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RESEARCH ARTICLE

The Toxic Effects of Combined Exposure to Copper Sulfate and Polystyrene Microplastics on Sperm Quality and Testicular Tissues in Male Mice

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Abstract

Both copper sulfate ($CuSO_4$) and polystyrene microplastics (PS-MPs) exhibit toxic effects on the male reproductive system. Notably, PS-MPs can adsorb copper ions, making their combined pollution-induced reproductive toxicity and underlying mechanisms urgent to clarify. This study aimed to investigate the toxic effects of $CuSO_4$ and PS-MPs co-exposure on sperm quality and testicular tissue in male mice, and to determine potential synergism, thereby providing a basis for assessing reproductive health risks. Male mice were randomly divided into control, $CuSO_4$, PS-MPs, and combined exposure groups, with continuous intragastric administration for 30 days. Sperm parameters, testicular histopathology, cell apoptosis, and apoptosis-related protein expressions [Bcl-2-associated X protein (Bax), B-cell lymphoma-2 (Bcl-2), cysteinyl aspartate specific proteinase-3 (Caspase-3), tumor protein p53 (p53)] were detected. Results showed that combined exposure most significantly inhibited body weight gain ($P<0.05$). Compared with single exposure, the combined group exhibited more severe sperm quality deterioration, severe testicular histopathological lesions, and a significant increase in Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells ($P<0.05$ or $P<0.01$). Western blot analysis confirmed upregulated expressions of Bax, Caspase-3, and p53, and downregulated Bcl-2 in the combined group, indicating activation of the apoptotic pathway. In conclusion, $CuSO_4$ and PS-MPs have aggravated toxic effects on the male reproductive system, and the mechanism involves the activation of apoptotic signaling.

Keywords: Combined exposure, $CuSO_4$, Male reproductive toxicity, PS-MPs, Sperm quality

INTRODUCTION

The rapid pace of industrialization and urbanization has led to the coexistence of various pollutants in the environment. Copper sulphate ($CuSO_4$) is a chemical commonly used in electroplating, pesticides and aquaculture; the copper pollution in water bodies caused by the discharge of wastewater containing this substance cannot be overlooked [1,2]. Meanwhile, polystyrene microplastics (PS-MPs) are one of the most frequently detected types of microplastics in the environment [3], and they can coexist with $CuSO_4$ in the environment [4]. Recent studies have shown that the combined exposure of PS-MPs and heavy metals such as cadmium can synergistically damage male reproductive functions through the ferroptosis pathway [5], suggesting that the combined pollution of microplastics and metal ions may have similar synergistic toxicity mechanisms. However, the toxic effects of $CuSO_4$ combined with PS-MPs on the male reproductive system, as well as the underlying molecular mechanisms, remain largely unexplored, representing a critical scientific gap in the field of environmental reproductive toxicology.

Previous studies have demonstrated that excessive exposure to $CuSO_4$ has significant toxicity to the male reproductive system [6,7]. The main target organs of excessive copper are the testes, especially the spermatogenic tubules and supporting cells, followed by the interstitial tissue. Notably, most existing studies have focused on copper-induced damage to spermatogenic tubules, while research on the effects of $CuSO_4$ exposure on testicular interstitial tissue remains scarce. Specifically, copper exposure first

leads to damage of the spermatogenic epithelium, causing disruption of the structure of the spermatogenic tubules, with the main characteristics being the detachment, disorder, and even vacuolation of spermatogenic epithelial cells [7,8]. The mechanism involves the accumulation of copper ions in the testicular tissue: excessive copper triggers oxidative stress and damages mitochondrial function, ultimately leading to apoptosis of reproductive cells [1]; at the same time, it can interfere with the function of the hypothalamus-pituitary-gonadal axis, inhibit the ERK signaling pathway, and reduce the levels of gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone, ultimately resulting in spermogenesis disorders and decreased fertility [9]. Particularly importantly, recent studies suggest that copper nanoparticles can also directly damage the interstitial tissue, inducing Leydig cell apoptosis and steroidogenesis disorders through activating the ERK1/2 signaling pathway [10]; this is different from the mechanism of $CuSO_4$ inhibiting the ERK pathway. However, whether traditional $CuSO_4$ exposure can directly damage interstitial tissue, and if so, how this damage interacts with spermatogenic tubule injury, remains unclear and requires further investigation.

PS-MPs are formed by the long-term weathering and mechanical fragmentation of polystyrene in the environment, with most of them having a diameter of less than 5 mm [3]. Due to their small particle size and large specific surface area, PS-MPs can adsorb and carry other pollutants for migration, and enter the animal body through ingestion or respiration [11,12]. After reaching the testes, PS-MPs can penetrate the blood-testis barrier and directly act on the epithelial cells of the spermatogenic tubules [11,12]. The disruption of the structure of the spermatogenic tubules leads to the inhibition of spermatogenic cell proliferation, resulting in a decrease in sperm concentration and motility, and an increase in the rate of sperm abnormalities. Deng et al. [13] confirmed that PS-MPs can damage the reproductive performance of male mice and disrupt the lipid homeostasis of the offspring, suggesting their transgenerational toxicity risk; Hou et al.[14] reported that continuous intragastric administration of 5 μm PS-MPs (5 mg/d) significantly reduced the testosterone level in mice. These studies indicate that PS-MPs can interfere with testosterone secretion [15,16], but compared to heavy metals, the damage effect of PS-MPs exposed alone is relatively mild, mainly showing chronic and progressive damage characteristics [17]. Heavy metals such as copper can cause significant oxidative stress and cell apoptosis within a short period of time [1,2]. However, the reproductive toxicity of PS-MPs often only becomes apparent after long-term low-dose exposure, through the continuous activation of the p38 MAPK signaling pathway [18], the upregulation of the Bax/Caspase-3 apoptotic cascade reaction, and the progressive disruption of the blood-testis barrier integrity [12,19], resulting in reproductive system damage. It is particularly important to note that there are differences in reproductive toxicity among different particle sizes of PS-MPs (0.1 μm vs 1 μm). The toxicity of small particle sizes is more significant when combined with cadmium exposure [20]. Additionally, the surface of PS-MPs can adsorb copper ions in the environment, altering the bioavailability and toxicity effects of copper [4,21]. This carrier effect may enhance the combined toxicity risk of the two [22]. Further research has found that the combined exposure of PS-MPs and arsenic has been proven to cause transgenerational reproductive toxicity [23], confirming that the combined exposure of microplastics and metal pollutants has a synergistic reproductive risk and cross-generational health hazards.

Given the combined exposure risk of $CuSO_4$ and PS-MPs and the carrier effect of PS-MPs, the synergistic toxicity of both on the male reproductive system and its mechanism need to be clarified. Therefore, in this study, male mice were used as the subjects, and models of $CuSO_4$ and PS-MPs exposure alone and in combination were established. The indicators such as sperm concentration, motility, and deformity rate were detected, and the pathological changes of testicular tissues were observed. The expressions of apoptosis-related proteins such as Bax, Bcl-2, Caspase-3, and p53 were analyzed to explore the toxic effects and synergistic effects of combined exposure, providing experimental basis for the reproductive health risk assessment of environmental complex pollutants.

Material and Methods

Ethical Approval

All experimental procedures conformed to the ethical standards for laboratory animals, and the animal experimental protocol was reviewed and approved by the Animal Ethics Committee of South China Agricultural University with the ethics approval number 2025F386. During the feeding process, care was taken to handle the mice gently to reduce their stress response.

Experimental Animals and Conditions

Twenty-four 6-week-old male C57BL/6J mice (weighing 20-22 g) were selected (purchased from Guangdong Zhiyuan Biomedical Technology Co., Ltd.). The experimental diet and bedding materials for the mice were also purchased simultaneously. Before the start of the experiment, the mouse cages were thoroughly cleaned and disinfected. All the experimental animals were housed in the Experimental Center of the Animal Hospital of South China Agricultural University. The

environmental conditions were strictly controlled at a temperature of 22°C-24°C, a relative humidity of 50±10%, and a 12-h light/dark cycle. The mice had free access to food and water. The body weight, food intake, and water intake of each group of mice were weighed and recorded before each intragastric administration. Fresh mouse food and water were changed daily, and the bedding material was replaced every 3 days. After 1 week of adaptive feeding, the mice were randomly divided into 4 groups (n=6) according to their body weight: the control group (Ctrl), the copper sulfate group (Cu), the polystyrene microplastic group (Ps), and the combined exposure group (Cu+Ps). The names of each group corresponded to the treatment methods the mice received. The Ctrl group did not undergo any special treatment and was given 0.1 mL of deionized water by intragastric administration. The Cu group was given 0.1 mL of $CuSO_4$ solution, the Ps group was given 0.1 mL of PS-MPs solution, and the Cu+Ps group was given 0.1 mL of $CuSO_4$ solution + 0.1 mL of PS-MPs solution. The treatment was continued for 30 days, and then the mice were sacrificed by cervical dislocation and tissue samples were collected.

Instruments and Reagents

The main experimental instruments used in this study are: Vortex TM-1F Vortex Mixer (WIGGENHAUSER, Germany), DM1000 Optical Microscope (Leica, Germany), KZ Tissue Grinder (Wuhan Sevier Biotechnology Co., Ltd.), TGL-16B Benchtop Centrifuge (Shanghai Anting Scientific Instrument Factory), ME204 Electronic Balance (Mettler Toledo), 5804R Refrigerated Centrifuge (Eppendorf, Germany), Nanodrop 2000 Micro-Volume UV-Vis Spectrophotometer (ThermoFisher Scientific, USA), HI1220 Slide Holder (Leica, Germany), DHG-9123A Forced Air Drying Oven (Shanghai Yiheng Technology Co., Ltd.), SLK-O3000-S Shaker (Sorvall, USA), ELx808 Microplate Reader (BIOTECK, USA), PB-10 pH Meter (Sartorius, Germany), Unique-R10 Ultrapure Water System (Wuxi Xinyi Science Instrument Co., Ltd.), UVPChemStudio515 Multi-Mode Imager (Jena Analytics Co., Ltd.), DW-86L626 -80□ Ultra-Low Temperature Freezer (Haier Home Appliance Co., Ltd.), IMS Automatic Snowflake Ice Maker (Shanghai Xueshuo Ke Electrical Appliance Co., Ltd.), RH Basic2 Heating Magnetic Stirrer (IKA, Germany), EPS600 Electrophoresis System (Shanghai Tianeng Co., Ltd.), BG-Power600 SDS-PAGE Protein Electrophoresis Apparatus (Shanghai Tianeng Technology Co., Ltd.), DMi8 Inverted Fluorescence Microscope (Leica, Germany), RM2235 Rotary Microtome (Leica, Germany).

The main reagents and consumables are as follows: 2.5% (w/v) Microplastic Monodisperse Suspension (Cat. SEQ01-00250, Tianjin Saierqun Technology Co., Ltd.), Hematoxylin-Eosin Staining Solution (Cat. G1005-500ML, Wuhan Sevier Biotechnology Co., Ltd.), Copper Sulfate (500 g, Tianjin Zhiyuan Chemical Reagent Co., Ltd.), Paraformaldehyde (Cat. EKKS1699, Shanghai Ika Biotechnology Co., Ltd.), Ethanol (500 mL, Guangzhou Chemical Reagent Factory), PBS Buffer (Cat. G0002-2L, Wuhan Sevier Biotechnology Co., Ltd.), PMSF (Cat. ST505, Wuhan Sevier Biotechnology Co., Ltd.), RIPA Lysis Buffer (Cat. P0013B, Shanghai Beyotime Biotechnology Co., Ltd.), Universal Antibody Diluent (Cat. WB500D, Shanghai Xinseme Biotechnology Co., Ltd.), SDS-PAGE Gel Preparation Kit (Cat. E304-01, Nanjing Novizan Biotechnology Co., Ltd.), PVDF Membrane (Cat. ISEQ15150, Sigma, USA), BCA Protein Concentration Assay Kit (Cat. E112-01, Shanghai Xinseme Biotechnology Co., Ltd.), Tris (Cat. T8060, Shanghai Solabao Technology Co., Ltd.), SDS (Cat. 227, Biosharp, USA), Chemiluminescent Substrate (Cat. P2100, Shanghai Xinseme Biotechnology Co., Ltd.), Protein Marker (Cat. RM19001, Beijing Kangwei Century Biotechnology Co., Ltd.), Primary Antibodies (Bcl-2, Cat. A19693; Bax, Cat. A19684; p53, Cat. A19585; Caspase-3, Cat. A19664, all from Wuhan Abiotech Biotechnology Co., Ltd.; GAPDH, Cat. A19056, from Beijing Boacon Biotechnology Co., Ltd.), Secondary Antibodies (Goat Anti-Mouse IgG, Cat. CW0102S; Goat Anti-Rabbit IgG, Cat. CW0103S, both from Beijing Kangwei Century Biotechnology Co., Ltd.).

The concentration settings of $CuSO_4$ and microplastic gavage solutions in this study were supported by published oral toxicological models of male mice [24-26]. The reagents used in this paper are prepared as follows: $CuSO_4$ solution: Weigh 1.4 g of anhydrous $CuSO_4$ precisely according to the experimental requirements and add it to 70 mL of deionized water. Finally, prepare a working solution of $CuSO_4$ with a concentration of 20 mg/mL and store it for later use [24]. Microplastic solution: Precisely measure 8.4 mL of the single-dispersed suspension of microplastics (2.5%, w/v) and add it to 26.6 mL of deionized water. Finally, prepare a microplastic mixture solution with a concentration of 6 mg/mL and store it for later use [25]. The grouping scheme of single exposure and combined co-exposure was designed with reference to Chang et al.[26]. Polyformaldehyde fixative solution (4%): Dissolve 40 g of polyformaldehyde in 800 mL of PBS, heat it and add NaOH to fully dissolve it. Let it cool naturally and then adjust the pH to 7.2-7.4 with 1 mol/L HCl. Finally, make up to 1000 mL with PBS. Transfer solution: Weigh 3.03 g of Tris and 14.41 g of glycine, mix them thoroughly with 800 mL of ddH_2O, and then add 200 mL of methanol to fully dissolve them. TBST working solution (1x): Mix 50 mL of TBST (10x) with 450 mL of deionized water thoroughly, prepare TBST (1x), store it at 4□ for later use. Blocking solution: Weigh 6 g of skimmed milk powder, add it to 120 mL of TBST (1×), and mix thoroughly to fully dissolve it.

General Physiological Indicators

The body weight, food intake and water intake of each mouse were measured and recorded before each gavage.

The average body weight of the mice in this group = total weight of all mice in the group/number of mice in the group

The average daily water intake of the mice in this group = total daily water intake of all mice in the group / number of mice in the group

Sample Collection

After continuous intragastric administration for 30 days, all the experimental mice were sacrificed by cervical dislocation. Then, the bilateral testicles and epididymis of the mice were removed, weighed precisely, and used to calculate the organ coefficient. One testicle from each mouse was taken out, rinsed with normal saline to remove surface impurities, and then one testis was fixed in 4% formaldehyde for subsequent histopathological examination and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, while the other was rapidly preserved in liquid nitrogen for subsequent Western blot analysis.

Sperm Count, Motility, and Abnormality Rate

After the animals are sacrificed, the epididymis is quickly collected and the surrounding tissues are removed. It is slowly placed into 1 mL of physiological saline at 37□, sterilized under high pressure, and then cut into pieces using an ophthalmic scalpel to fully disperse the sperm at the tail end of the epididymis in the physiological saline. The sperm count is determined using a sperm counting plate under an optical microscope. The number of sperm within the grid is counted using a cell counting plate to determine the sperm count. The proportion of live sperm to the total sperm count is defined as sperm motility, and the proportion of abnormal sperm to the total sperm count is defined as sperm abnormality rate. Then, according to the manufacturer's instructions, the slides are stained with sperm staining solution. Each sample is observed three times. To reduce subjective bias, the relevant observations and counts are completed by personnel who are not aware of the experimental grouping.

Testicular Index

After the 30-day trial period ended, the body weight of the mice was measured. Then, the mice were dislocated and processed, and their testicles were immediately removed, with the surrounding fat and connective tissues being removed and weighed. The ratio of testicle weight to mouse weight (testicle coefficient) was expressed as (testicle weight/mouse weight) x 100%.

Observation of Testicular Histopathology

The fixed testicles were cut into small blocks measuring 5 mm x 5 mm x 3 mm, which were then subjected to stepwise ethanol dehydration, xylene clearing and paraffin infiltration before being embedded to produce paraffin blocks. Using a microtome, the paraffin block was sectioned into a series of 3-5 μm thick sections. The sections were mounted onto slides and heat-set at 60°C for 2-4 h to fix them. Following dewaxing in xylene and a gradient of ethanol, the sections were rinsed twice with distilled water; hematoxylin-eosin (HE) staining was then performed, involving sequential treatment with hematoxylin solution for 5-10 min, followed by rinsing with water, differentiation with hydrochloric acid-alcohol for 30 sec to 1 min, re-staining with ammonia solution, and staining with eosin solution for 1-3 min. Following staining, the sections were rapidly dehydrated using a gradient of ethanol and cleared with xylene, before being mounted with neutral resin. Upon completion of mounting, the sections were examined under a microscope and photographed.

TUNEL Staining

After paraffin sections have been deparaffinised using an environmentally friendly deparaffinising solution, they are sequentially rehydrated using a gradient of anhydrous ethanol, rinsed with distilled water, and a hydrophobic ring is drawn around the specimen using an immunohistochemistry waterproof pen. Proteinase K working solution (1:9) is then added, and the sections are incubated at 37°C for 20 min; Membrane lysis was performed as required (20 min at room temperature), followed by three 5-min washes in PBS and a 10-min incubation in equilibrated buffer at room temperature; Incubate with TUNEL reaction mixture (TdT enzyme: dUTP: buffer = 2:5:50) at 37°C in the dark for 1 h; wash with PBS, counterstain with DAPI for 10 min, mount with anti-fade mounting medium, observe under a fluorescence microscope to capture images, and analyse positive expression using ImageJ software.

Extraction of Total Protein and Western Blotting

Remove testicular tissue from the -80°C freezer, cut 0.2-0.3 g into an Eppendorf tube, add 250 μL of RIPA lysis buffer containing protease inhibitors, homogenise on an ice bath, and centrifuge at 4°C and 12.000 rpm for 10 min; collect the supernatant. Determine protein concentration using the BCA method: Dispense 25 μL of sample into a 96-well plate, add 200 μL of BCA working solution, incubate at 37°C for 30 min, and measure absorbance at 562 nm.

Western blotting: Prepare a 12% SDS-PAGE gel; load biological replicate samples from six individual mice

per group (n = 6), with each sample run once without technical replication (4 μL marker + 10 μL sample); run on a 80 V focusing gel for 30 min, followed by a 120 V separating gel for 60 min; after methanol-activating the PVDF membrane, transfer at 150 mA for 90 min; Block with 5% skimmed milk powder at room temperature for 1 h, wash three times with TBST for 10 min each; incubate with primary antibody at 4°C for 16 h, wash three times with TBST for 10 min each; incubate with the appropriate secondary antibody at room temperature for 1 h, wash three times with TBST for 5 min each; develop with developing solution, expose on an imaging system, and normalize band grayscale values to the corresponding internal reference (β-actin) prior to analyse grayscale values using ImageJ.

Statistical Analysis

All the data were sorted by Microsoft Office 2024 software and analyzed statistically using GraphPad Prism 9.5.0 software. The number of repetitions for each group of experiments was ≥3. The data were expressed as the mean ± standard deviation. When comparing between two groups, Student's t test or Mann–Whitney non-parametric test was used according to the type of data distribution to determine whether the differences between the groups were statistically significant. In multi-group comparisons, one-way ANOVA was used for continuous variables and chi-square test for categorical variables. Pairwise comparisons between groups were conducted based on Bonferroni method. When $P<0.05$, it was considered statistically significant.

Results

Changes in Mouse Body Weight, Feed Intake, and Water Intake

To evaluate the effects of $CuSO_4$, PS-MPs, and combined exposure on the general growth status of mice, the body weight changes of each group of mice were continuously recorded during the 30-day intragastric administration period, and the feed intake and water intake were statistically analyzed. The results are shown in *Fig. 1-A*. Compared with the control group, the inhibition of weight gain in the combined exposure group was the most significant, and the weight of the $CuSO_4$ group and the microplastics group also showed a certain downward trend. *Fig. 1-B, C* show that there were no statistically significant differences in feed intake and water intake among the groups ($P>0.05$

Analysis of Changes in Sperm Quantity, Vitality, Abnormality Rate and Testis Index

As shown in *Fig. 2-A*, compared with the control group, the sperm quantity in the combined exposure group significantly decreased ($P<0.01$), and the $CuSO_4$ group and the microplastic group also showed a decreasing trend; *Fig. 2-B*, the sperm vitality in the combined exposure group significantly decreased ($P<0.01$), and the $CuSO_4$ group was also lower than the control group ($P<0.05$); *Fig. 2-C*, the sperm abnormality rate in the combined exposure group significantly increased ($P<0.05$). *Fig. 2-D*, there was no statistically significant difference in the testis coefficient among the groups ($P>0.05$).

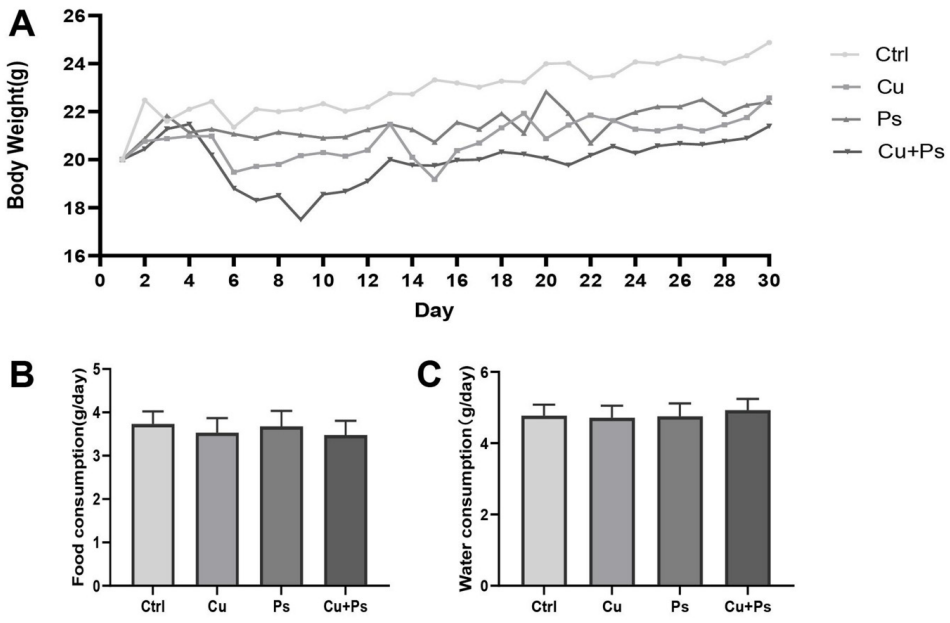

Fig 1. Changes in body weight, feed intake, and water intake of mice in each treatment group. **(A)** Body weight; **(B)** Feed intake; **(C)** Water intake. All data are presented as mean ± standard deviation (SD), n = 6 per group

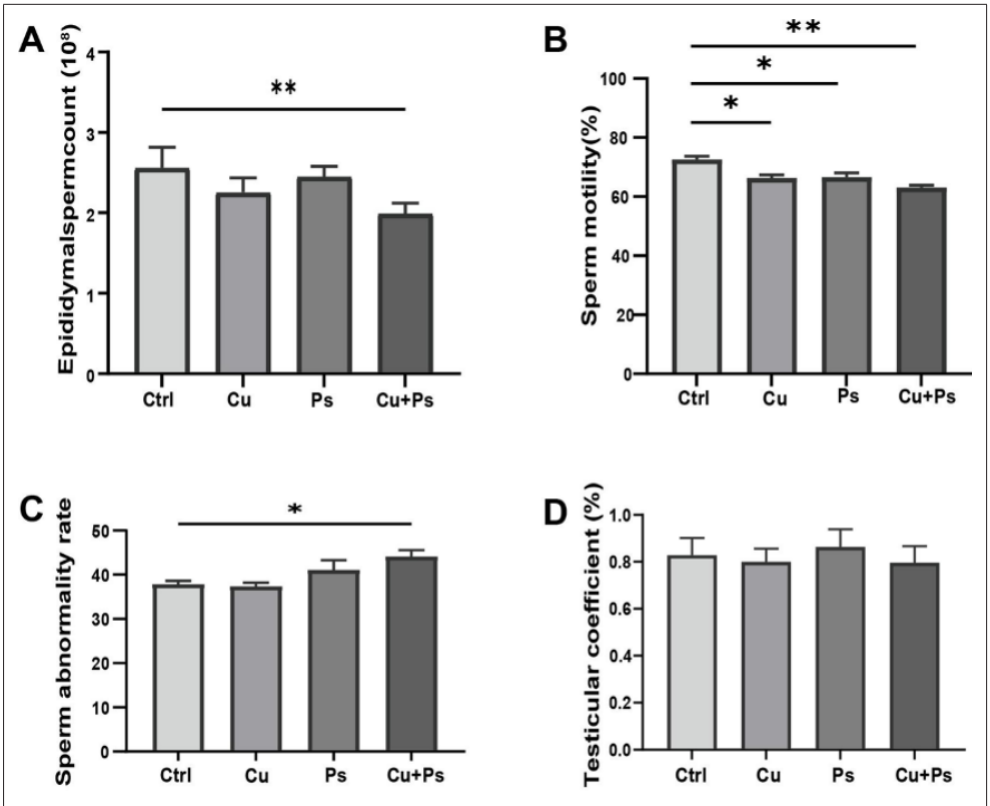

Fig 2. Sperm quality and testicular coefficient of male mice in each treatment group. (A) Seminal fluid sperm count (×108); (B) sperm motility (%); (C) sperm abnormality rate (%); (D) testicular coefficient (%). All data are presented as mean ± SD, n = 6 per group. Asterisks indicate significant differences: *P<0.05, **P<0.01

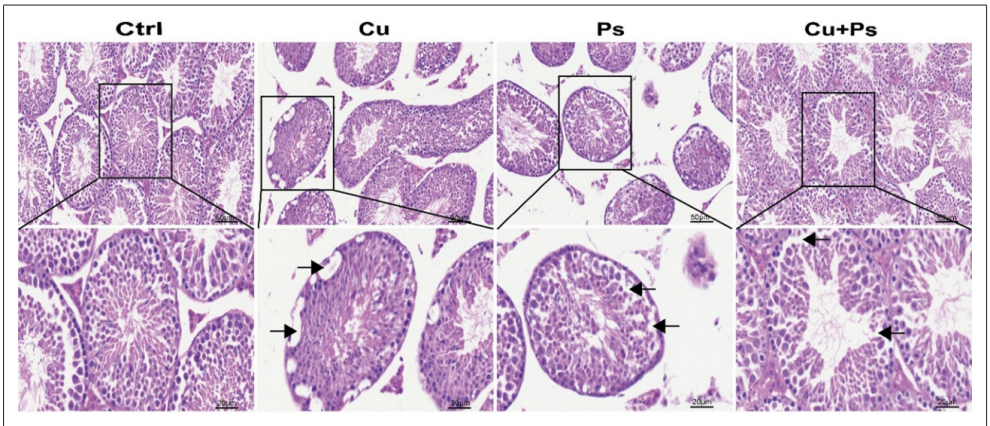

Fig 3. Morphology of mouse testicular tissue. Control group (Ctrl); copper sulfate group (Cu); polystyrene microplastics group (PS); combined exposure group (Cu + PS). Representative hematoxylin-eosin (HE) staining sections of testicular tissue. The upper row has a scale bar of 50 μm, and the lower row has a scale bar of 20 μm. Black arrows indicate typical pathological changes in testicular tissue, including vacuolation of seminiferous tubules, loose tubular wall structure, increased cell detachment, and reduced cellular components in the lumen

Pathological Observation of Testicular Tissue

H&E staining was used to observe the pathological changes of testicular tissue in each group of mice. The results are shown in *Fig. 3*. The testicular tissue structure of the control group was intact, with regular arrangement of seminiferous tubules, clear basement membrane, and distinct layers of spermatogenic epithelium. Both the $CuSO_4$ group and the microplastic group showed varying degrees of tissue damage, mainly manifested as atrophy of seminiferous tubules, disordered arrangement, shedding and vacuolation of spermatogenic epithelial cells, etc. Among them, the $CuSO_4$ group showed more obvious damage. Compared with the single exposure group, the testicular tissue damage in the combined exposure group

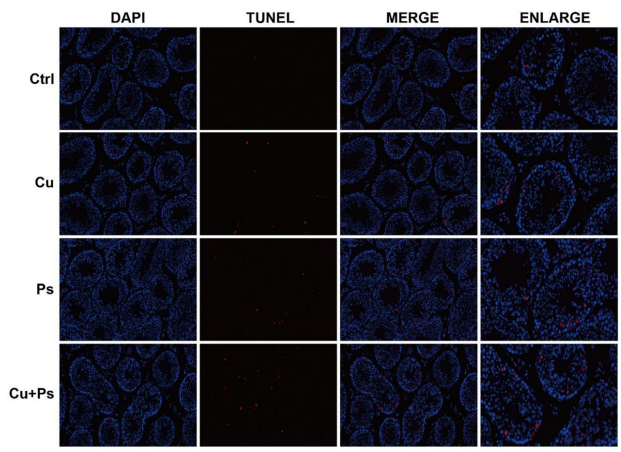

Fig 4. Results of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) fluorescence staining of testicular tissues in each treatment group. Cell nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) appear blue under ultraviolet excitation, while positive apoptotic cells are red

was the most severe, manifested as significant atrophy of seminiferous tubules, disordered structure, damage to the continuity of spermatogenic epithelium, and layer separation.

TUNEL Staining Results

TUNEL staining was used to detect the apoptosis of testicular cells in each group of mice. The results are shown in *Fig. 4*. In the control group (Ctrl), only a few red fluorescent positive cells were observed; in the $CuSO_4$ group, the microplastic group, and the combined exposure group, red fluorescent positive signals were visible, among which the positive signal in the combined exposure group was the most obvious.

Expression of Testicular Apoptosis Proteins

As shown in *Fig. 5-A*, the relative expression level of Bax protein in the testicular tissue of the combined exposure group was higher than that of the control group, and the difference was statistically significant (P<0.05). As shown in *Fig. 5-B*, compared with the control group, the relative expression level of Bcl-2 protein in the testicular tissue of the combined exposure group significantly decreased (P<0.01); although there was no significant difference between the copper group and the microplastic group (P>0.05), both showed a downward trend. As shown in *Fig. 5-C*, compared with the control group, the relative expression level of Caspase-3 protein in the testicular tissue of the combined exposure group significantly increased (P<0.05). As shown in *Fig. 5-D*, compared with the control group, the relative expression level of p53 protein in the testicular tissue of the combined exposure group significantly increased (P<0.05).

Discussion

This study established a gastric gavage exposure model to evaluate the effects of $CuSO_4$ and PS-MPs, either alone or in combination, on the reproductive system of male mice. The focus was on analyzing changes in sperm quality, pathological damage to testicular tissue, and the expression of apoptosis-related molecules. After 30 days of exposure, all the mice in each treatment group showed varying degrees of body weight reduction. Among them, the combined exposure group showed the most significant growth inhibition, followed by the $CuSO_4$ group and the microplastic group; however, there were no significant differences in food intake and water intake among the

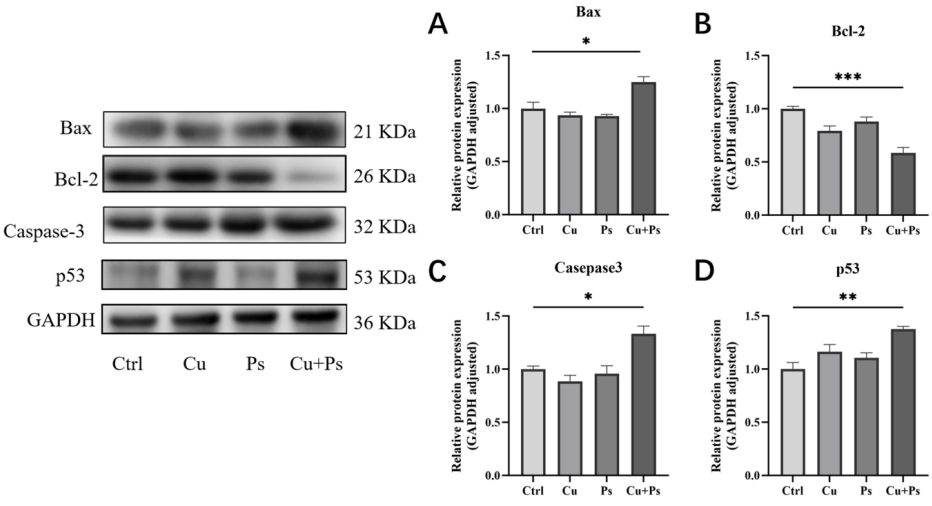

Fig 5. Expression of apoptosis-related proteins. (A) Relative expression level of Bcl-2-associated X protein (Bax); (B) Relative expression level of B-cell lymphoma-2 protein (Bcl-2); (C) Relative expression level of cysteinyl aspartate specific proteinase-3 (Caspase-3); (D) Relative expression level of tumor protein p53 (p53). All data are presented as mean ± SD, n = 6 per group. Significant differences are indicated by the symbols within the figure: *P<0.05, **P<0.01, ***P<0.001

groups. Notably, no significant inter-group differences were detected in food or water consumption, suggesting that the observed weight suppression was attributable to toxicant-induced metabolic perturbations rather than reduced caloric intake. Weight changes are often used as an indicator to reflect the overall health status of the body, so this study used it as a reference indicator for evaluating systemic toxic effects and conducted a comprehensive analysis in combination with subsequent histological and molecular detection results.

The evaluation results of sperm quality showed that both $CuSO_4$ and microplastics alone could have adverse effects on the reproductive function of male mice. Moreover, the combined exposure had a more significant impact. Compared with the control group, the combined exposure group exhibited more pronounced reductions in sperm count, decreased sperm motility, and increased sperm deformity rate, indicating a stronger interference with the process of sperm generation and maturation under the condition of combined exposure. Previous studies have shown that copper exposure can cause damage to the ultrastructure of the testis and epididymis, reduce sperm count, and accompany the occurrence of various forms of cell death [27]. While copper primarily disrupts mitochondrial electron transport and metal ion homeostasis through redox cycling, microplastics may exert toxicity via physical particle irritation, inflammatory cytokine induction, and gut-liver-testis axis perturbation. These distinct yet potentially convergent mechanisms may account for the aggravated reproductive impairment observed under co-exposure conditions, although whether this constitutes additive potentiation or true synergism remains to be statistically validated. In the cadmium testicular toxicity study by Wang et al.[28], it was also confirmed that heavy metals can cause disordered arrangement of spermatogenic cells and decline in sperm quality by inducing the accumulation of ROS, which is similar to the copper toxicity effect observed in this study. Furthermore, in the study by Elsayed et al.[29] on the reproductive toxicity of acrylamide, changes in functional indicators (sperm quality, sex hormone levels) occurred earlier than changes in testicular weight, confirming that the damage caused by environmental pollutants to the reproductive system often follows the temporal sequence of "function before structure". In the study by Ullah et al.[30] on calcium nanoparticles, it was also confirmed that particulate pollutants can induce sperm arrest and reduction of spermatogenic cells through oxidative damage, which is consistent with the reproductive damage characteristics observed in this study of PS-MPs. Although there was no significant difference in testicular index among the various treatment groups in this study, functional indicators such as sperm quantity, vitality, and deformity rate have changed significantly, indicating that at the doses and periods examined in this study, the exposure effect may initially manifest as damage to the spermatogenic process and sperm maturation quality rather than a synchronous change in testicular weight. The phenomenon of functional changes preceding structural changes is not uncommon in reproductive toxicology research. In conclusion, in subsequent studies, one can examine the effects of higher doses or longer exposure times on testicular weight, as well as assess whether there is a dose-effect relationship.

The mild dilation of the seminiferous tubule's lumen, reduced number of spermatogenic cells, and widened interstitial tissue are common pathological changes in this organ of mice after exposure to copper and microplastics [31]. In this experiment, the reproductive tissues of mice were observed through H&E staining, and it was found that the structure of the organ in the control group was intact, the seminiferous tubules were arranged neatly, and the layers of spermatogenic cells were distinct, reflecting the normal spermatogenic process. However, both the $CuSO_4$ group and the microplastic group showed varying degrees of tissue damage, such as atrophy of the seminiferous tubules, vacuolation, and shedding of spermatogenic cells. In contrast, the combined exposure group showed more obvious tissue lesions, in addition to the above changes, there were also disordered structures of the spermatogenic epithelium and local disruption of continuity, indicating that the spermatogenic function was significantly affected. Previous studies have shown that copper ions can promote the production of reactive oxygen species (ROS) and induce mitochondrial damage, thereby causing degenerative changes in reproductive tissues. While microplastics, as exogenous particles, can induce inflammatory responses and exacerbate oxidative stress levels [17]. Abduallah et al.[32] further demonstrated that metallic nanocomposites induce testicular oxidative stress, spermatogenic tubule degeneration, and epithelial detachment, suggesting that particulate pollutants may converge on oxidative stress pathways to damage testicular tissue. Additionally, Ijaz et al.[33] documented Caspase-3 activation and seminiferous tubule disruption following acrylamide exposure, while stigmasterol-mediated alleviation of PS-MP nephrotoxicity implicated the NF-κB inflammatory pathway and Bax/Caspase-3 apoptotic cascade in microplastic-induced tissue injury. Collectively, these reports suggest that under co-exposure conditions, $CuSO_4$ and PS-MPs may amplify testicular damage through the convergence of oxidative stress and inflammatory signaling pathways, rather than through isolated, independent toxic actions.

Apoptosis is one of the important pathological processes caused by environmental exposure leading to damage to

the reproductive system. Spermatogenic cells are more sensitive to oxidative stress, inflammatory responses, and barrier damage, so the level of apoptosis is often used as an indicator to evaluate reproductive toxicity [19]. The TUNEL staining results of this study showed that positive apoptotic signals appeared in the $CuSO_4$ group, microplastic group, and combined exposure group. Among them, the positive cell number in the combined exposure group was the most obvious, reflecting that the programmed cell death level of spermatogenic cells further increased under the condition of combined exposure. Further protein expression detection results showed that compared with the control group, the pro-apoptotic related proteins Bax, Caspase-3, and p53 in the combined exposure group were significantly upregulated, while the anti-apoptotic protein Bcl-2 expression decreased, indicating that the cell death-related signals in the testicular tissue were further activated. These expression patterns are consistent with the canonical intrinsic apoptotic pathway: p53 activation transcriptionally induces Bax, which oligomerizes on the mitochondrial outer membrane to form pores, triggering cytochrome c release and subsequent Caspase-3 activation, while Bcl-2 downregulation alleviates its inhibitory constraint on mitochondrial permeabilization. Studies on the toxicity of environmental toxins to the testis have also observed similar molecular expression patterns: oxidative stress activates the p53 pathway, which then upregulates pro-apoptotic proteins and inhibits the anti-apoptotic protein Bcl-2, ultimately leading to the activation of effect caspase and causing spermatogenic cell apoptosis [34]. Previous studies have shown that microplastics can promote the death of reproductive cells by inducing oxidative stress and lipid peroxidation [35], and copper homeostasis disorder may also be involved in the process of spermogenesis cell death [36]. Mechanically, oxidative stress can upregulate Bax and inhibit Bcl-2 through the p53-mediated pathway, thereby causing a decrease in mitochondrial membrane potential and the release of cytochrome C, activating the Caspase cascade reaction, and ultimately leading to reproductive cell apoptosis and testicular tissue damage [37,38]. Given the central role of oxidative stress in the reproductive toxicity of copper and microplastics, the study by El-Sayed et al.[39] on the alleviation of thiamethoxam toxicity by vitamin C suggests that antioxidant intervention may provide a potential strategy for reducing the reproductive damage caused by such environmental pollutants.

Several methodological and interpretive limitations of this study should be acknowledged. First, the sample size (n = 6 per group) is relatively modest, which may have limited the statistical power to detect subtle inter-group differences and increases the risk of type II errors. Second, although oxidative stress was repeatedly invoked as the putative underlying mechanism, the absence of direct oxidative stress biomarkers (e.g., MDA, SOD, CAT, GPx, and GSH) precludes definitive mechanistic conclusions and represents a significant gap that future studies should address. Third, no formal interaction analysis -such as two-way ANOVA interaction terms or combination index calculations- was conducted to statistically discriminate between additive, synergistic, or antagonistic effects. Therefore, descriptions implying synergistic interaction should be interpreted cautiously, and the observed exacerbation may reflect additive toxicity rather than true synergism. Fourth, the 30-day exposure duration may be insufficient to capture delayed or chronic reproductive sequelae, and the lack of a dose-response assessment limits the environmental relevance and extrapolation of these findings.

In conclusion, this study demonstrates that individual exposure to $CuSO_4$ or PS-MPs induces spermatogenic cell apoptosis and compromises sperm quality, whereas combined exposure exacerbates these adverse outcomes. The co-exposure-mediated enhancement of apoptotic responses is associated with activation of the p53-Bax/Bcl-2-Caspase-3 signaling axis, indicative of mitochondrial-dependent apoptotic pathway engagement. These findings provide experimental evidence that complex environmental pollutant mixtures pose enhanced risks to male reproductive health through the convergent activation of pro-apoptotic signaling cascades. Future investigations should incorporate larger cohorts, comprehensive oxidative stress profiling, formal interaction analyses, and extended exposure durations to substantiate the mechanisms underlying $CuSO_4$ and PS-MP co-toxicity and to inform the development of targeted antioxidant intervention strategies.

Declarations

Availability of Data and Materials: Data and materials are available from the corresponding author (ZC) upon reasonable request.

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