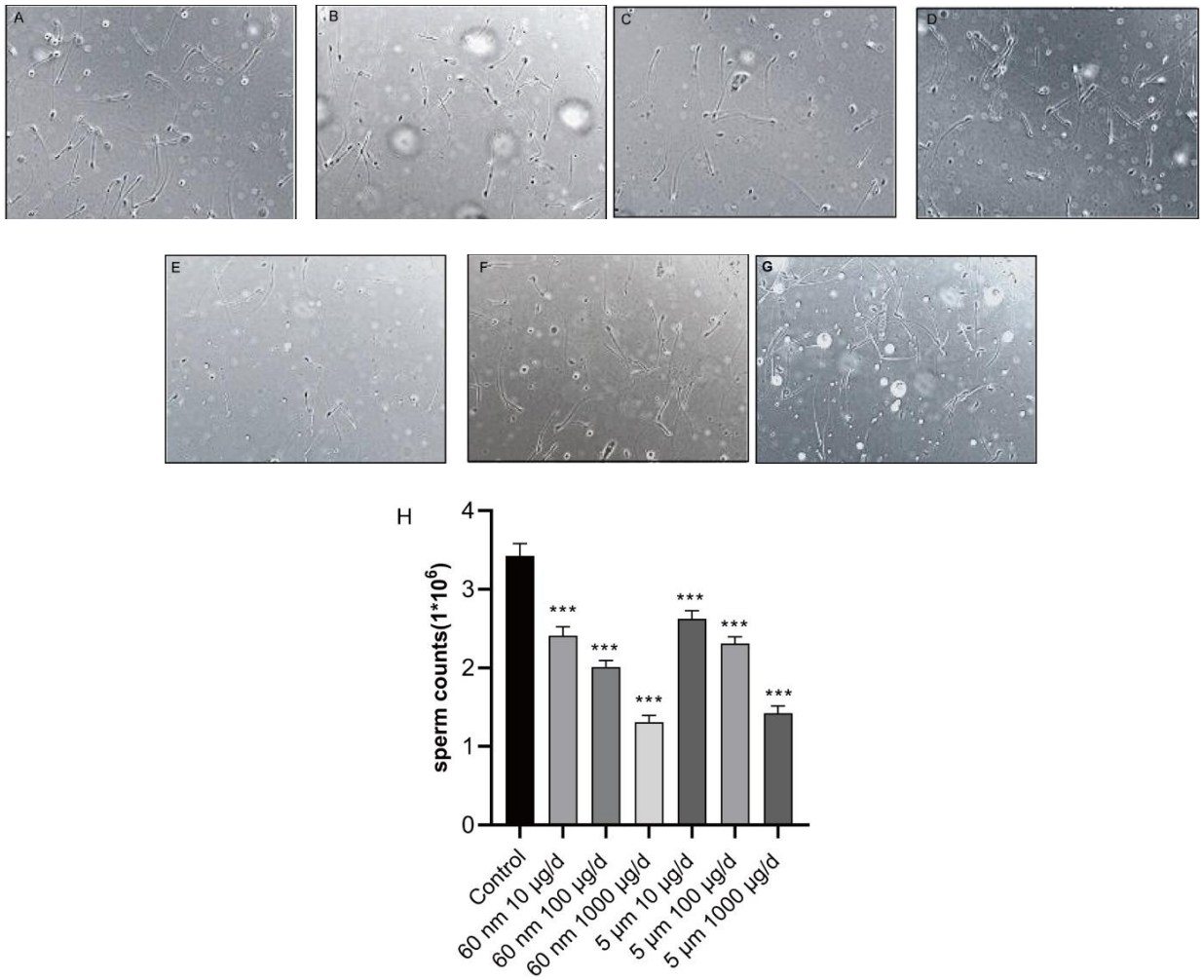

Figure 3. Effects of PS-MPs on sperms of mice. **A.** 5 μm 10 μg/d PS-MPs. **B.** 5 μm 100 μg/d PS-MPs. **C.** 5 μm 1,000 μg/d PS-MPs. **D.** 60 nm 10 μg/d PS-MPs. **E.** 60 nm 100 μg/d PS-MPs. **F.** 60 nm 1,000 μg/d PS-MPs. **G.** Control. **F.** Quantification of A-G. Date was presented as the mean ± SEM from 8 randomly chosen pictures. $^{***}P < 0.001$.

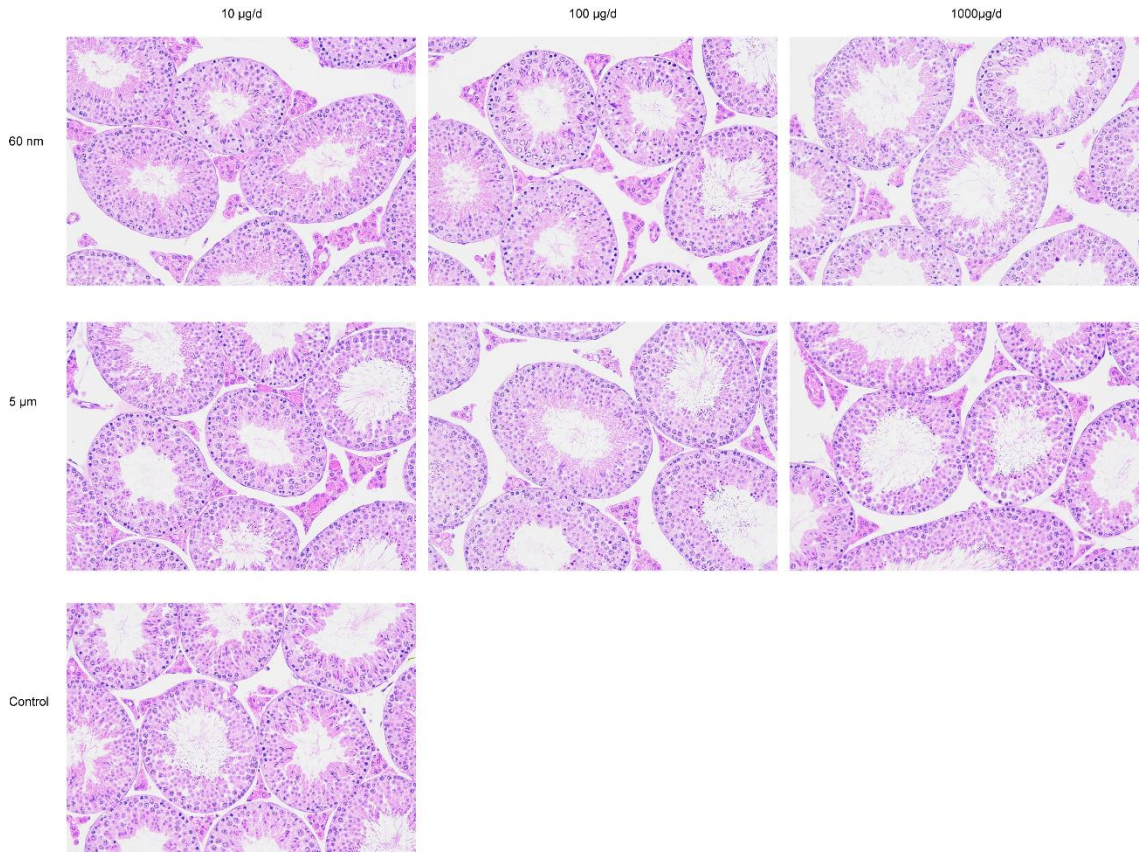

Figure 4. Effects of PS-MPs on testicular structures. HE sections of mice testes (200X).

The effect of PS-MPs with different particle sizes and doses on mouse testicular tissue

HE staining of the tissue section showed that the testicular tissue of mice in the groups exposed to PS-MPs with different particle sizes and doses exhibited varying degrees of pathological damage including inflammation and apoptosis. Moreover, the number of both spermatogenic cells and sperm in mouse testicular tissue was significantly lower in the microplastic-treated group than that in the control group. Compared with the control group, the testicular tissue of mice treated with microplastics displayed damage and atrophy of the seminiferous tubules, loose arrangement of spermatogenic cells, and the appearance of vacuoles in the damaged tissue (Figure 4). Notably, the effects of testicular tissue damage in the groups treated with different doses of 60 nm MPs and 5 μm MPs were more significant than that in control group. These observations demonstrated that PS-MPs with different particle sizes and doses could cause varying degrees of damage to mouse testicular tissue.

Discussion

Microplastics (MPs) mainly come from the degradation of plastic products and the emission of plastic particles [29]. It has been shown that microplastic pollution is deteriorating more rapidly on land than in the oceans due to human activities [30, 31]. In recent years, with the intensification of microplastic pollution, the adverse effects of environmental pollutants on reproductive health have attracted widespread social attention. As a new type of environmental pollutant, microplastics have recently been proven to have certain toxicity to the gastrointestinal tract and liver of aquatic organisms and mammals [20]. Research on

microplastics mainly focuses on aquatic organisms such as algae, zooplankton, crustaceans, fish, and other invertebrates, and it has been demonstrated that microplastics have damaging effects on their reproductive systems [32]. A limited number of studies have found that PS-MPs impair mammalian reproductive development. Specifically, exposure of male and female mice to MPs can cause changes in the sex ratio at birth, affect the weight of offspring, and induce metabolic disorders of lipids and amino acids in offspring, suggesting that MPs affects the health of offspring [33, 34]. However, there are relatively few studies on the damage of microplastics of different particle sizes to the mammalian reproductive system. This study investigated the effects of PS-MPs of different particle sizes and doses on the reproductive damage of male Kunming mice by using two types of microplastics with different particle sizes of 5 μm and 60 nm, and three dose gradients based on previous toxicological studies on MPs in aquatic organisms. Among the dose gradients, 10 μg/d was the low-dose exposure concentration, while 100 μg/d was the medium-dose exposure concentration, which was equivalent to the actual environmental concentration of MPs in natural rivers, and 1,000 μg/d was the high-concentration microplastic exposure. Both body weight and coefficient were commonly used indicators in toxicology and were also sensitive indicators of systemic toxicity [35]. The results showed that the body weight of mice exposed to microplastics with different doses of 5 μm and 60 nm particle sizes increased slowly compared with the control group. The body weight of mice was lower in the microplastic-exposed groups than in the control group at the end of the experiments, suggesting that microplastics might have a certain impact on the body weight of mice. The testis weight of mice exposed to microplastics were measured and no significant difference in the testis weight between the microplastic-treated groups and the control group was observed, implying that microplastics of the two particle sizes had no adverse effects on testicular development in mice.

The testes are an important male reproductive organ and are also the site of sperm production. Normal spermatogenesis is an important guarantee for sperm quality and even the maintenance of normal reproductive function. In this study, microplastics with different particle sizes at different doses showed varying degrees of damage to the mouse sperm quality. This observation was consistent with the results of previous studies showing that exposure of male Wistar rats to PS-MPs led to the damage of seminiferous tubule, resulted in apoptosis of spermatogenic cells, and decreased the motility and concentration of sperm [36]. It was also shown that exposure of ICR mice to 5 μm PS-MPs through free drinking water could cause certain damage to sperm count and testicular tissue [37]. The present study found that microplastics of different particle sizes and doses could impair sperm development in male Kunming mice.

The histological examination of testicular tissue revealed that germ cells at various developmental stages in the seminiferous tubules of mice exposed to different doses of microplastics with different particle sizes exhibited nuclear shrinkage, rupture, and vacuolization, while the sperm density in the tubules declined. Moreover, the above damaging effects became more pronounced with the increase in the dose of microplastics. This finding suggested that PS-MPs might cause a certain degree of damage to sperm production in mice through impairing testicular tissue, leading to a decrease in sperm quality. Other studies have shown that endometrial decidualization was impaired in female mice exposed to microplastics, resulting in adverse pregnancy outcomes such as fetal intrauterine growth restriction, premature birth, and miscarriage [38]. In addition, microplastic exposure could lead to cytotoxicity in mice, as well as oxidative stress and inflammatory responses in male mice [26]. In terms of sperm production, microplastics could disrupt the blood-testis barrier, affecting testicular health, and reducing sperm quality. They could also increase the ubiquitination of RAC1 and CDC42 in the sperm of infertile mice

and interfere with RAC1- and CDC42-dependent F-actin polymerization, inhibiting sperm capacitation and capacitation-dependent sperm function, and ultimately leading to reduced male fertility [39]. Thus, it can be inferred that microplastics have damaging effects on the reproductive system of both male and female mice. All previous findings are in accordance with the conclusion drawn from the present study that microplastics of different doses and particle sizes cause damage to the sperm of mice. This study still has its limitation that the specific mechanism underlying microplastic-caused reproductive damage in mammals has not yet been elucidated. Consequently, future research will focus on addressing this issue at the molecular level using transcriptomics, metabolomics, and intestinal microbiological approaches.

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