RESEARCH ARTICLE

Combined effect of polystyrene nanoplastic and di-n-butyl phthalate on testicular health of male Swiss albino mice: analysis of sperm-related parameters and potential toxic effects

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Abstract

Plastics, especially polystyrene nanoplastic particles (PSNPs), are known for their durability and absorption properties, allowing them to interact with environmental pollutants such as di-n-butyl phthalate (DBP). Previous research has highlighted the potential of these particles as carriers for various pollutants, emphasizing the need to understand their environmental impact comprehensively. This study focuses on the subchronic exposure of male Swiss albino mice to PSNP and DBP, aiming to investigate their reproductive toxicity between these pollutants in mammalian models. The primary objective of this study is to examine the reproductive toxicity resulting from simultaneous exposure to PSNP and DBP in male Swiss albino mice. The study aims to analyze sperm parameters, measure antioxidant enzyme activity, and conduct histopathological and morphometric examinations of the testis. By investigating the individual and combined effects of PSNP and DBP, the study seeks to gain insights into their impact on the reproductive profile of male mice, emphasizing potential synergistic interactions between these environmental pollutants. Male Swiss albino mice were subjected to subchronic exposure (60 days) of PSNP (0.2 mg/m, 50 nm size) and DBP (900 mg/kg bw), both individually and in combination. Various parameters, including sperm parameters, antioxidant enzyme activity, histopathological changes, and morphometric characteristics of the testis, were evaluated. The Johnsen scoring system and histomorphometric parameters were employed for a comprehensive assessment of spermatogenesis and testicular structure. The study revealed non-lethal effects within the tested doses of PSNP and DBP alone and in combination, showing reductions in body weight gain and testis weight compared to the control. Individual exposures and the combination group exhibited adverse effects on sperm parameters, with the combination exposure demonstrating more severe outcomes. Structural abnormalities, including vascular congestion, Leydig cell hyperplasia, and the extensive congestion in tunica albuginea along with both ST and Leydig cell damage, were observed in the testis, underscoring the reproductive toxicity potential of PSNP and DBP. The Johnsen scoring system and histomorphometric parameters confirmed these findings, providing interconnected results aligning with observed structural abnormalities. The study concludes that simultaneous exposure to PSNP and DBP induces reproductive toxicity in male Swiss albino mice. The combination of these environmental pollutants leads to more severe disruptions in sperm parameters, testicular structure, and antioxidant defense mechanisms compared to individual exposures. The findings emphasize the importance of understanding the interactive mechanisms between different environmental pollutants and their collective impact on male reproductive health. The use of the Johnsen scoring system and histomorphometric parameters provides a comprehensive evaluation of spermatogenesis and testicular structure, contributing valuable insights to the field of environmental toxicology.

Keywords Combined toxicity · DBP · Histological observation · Morphometric analysis · PSNP · Sperm parameters

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Introduction

Plastic production has surged in recent years, reaching an annual output of 335 million tons from 1.5 million tons (Alimba and Faggio 2019). In 2010, 192 countries produced 275 million metric tonnes of plastic waste (Jambeck et al. 2015). Over time, plastic waste breaks down into

microplastic (MPs; 5 mm to 5 μm) and nanoplastic (NPs; 1 to 1000 nm) due to various activities (Da Costa Filho et al. 2021; Molenaar et al. 2021). The small size and large surface area of micro- and nanoparticles contribute to diverse applications, raising concerns about increased human exposure through inhalation, ingestion, and dermal absorption (Altammar 2023). Exposure to micro- and nanomaterials poses potential hazards to human health and the environment (Yee et al. 2021). Microplastics and nanoplastics, such as polyethylene terephthalate (PET), polystyrene (PS), polyethylene (PE), and polyvinyl chloride (PVC), have been detected in various environments and common food items, presenting oral ingestion as the primary exposure route (Prata et al. 2020). These particles have been found in biological tissues and organs of aquatic and terrestrial organisms, leading to detrimental effects such as decreased feeding activities, intestinal barrier failure, impaired health, inflammation, oxidative damage, reproductive effects, neurotoxicity, and energy imbalance (Corinaldesi et al. 2021; Massier et al. 2021; Jankowska-Kieltyka et al. 2021; Jewett et al. 2022; Koelmans et al. 2022).

Recent studies found the effects of polystyrene microplastics on marine medaka and frogs' reproductive systems, impacting oysters fertilization and sperm quality (Wang et al. 2019; Wei et al. 2022). In mice and rats, these microplastics cause testicular inflammation, reduced testosterone, and impaired sperm quality (Amereh et al. 2020; Jin et al. 2021). Smaller size polystyrene nanoplastics (PSNPs) also affected the male reproductive system by impacting testosterone levels in mice (Jin et al. 2022).

Microplastic and nanoplastic particles have high surface areas and can act as carriers for various contaminants, including hydrophobic organic chemicals, pathogens, and heavy metals (Lin et al. 2019; Lins et al. 2022; Xie et al. 2023). Among the contaminants of concern, phthalates, commonly used additives in plastics, have attracted attention. Dibutyl phthalate (DBP), a prevalent phthalate, can easily diffuse from plastics into the environment and has been detected in air, soil, water bodies, vegetables, and packaged foods (Sakhi et al. 2014; Zhang et al. 2019; Ai et al. 2021; Feng et al. 2021; Zhou et al. 2021). DBP is classified as an endocrine-disrupting chemical (EDC) by the European Chemical Agency (ECHA) due to its ability to disrupt hormonal balance. DBP's reproductive impacts on males include developmental problems such as hypospadias, poor sperm motility, cryptorchidism, and spermatogenesis dysfunction (Chu et al. 2013; Porras et al. 2014; Kirti et al. 2023).

Coexposure to micro- and nanoplastics and pollutants has been also found to result in hazardous outcomes, although the specific effects can vary. For instance, coexposure to microplastics and cadmium or phthalates yielded different responses in blue mussels compared to individual

contaminant exposure (Magara et al. 2018; Zhang et al. 2020). Studies on the coexposure of polystyrene microplastics and DBP are limited, but evidence suggests the generation of phytotoxicity in red lettuce crop plants due to coexposure (Gao et al. 2021). Recently, a study even reported antagonistic effects between these contaminants in marine species (*Chlorella pyrenoidosa*) (Li et al. 2020a, b).

The accumulation of PSNP (50 nm) is already well reported in different studies conducted (Xu et al. 2023; Zhou et al. 2022; Contino et al. 2023). As a large amount of literature confirmed that NPs of this diameter range can easily cross the blood-testis barrier, we postulated to find reprotoxic effects of PSNP combined with a major environmental pollutant, DBP.

Given the limited understanding of the combined effects of nanoplastics and DBP, it is crucial to investigate the potential consequences of their co-exposure to the male reproductive system. While research has been conducted on the risk of coexposure to contaminants like plastic particles, the specific focus on polystyrene nanoplastics and DBP is lacking. Furthermore, it remains unclear if plastic particles could enhance toxicity when they are co-occurring with other contaminants.

Our study focuses on the effects of DBP and polystyrene nanoplastic particle exposure, both individually and in combination, on the male reproductive system of Swiss albino mice. We aim to provide insights into how these pollutants, alone and in combination, affect sperm quality, biochemical parameters, and testicular pathology. By investigating the combined impact of these contaminants, we hope to shed light on their potential interactions and provide valuable insights for further research and risk assessment.

Material and method

Chemicals

DBP (Cat. No. 84–74-2, 99% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA). PSNPs with a size of 50 nm were purchased from Kisker, Germany (cat#PPS-0.050). The PSNPs were supplied as a 2.5% suspension in deionized water. Stock suspension had mass concentrations of 25 mg/mL (w/v). Throughout the investigation, the particle suspensions were maintained at 4 °C. All other chemicals and solvents used in this study were of analytical reagent grades.

Experimental animals

Adult Swiss albino mice (*Mus musculus*) were obtained from the Indian Veterinary Research Institute, Izetnagar Bareli UP. The animals were housed in a well-ventilated

vivarium at a temperature of around 29 ± 2 °C (relative humidity 33–40%) with natural light and dark cycles. The animals were kept on standard mice feed procured from Hindustan Level Ltd., New Delhi, India, and housed in propylene cages with wood shavings evenly distributed on the floor along with drinking water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee, and all the experiments were carried out according to the guidelines of the CPCSEA (Committee for Control and Supervision of Experiments on Animals, Government of India, New Delhi). The protocol of the study was approved by the Institutional Animal Ethics Committee (IAEC), CPCSEA Regd No: 1689/PO/Re/S/13/CPCSEA. All the animals were acclimated for 2 weeks before initiating the studies.

Preparation of PSNP suspensions and characterization

Nanoplastic suspensions were freshly prepared by adding predetermined volumes of 50 nm PSNP to distilled water (pH 6.9 ± 0.1) to achieve 0.2 mg/mL NPs. The dosage of PSNP was chosen based on a previous study conducted (Sharma et al. 2023).

Experimental design

Male Swiss albino mice aged 10 weeks (30–35 g) were selected and exposed to DBP at a dosage of 900 mg/kg bw (1/20 LD50) and PSNP (0.2 mg/mL of 50 nm size) alone and in combination via oral gavage for a sub-chronic period of 60 days. The body weight of the animals was recorded twice a week during the experiment.

The dosage of DBP in this study was selected based on a previous study that focused on the effects of DBP on sperm parameters (Hao et al. 2013) and to further understand the individual and combined effect of DBP on the reproductive profile of Male Swiss albino mice (Hao et al. 2013). PSNP doses have been selected according to our previous published work results (Sharma et al. 2023), and study reporting the accumulation and toxicity of microplastics in various tissues and organs (Deng et al. 2017).

The mice in each group (I–IV, $n = 6$) were given the following treatments (Table 1):

GROUP I: Control group (olive oil). Animals were administered 100 μl/mouse of olive oil through oral gavage once a day for 60 days.
GROUP II: DBP Group. Animals were administered 900 mg/kg bw DBP (1/20 of LD50) through oral gavage once a day for 60 days (Hao et al. 2013).
GROUP III: Polystyrene nanoplastic particles (PSNPs) group. Animals were administered 0.2 mg/mL of polystyrene nanoplastic particles with a diameter of 50 nm through oral gavage once a day for 60 days (Deng et al. 2017).
GROUP IV: DBP + polystyrene nanoplastic particles. Animals were given DBP (900mg/kg bw of DBP) and polystyrene nanoplastic particles (0.2 mg/mL, 50 nm) through oral gavage once a day for 60 days, with a half-hour gap between the administrations.

At the end of the experiment, the animals were anesthetized with ketamine (60 mg/kg, i.p., Themis Medicare Ltd., India) and xylazine (10 mg/kg, i.p., Indian Immunologicals Ltd., India) followed by cervical dislocation. Both the testes and cauda epididymis were removed and weighed. Cauda epididymis from each mouse was processed for the sperm count, sperm motility, and sperm morphology parameters. From one set of animals, the testes from each animal were processed for biochemical assay and histological observations.

Sperm analysis

Sperm motility and density

A 100-mg sample of cauda epididymis was minced with a sharp razor blade and suspended in 2.0 mL of normal saline (0.9% NaCl, 37 °C) to assess sperm motility and density (sperm counts). The suspension was filtered through a nylon mesh to separate the sperm from the tissue. A drop of the well-mixed sample was placed under a cover slip in Neubauer's counting chamber (Prasad et al. 1972).

Table 1 Outline of animal distribution used in the study according to experimental groups

Group	Treatment	Mode of exposure	Duration of exposure	Dose	Reference
Group I	Control group (olive oil)	Via oral gavage	60 days	100 μL/mouse of olive oil	Hao et al. (2013)
Group II	DBP			900 mg/kg bw DBP (1/20 of LD50)	Hao et al. (2013)
Group III	PSNP			0.2 mg/mL (50 nm diameter)	Sharma et al. (2023)
Group IV	DBP + PSNP			900 mg/kg bw DBP + 0.2 mg/mL PSNP	Hao et al. (2013), Sharma et al. (2023)

Quantitative motility was calculated as a percentage by counting both mobile and immobile spermatozoa in distinct regions using a light microscope at a magnification of $\times 100$. The standard procedure was used to count the sperm, which was quantified as a million mL^{-1} of suspension (Prasad et al. 1972).

Sperm viability

To assess sperm viability, the study followed the guidelines provided in the WHO Laboratory Manual. A sample of 100 mg of cauda epididymal tissue was taken and mixed with 2 mL of physiological saline. Next, 50 μL of the sperm suspension was combined with 100 mL of eosin stain. After a 30-s incubation period, an equal volume of Nigrosin stain was added. The stained sperm were then examined, and approximately 200 sperm were observed and analyzed to determine the percentage of live and dead cells. Live cells appeared colorless, while dead cells were identified by their red or pink coloration.

Sperm abnormality

The percentage of sperm abnormality in the cauda epididymis was calculated using the protocol described by Wyrobek et al. (1983). A 100-mg sample of cauda epididymal tissue was dissolved in 2 mL of saline. The sperm suspension was smeared onto albumin-greased glass slides. The slides were stained for 45 min with 1% eosin Y stain. The abnormalities were observed in 200 sperm/slides.

Biochemical assessment

The testis of the animals was washed in cold isotonic saline and further processed for the estimation of antioxidant activity.

Lipid peroxidation activity in testicular tissues

Thiobarbituric acid reactive substances (TBARS) were estimated as a measure of malondialdehyde (MDA) formation, following the method described by Ohkawa et al. (1979).

CAT, SOD, and GSH activity in testicular tissues

The activity of catalase (CAT) in the testis tissue was assessed spectrophotometrically, using hydrogen peroxide (H_2O_2) as the substrate following the method of Luck (1965). The superoxide dismutase (SOD) assay was performed by the pyrogallol autoxidation method, as described by Marklund and Marklund (1974). The activity of glutathione peroxidase (GSH) was measured according to the procedure suggested by Moron et al. (1979).

Histopathological assessment and morphometric analysis of testicular cells

Staining For histopathological observations, the testicles were removed, washed in normal saline, fixed in 10% neutral buffered formalin (Ellenburg et al. 2020), dehydrated in a graded series of alcohol, and thoroughly cleared in xylene. The tissue was sectioned at a thickness of 5 μm using a microtome (Leica VT 1200 Model), fixed in paraffin, and stained with hematoxylin and eosin (H & E). A light microscope (Nikon Eclipse E200) fitted with a Nikon DS-Fi1 digital camera was used to examine the sections and capture photomicrographs for histological examination. (Gamble 2008; Sharma et al. 2023).

Scoring criteria for histopathology

A semiquantitative scoring system was employed to assess histopathological observations in different experimental groups. The scoring system aimed to evaluate various histopathological parameters, including vascular congestion, Leydig cell hyperplasia, tubular edema, thinning and thickening of tubular lumen, hyalinization, vacuolization, tumor presence, and structural abnormalities in seminiferous tubules (S.T.). The scores were assigned based on the severity of each parameter, with higher scores indicating more pronounced effects. The scoring criteria were as follows: no observation (0), mild (1), moderate (2), and severe (3). These scores were then used to quantitatively represent the histopathological changes observed in each experimental group, facilitating a comprehensive analysis of the effects of the studied compounds on testicular morphology.

Morphometric analysis Quantification of Leydig cells, seminiferous tubule wall length and diameter, and width of germ cells showing nuclear and cytoplasmic staining in the testicular seminiferous tubule regions was performed using Image J software. The data are presented as mean $\pm$ SEM, and distinct superscripts within the identical row depict significant variances ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Johnsen's scoring The testicular tissue was evaluated using a light microscope photomicrograph. Histological assessment of testicular damage and spermatogenesis utilizing Johnsen's mean testicular biopsy score (MTBS) (Johnsen 1970) was performed. Under 400c magnification, ten tubules were evaluated for each animal. Each tubule received a score from 1 to 10 based on the presence or absence of germ cell types in the testicular seminiferous tubules, including spermatozoa, spermatids, spermatocytes, spermatogonia, germ cells, and Sertoli cells to assess histology. A higher Johnsen score indicates a better status of spermatogenesis, while a lower score suggests more severe dysfunction. A score of 1 implies no epithelial

maturation, considered for tubules with complete inactivity, whereas a score of 10 signifies full epithelial maturation, considered for tubules with maximum activity (Table 3).

Statistical analysis

The results were analyzed using GraphPad Prism 8.4.0. The data set was subjected to a one-way analysis of variance (ANOVA) and a post hoc test (Tukey's multiple comparisons tests) (Sharma et al. 2014, 2023). Johnsen score data were analyzed by Kruskal–Wallis followed by Dunn's post hoc test. The data are represented as mean + SEM, and differences were considered significant when $*p < 0.05$, highly significant when $**p < 0.01$, and very highly significant when $***p < 0.001$.

Results

Characterization of PSNP particles

The size of the particle was confirmed by a scanning electron microscope (SEM). The hydrodynamic diameter (dh) and zeta potential of PSNP were characterized using a particle size analyzer (Malvern Panalytical) (Fig. 1).

Body Weight and Testis Weight

Mice administered DBP, PSNP alone, and in combination for 60 days showed a marked decrease in body weight gain in all the treated groups as compared to the control group (Fig. 2a). The effect was found to be significant in the DBP-treated group ($p < 0.05$). However, the weight of the testis was not much affected in DBP- and PSNP alone–treated groups as compared to the control. However, we observed a significant decrease in
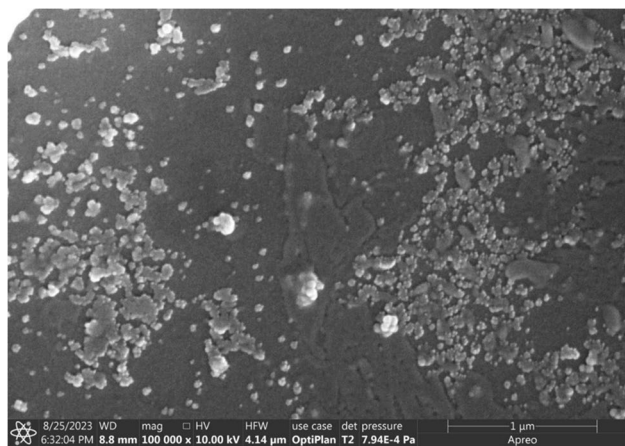

Fig. 1 Representative scanning electron microscopy image of polystyrene nanoparticle experimental design

the weight of the testis in the animals treated with both DBP and PSNP in the combination DBP+PSNP ($p < 0.05$)–treated group as compared to the control group (Fig. 2b).

Effects of DBP, PSNP alone, and in combination on the sperm parameters

The treatment of mice with DBP and PSNP alone at 900 mg/kg bw and 0.2 mg/mL dosage respectively resulted in a significant decrease ($p < 0.05$; $p < 0.01$; $p < 0.001$) in sperm motility, sperm count, and sperm viability after 60 days exposure. Moreover, a highly significant decrease in sperm motility (Fig. 3a), sperm count (Fig. 3b), and sperm viability (Fig. 3c; $p < 0.001$) was observed when exposed to the DBP+PSNP combination group as compared to their individual treated and control group.

Abnormal sperm percentages were accessed in all the treated groups to observe the sperm morphology. In comparison to the control group, mice that were exposed to DBP ($p < 0.01$) and PSNP ($p < 0.05$), individually, had more sperms with abnormal structure, while mice who received DBP+PSNP ($p < 0.001$) in combination had greater sperm malformation incidences (Fig. 2d). The abnormalities of sperm primarily occurred in the sperm head, midsection, or tail and were characterized by the following changes like a banana head, detached head, double head, headless sperm, head folded on itself, amorphous head, double head attached, bent in midpiece, stump, and short tail, bent tail, and coiled tail (Fig. 3, Fig. 4).

Effects of DBP, PSNP alone, and in combination on the activity of antioxidant enzymes and oxidative stress of testicular tissue

Effect on catalase activity

CAT activity showed a significant decrease in DBP ($p < 0.05$) and PSNP ($p < 0.05$) groups as compared to control. The decrease was also found to be significant in the DBP+PSNP combination group ($p < 0.05$) as compared to the control (Fig. 5a).

Effect on SOD activity

A decrease in the SOD activity in the testis was observed in DBP, PSNP alone, and in combination-treated groups. However, the decrease was significant in PSNP alone ($p < 0.01$) and DBP+PSNP ($p < 0.01$) combination groups as compared to the control (Fig. 5b).

Effect on GSH activity

A decrease in the GSH activity of the testis was observed in the PSNP alone group ($p < 0.05$). Moreover, the

Fig. 2 Effect of DBP and PSNP sub-chronic (60 days) exposure alone and in combination on body weight (**a**) and weight of testis (**b**) of Swiss albino mice. Data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. Values representing mean ± SEM bearing different superscripts in the same rows differ significantly ($p < 0.05$). DBP, dibutyl-n-phthalate; PSNP, polystyrene nanoplastic
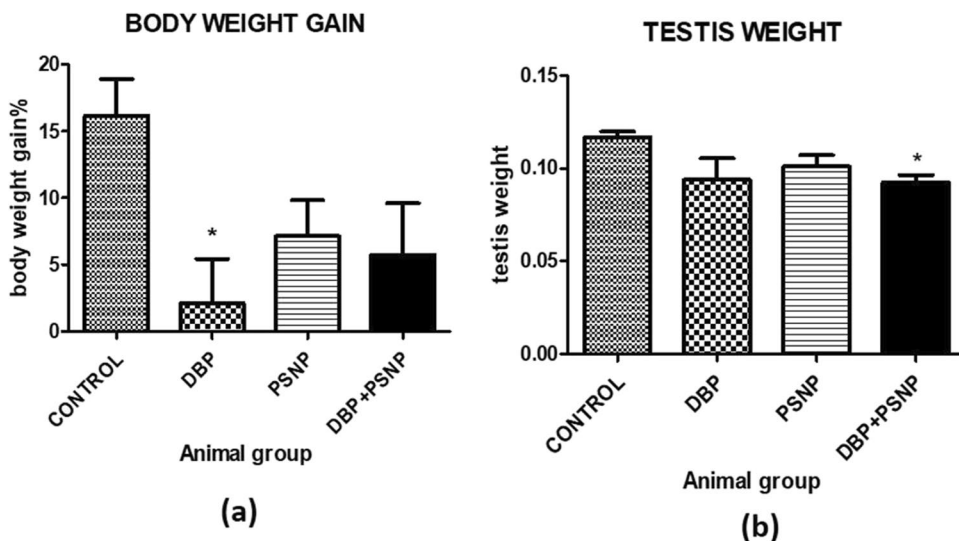

Fig. 3 Effect of DBP and PSNP alone and in combination sub-chronic exposure (60 days) in Swiss albino mice on sperm parameters; sperm motility (**a**), sperm count (**b**), sperm viability (**c**), sperm abnormality (**d**). Data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. Values represent mean ± SEM bearing different superscripts in the same rows differ significantly (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). $n = 6$
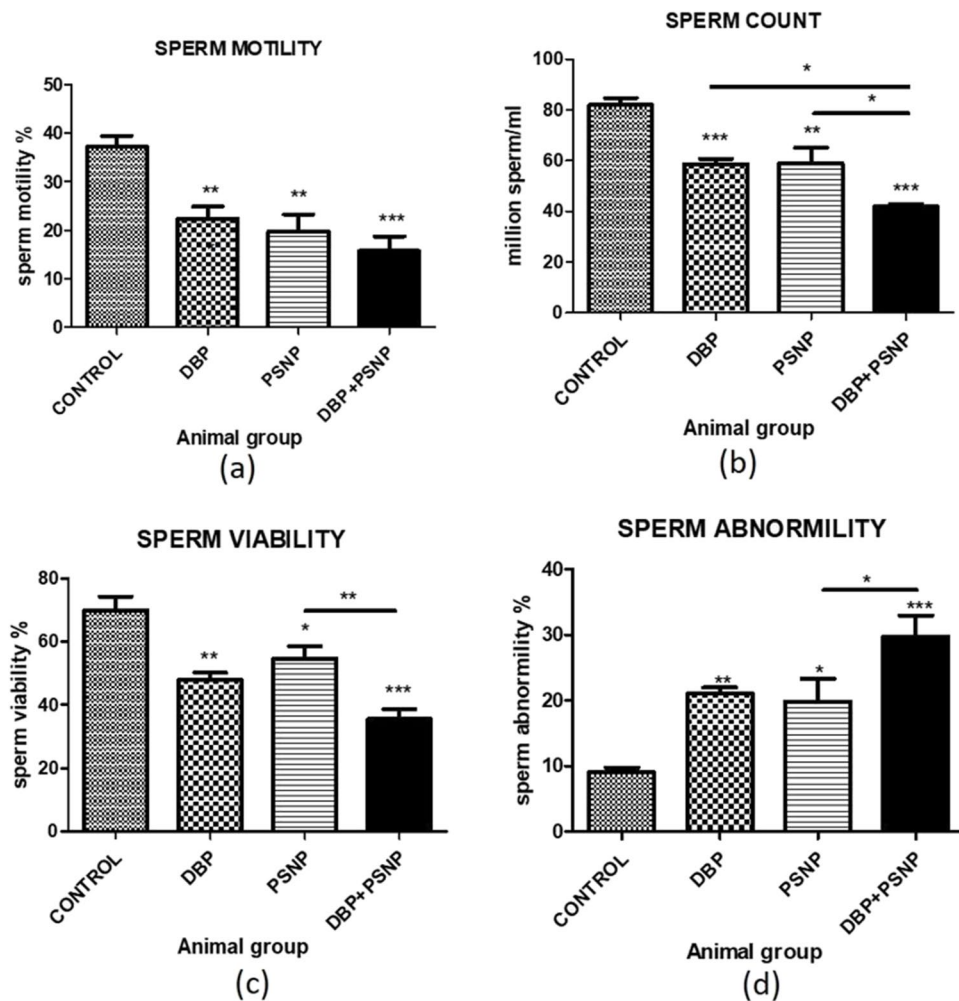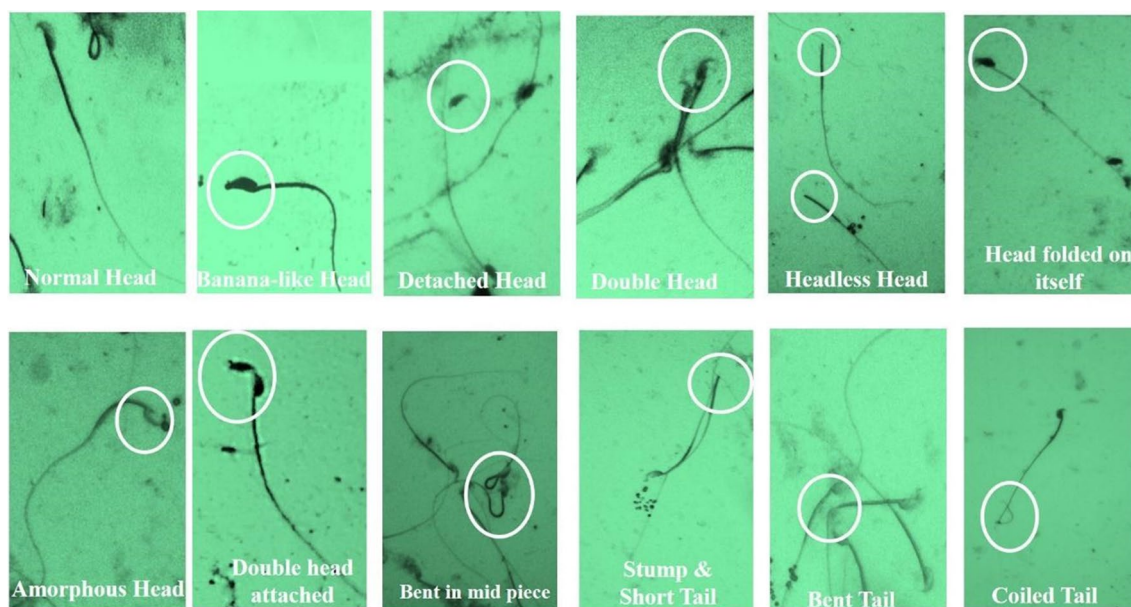
Fig. 4 Histopathological abnormalities in sperms primarily occurred in the sperm head, midsection, or tail and were characterized by the following changes like banana head, detached head, double head, headless sperm, head folded on itself, amorphous head, double head attached, bent in midpiece, stump, and short tail, bent tail, and coiled tail (HE×100). Scale bar = 100 μm. N per group: control (6), DBP (6), PSNP (6) DBP + PSNP (6)

decrease was highly significant in the DBP + PSNP ($p < 0.01$) combination group as compared to the control (Fig. 5c).

Effect on LPO activity

DBP ($p < 0.001$)-, PSNP alone ($p < 0.001$)–, and in combination ($p < 0.001$)–induced oxidative stress was noticeable by the highly significant increased lipid peroxidation activity measured in the testis (Fig. 5d) as compared to the control group.

Histopathological changes in mice testis

Histological examinations of the control testis (Fig. 6a, b, c, d) showed no histopathological alterations. However, mice exposed to DBP and PSNPs, both individually and combined, showed disruptions in testicular tissue, including Leydig cell hyperplasia and vascular congestion with thinning of tubule lumen and vacuolization (Fig. 7a, b, c, d). Moreover, exposure to PSNP led to the extensive congestion in tunica albuginea along with both ST and Leydig cell damage (Fig. 8a, b, c, d). The combined exposure of DBP and PSNP exacerbated these effects, causing vascular congestion, tubular edema, and reduced sperm development (Fig. 9a, b, c, d) (Table 2).

Histomorphometric analysis

Histological evaluation (Johnsen's score) of seminiferous tubules in treated mice GraphPad Prism 8.4.0 was used to depict the Johnsen scores of seminiferous tubule cross-sections in both the normal and treated groups. The results indicated a decrease in all treated groups. Specifically, a significant decrease was observed in the DBP ($p < 0.05$), while a substantial increase was noted in the combination group (Fig. 10, Fig. 11; $p < 0.001$) (Table 3).

Leydig cell count A decrease was observed in all treated groups, with a particularly significant reduction in the number of Leydig cells compared with the control (Fig. 10a; $p < 0.01$).

Seminiferous tubule wall length There was found to be a significant decrease in tubule wall length in all the treated groups compared with the control (Fig. 10b; $p < 0.001$; $p < 0.01$; $p < 0.001$).

Seminiferous tubule diameter There was found to be a very highly significant decrease in the tubule diameter in all the treated groups compared with the control (Fig. 10c; $p < 0.001$; $p < 0.001$; $p < 0.001$).

Width of germ cell layers There was found to be a highly significant decrease in the the germ cell width in the combination groups compared with the control (Fig. 10d; $p < 0.001$; $p < 0.001$; $p < 0.001$).

Fig. 5 Effect of DBP and PSNP alone and in combination sub-chronic exposure (60 days) in Swiss albino mice on catalase (CAT; Fig. 4a), superoxide dismutase (SOD; **b**), glutathione peroxidase (GSH; **c**), lipid peroxidation (LPO; **d**) of testis. DBP, dibutyl-n-phthalate; PSNP, polystyrene nanoplastic. Data were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison test. Values represent mean ± SEM bearing different superscripts in the same rows differ significantly (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). $N = 6$
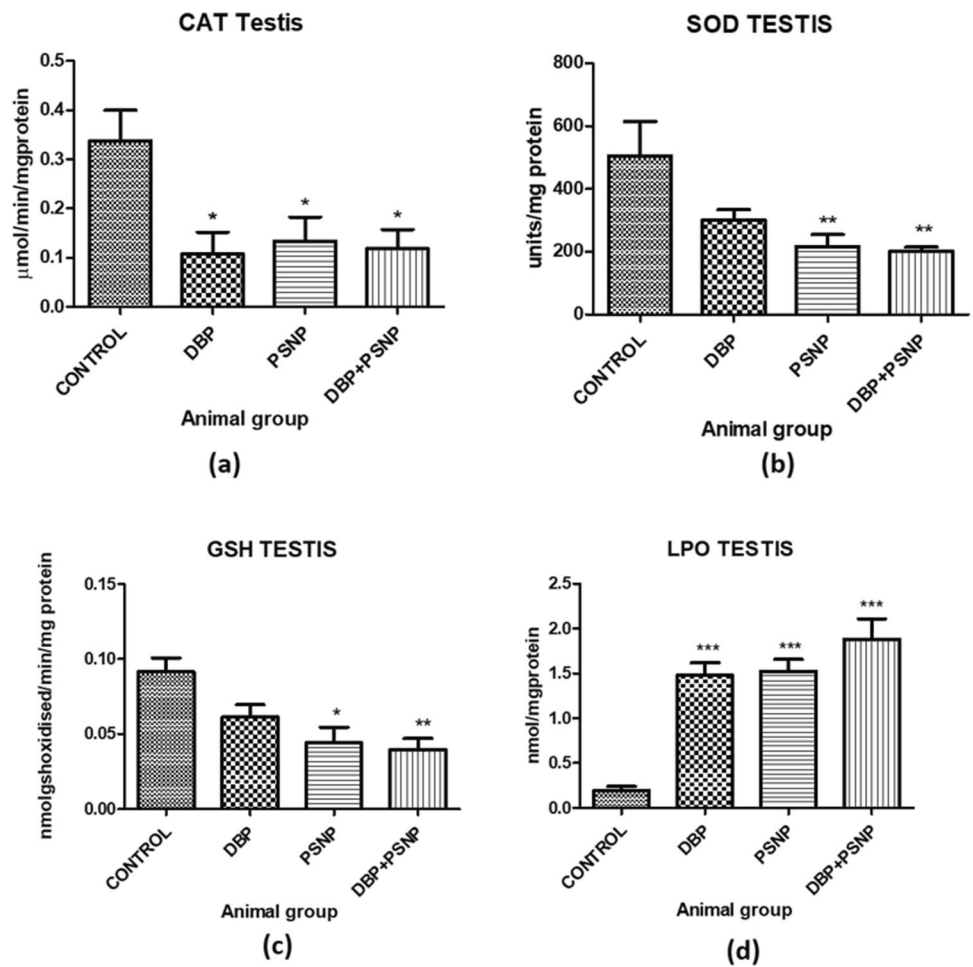
Fig. 6 Histopathological evaluation (**a**, **c**) shows normal histological observations of testis in the control group. **a**, **c** × 400 and **b**, **d** (HE × 100). Scale bar = 100 μm and 1000 μm. N per group: control (6)
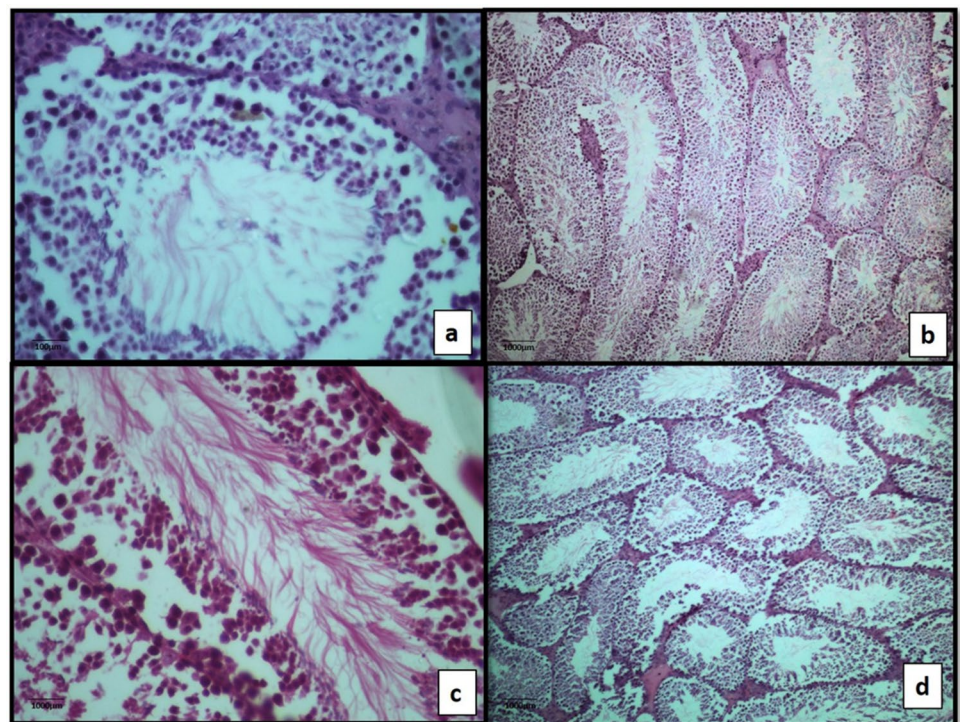
Fig. 7 Histopathological evaluation of testis in DBP-treated group shows vascular congestion and slight Leydig cell hyperplasia (**a**; arrow); thinning of tubule lumen (**b**; H). Thickening and hyalinization of the interstitial septa (arrowhead) and widening of the interstitial septa with extensive ST and Leydig cell damage (**d**; arrowhead) with vacuolization (**c**; v). **a**, **c** ×400 and **b**, **d** (HE×100). Scale bar = 100 μm and 1000 μm. N per group: DBP (6)
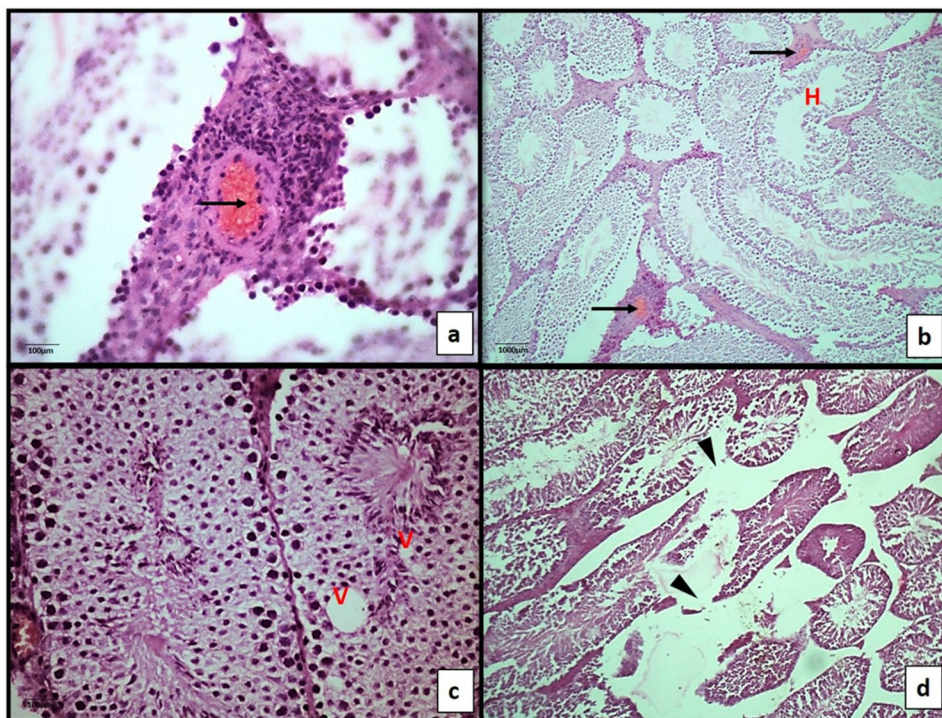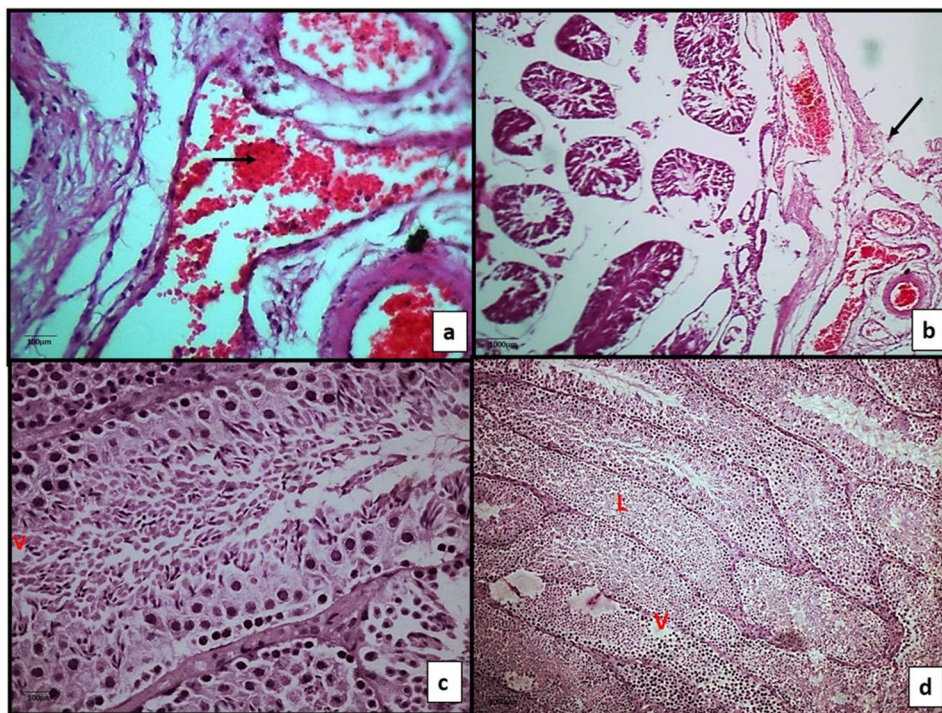

Fig. 8 Histopathological evaluation of the testis in PSNP-treated group shows an extensive congestion in tunica albuginea along with both ST and Leydig cell damage (**a**, **b**; arrow); obstruction of tubules lumen (**d**; L) with vacuolization (**c**; V). **a**, **c** ×400 and **b**, **d** (HE×100). Scale bar = 100 μm & 1000 μm. N per group: PSNP(6)

Discussion

Plastics, particularly PSNPs, exhibit durability and absorption properties, enabling interaction with pollutants such as DBP in the environment (Law and Thompson 2014). The adsorption and release processes of DBP on plastic particles underscore their potential as carriers of various pollutants (Barus et al. 2021), emphasizing the importance of acquiring a comprehensive understanding of their environmental impact.

Fig. 9 Histopathological evaluation of testis in DBP + PSNP–treated group shows vascular congestion with tubular edema (**c, d**; arrowhead); thickening of tubule lumen (**b**; L) with vacuolization (V) and rupturing of seminiferous tubule (arrow). **a, c** ×400 and **b, d** (HE×100). Scale bar = 100 μm and 1000 μm. N per group: DBP + PSNP (6)
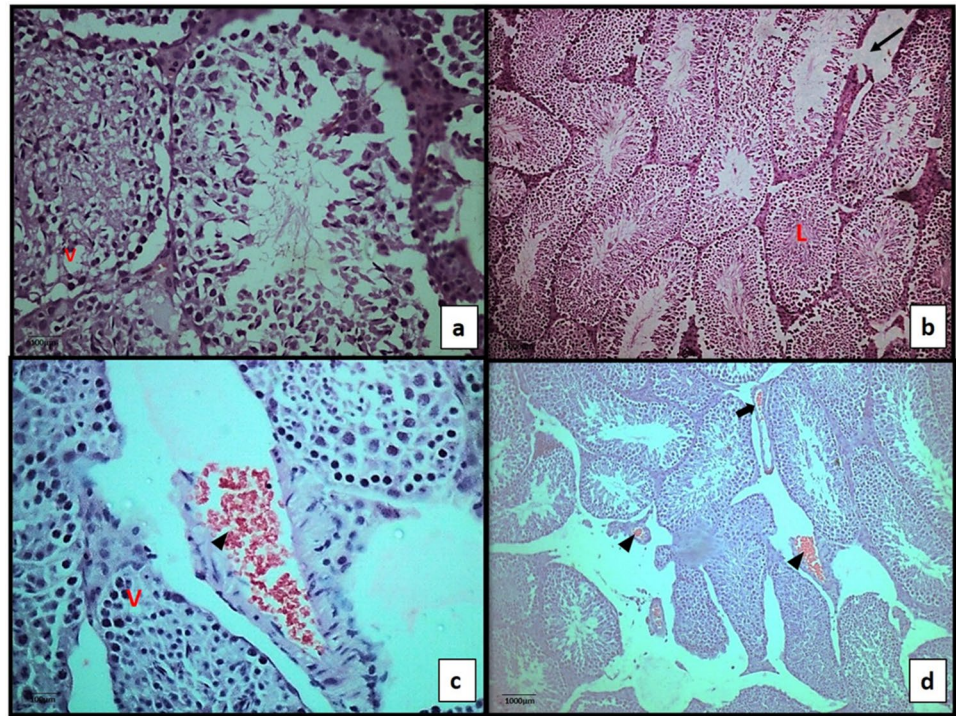

Table 2 Scoring criteria for testicular histopathological observations represent the increasing severity of the observed change as no observation (0); mild (1); moderate (2); and severe (3)

Groups	Morphological observations								
	Vascular congestion	Leydig cell hyperplasia	Tubular edema	Tubule lumen		Hyalinization	Vacuolization	Tumor	Structural abnormalities in S.T
				Thinning of tubular lumen	Thickening of tubular lumen				
CONTROL	0	0	0	0	0	0	0	0	0
DBP	3	1	1	2	1	2	3	0	3
PSNP	1	0	0	3	1	0	3	2	2
DBP + PSNP	2	1	2	3	3	1	3	1	3

In this study, our objective was to examine the reproductive toxicity resulting from simultaneous exposure to PSNP and DBP in male Swiss albino mice. The toxicity was assessed by analyzing sperm parameters in the cauda epididymis, measuring antioxidant enzyme activity, and conducting a histopathological and morphometric examination of the testis. The primary objective was to gain insights into how DBP and PSNP, both individually and in combination, affect the reproductive profile of male Swiss albino mice.

No mortality was observed in mice exposed to PSNP (0.2 mg/mL, 50-nm size) and DBP (900 mg/kg bw), indicating non-lethal effects within tested doses. However, all treated groups showed reductions in body weight gain and testis weight compared to the control group. The decline in body weight gain may be linked to disturbances

in metabolic, hormonal, and inflammatory processes, potentially exacerbated by the synergistic effects of PSNP and DBP (Farhana and Rehman 2023). The combination group displayed a significant decrease in testis weight, contrary to previous studies (Li et al. 2022). The additive effects observed in alterations of testis weight suggest that the combination of these pollutants can lead to more substantial changes in reproductive parameters compared to PSNP and DBP individual exposures (Hussain et al. 2023). Individual exposure to DBP and PSNP also resulted in a decrease in testis weight, consistent with previous research highlighting the adverse impact of DBP on body weight gain. This supports the notion that reductions in body weight and organ weight can serve as reliable indicators of toxicity (Singh and Lata 2015; Qin et al. 2017).

Fig. 10 Effect of DBP and PSNP alone and in combination sub-chronic exposure (60 days) in Swiss albino mice on Leydig cell count (**a**); seminiferous tubule wall length (**b**); seminiferous tubule diameter (**c**); width of germ cell layers. DBP, dibutyl-n-phthalate; PSNP, polystyrene nanoplastic. Data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. Values that represent mean ± SEM bearing different superscripts in the same rows differ significantly (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). $n = 6$
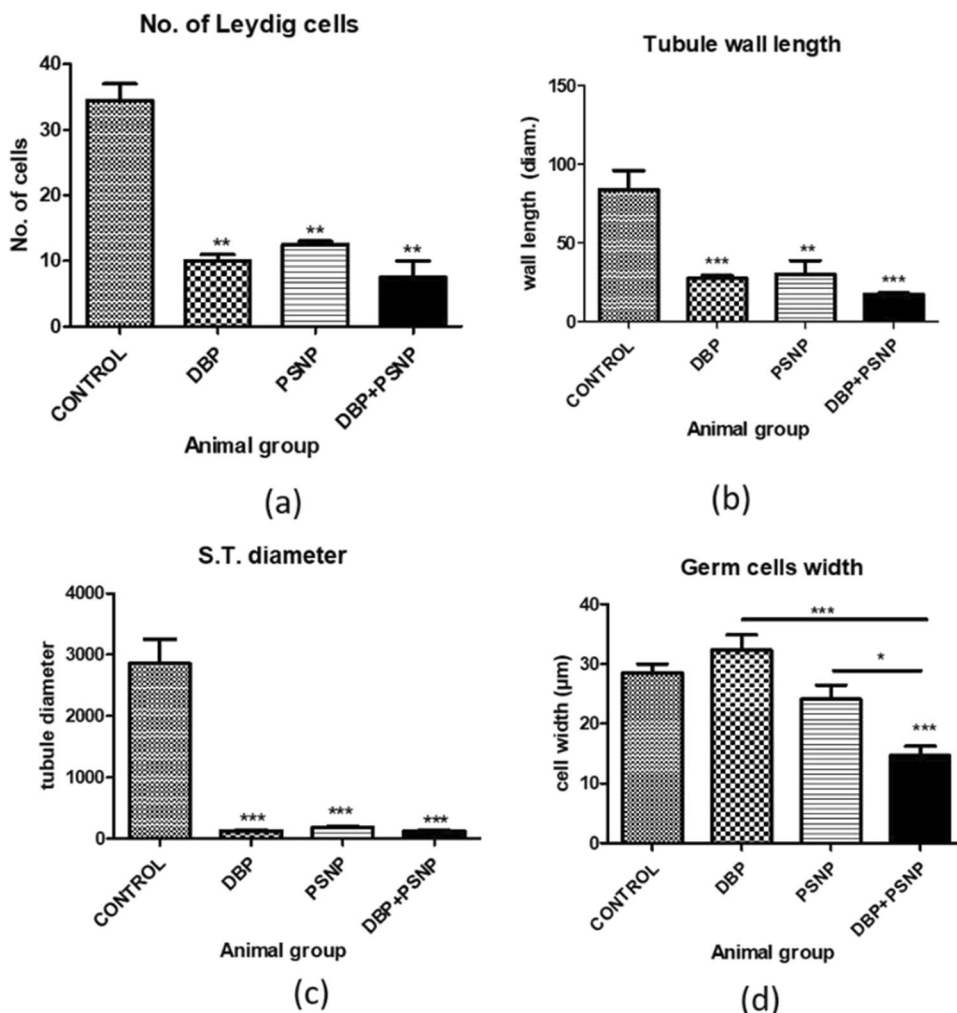
This study extends our understanding of the reproductive impacts of nanoplastic and microplastic exposure by investigating the effects of PSNP and DBP on sperm quality in Swiss albino mice. Individual exposures to PSNP and DBP at 900 mg/kg bw and 0.2 mg/mL dosage, respectively, resulted in a significant decrease in sperm motility, sperm count, and sperm viability after 60 days. Interestingly, combined exposure demonstrated a highly significant decrease in sperm parameters, revealing synergistic effects between these chemicals. Co-exposure intensified adverse effects on sperm physiology compared to individual exposure, suggesting an interactive mechanism amplifying their detrimental impact on sperm quality.

In addition to the functional impacts on sperm, our study revealed significant histopathological defects under experimental conditions. Abnormalities observed in the treated group, such as banana-shaped heads, detached heads, double heads, headless sperm, and various tail abnormalities, could hinder fertilization and impact sperm vitality and motility, aligning with findings reported by Alabi and Bakare (2011). Simultaneous exposure to both DBP and PSNPs amplified

adverse effects on sperm morphology, indicating a synergistic effect that exacerbates the disruption of normal sperm structure.

The underlying mechanisms involve interference with sperm development and maturation processes. DBP disrupts Sertoli cell functioning, leading to abnormalities in sperm structure (Xie et al. 2022). Additionally, the accumulation of nanoplastics, such as PSNP, directly impacts germ cells, disrupting crucial cellular processes and resulting in histopathological abnormalities (Wang et al. 2018). These findings underscore the importance of considering combined exposures and provide insights into the potential mechanisms through which PSNP and DBP exert their adverse effects on sperm quality, with implications for reproductive health and environmental risk assessment.

Li et al. (2022) and other researchers have also previously demonstrated the accumulation of nanoplastic and microplastics in the testis, causing mitochondrial damage and disrupting spermatogenesis (Xu et al. 2021). DBP, a phthalate, disrupts the blood-testis barrier (BTB) and Sertoli cell functioning, leading to harmful substance entry, germ cell
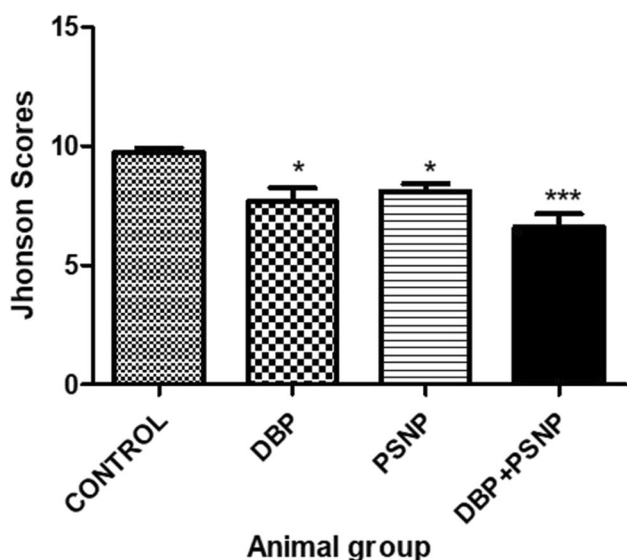
Fig. 11 Effect of DBP and PSNP alone and in combination sub-chronic exposure (60 days) in Swiss albino mice on Johnsen's score of seminiferous tubule cross-sections. DBP, dibutyl-n-phthalate; PSNP, polystyrene nanoplastic. Data were analyzed by Kruskal–Wallis followed by Dunn's post hoc test. Values that represent mean ± SEM bearing different superscripts in the same rows differ significantly ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). $n = 6$

enzymes (SOD, CAT, GSH), GSH, and lipid peroxidation (LPO) significantly decreased in testicular tissues after exposure to DBP and PSNP alone or combined, indicating disruption in the testis' antioxidant defense system (Asadi et al. 2017; Pizzino et al. 2017).

Research consistently highlights the intricate relationship between oxidative stress and adverse reproductive outcomes. Studies have demonstrated a compelling association between increased oxidative stress markers within the testicular microenvironment and impaired sperm function (Asadi et al. 2017). For instance, investigations by Agarwal et al. (2014) and Aitken et al. (2016) have elucidated how increased levels of ROS can lead to oxidative damage to sperm DNA, membrane, and proteins, consequently compromising sperm motility, morphology, and fertilization capacity (Mannucci et al. 2022). These findings underscore the critical role of oxidative stress in influencing key parameters of sperm quality. Moreover, Hussain et al. (2023) delve into the mechanistic aspects of oxidative stress-induced sperm dysfunction, providing valuable insights into the molecular pathways that link oxidative stress to reproductive impairments. Therefore, our study's focus on oxidative stress parameters in the evaluation of the reproductive effects of DBP and PSNP aligns with the well-established literature connecting oxidative stress to compromised sperm function and overall reproductive health.

The histopathological findings in this study provide valuable insights into the reproductive toxicity induced by DBP and PSNPs, both individually and in combination, in mammals. The ability of DBP and PSNP to disrupt the male reproductive system stems from their classification as endocrine-disrupting chemicals (EDCs). As demonstrated in our study, these substances, individually and combined, adversely affect the testis, a pivotal male reproductive organ responsible for spermatogenesis and testosterone synthesis (Kallsten et al. 2022).

Germ, Leydig, and Sertoli cells, crucial for sperm production, were impacted by exposure to PSNP and DBP. Sertoli cells play a vital role in nourishing germ cells and

loss, and apoptosis (Kallsten et al. 2022). Higher phthalate levels correlate with lower sperm quality (Jenardhanan et al. 2016). Increased microplastic accumulation in testicular tissues persists and causes damage to germ cells, interfering with normal spermatogenesis processes (Deng et al. 2020). The easy access of nanoscale plastics to the bloodstream facilitates their distribution throughout the body, potentially affecting various organs, including the testis (Leslie et al. 2022).

This study found that exposure to DBP and PSNP, either alone or in combination, induces oxidative stress in mouse testes, marked by an imbalance between reactive oxygen species (ROS) and antioxidants, leading to cellular damage (Pizzino et al. 2017). Activities of major antioxidant

Table 3 Histological classification of seminiferous tubule according to Johnsen's scoring system

Johnson score	Description
10	Complete spermatogenesis and perfect tubules
9	Many spermatozoa present but disorganized spermatogenesis
8	Only a few spermatozoa present
7	No spermatozoa but many spermatids present
6	Only a few spermatids present
5	No spermatozoa or spermatids present but many spermatocytes present
4	Only a few spermatocytes present
3	Only spermatogonia present
2	No germ cell present
1	Neither germ cells nor Sertoli cells present

contributing to the development of the blood-testis barrier, while Leydig cells are responsible for testosterone production, essential for spermatogenesis (Svechnikov et al. 2010). Exposure to PSNP negatively affected male reproduction in mice, causing abnormalities in spermatogenesis and disruption of the blood-testis barrier. Being EDCs, these substances interfere with hormonal regulation, particularly testosterone and follicle-stimulating hormones (FSH), crucial for proper spermatogenesis.

Our examination of testicular morphology in mice exposed to DBP and PSNP, individually and in combination, raised concerning implications for physiological processes. Leydig cell hyperplasia and vascular congestion indicate abnormal blood accumulation within testicular vessels, potentially hindering proper oxygen and nutrient distribution (Sheweita et al. 2016; Al-Zubi et al. 2022). Thinning of the tubular lumen suggests reduced space for spermatozoa development, potentially disrupting sperm maturation and production (Sharma et al. 2018). Hyalinization signifies structural damage, and disarrangement of spermatogonial layers with vacuolization suggests cellular damage or death (Sheweita et al. 2016).

Extensive congestion and damage to Leydig cells and seminiferous tubules in response to PSNP exposure signify substantial adverse effects on testicular morphology and function. The congestion observed in the tunica albuginea suggests abnormal blood vessel accumulation, potentially hindering proper blood flow and compromising overall testicular health. Damage to Leydig cells, responsible for testosterone production, implies a potential disruption in hormonal balance, affecting spermatogenesis and reproductive function. Concurrent damage to seminiferous tubules indicates a direct impact on sperm development, potentially impairing sperm production and quality (Titi-Lartey and Khan 2023.

Structural abnormalities within the testicular tubules contribute to the complexity of these tumors, with exfoliation of germinal layers, thickening of the lumen, and vacuolization indicating disruptions in normal testicular architecture (Cai et al. 2016; Sharma et al. 2018). These structural irregularities implicate potential disturbances in spermatogenesis and highlight the overall impact of PSNP-induced tumors on testicular tissue integrity.

The synergy between DBP and PSNP exacerbated disruptions, revealing vascular congestion, and implying impaired blood circulation affecting oxygen and nutrient supply to testicular cells. Tubular edema indicates inflammation or tissue damage, disrupting normal testicular structure and function (Creasy et al. 2012). Thickening of the tubule lumen suggests altered conditions for sperm development, possibly reducing sperm production and quality. Vacuolization denotes cell damage or death, and the rupturing of seminiferous tubules signifies trauma, potentially leading to infertility.

These findings underscore the complex interplay of DBP and PSNP in compromising the structural and functional integrity of the testis, highlighting the urgent need for comprehensive assessments of the reproductive risks associated with nanoplastic exposure.

The morphometric analysis conducted in this study, encompassing Johnsen score, Leydig cell count, seminiferous tubule wall length, and diameter, as well as the width of the germ cell layer, provides valuable insights into the quantitative aspects of testicular morphology and function in response to DBP and PSNP exposure.

The Johnsen scoring system employed to evaluate the histological structure and spermatogenesis revealed distinct alterations in seminiferous tubules across control and treated groups. Scores of 10, 9, and 8, indicating active spermatogenesis, showed a few to many spermatozoa present in the seminiferous tubules. Scores 6 and 7 denoted tubules with no spermatozoa but spermatids, while scores 4 and 5 indicated the absence of spermatozoa or spermatids but the presence of spermatocytes.

The observed decrease in Johnsen scores and alterations in spermatogenic cells' distribution indicate that exposure to DBP and PSNP disrupts spermatogenesis by interfering with germ cell development and differentiation (Franca et al. 2016). The reduction in Leydig cell count signifies disrupted testosterone production, a known effect of DBP, crucial for spermatogenesis and overall testicular function (Robaire et al. 2020).

Structural alterations in seminiferous tubules, reflected in decreased wall length and diameter, suggest histopathological changes induced by DBP and potentially exacerbated by PSNP (Sertoli2018). The significant decrease in germ cell layer width, particularly in the combination group, implies disruptions in layers crucial for spermatogenesis, influenced by the combined impact of DBP and PSNP (Wang et al. 2021).

These mechanisms collectively contribute to the observed quantitative alterations in testicular morphology and function, highlighting the complex interplay of DBP and PSNP in compromising male reproductive health. Understanding these mechanisms is crucial for developing targeted interventions and strategies to mitigate the reproductive risks associated with environmental exposures.

Conclusion

In conclusion, this comprehensive study sheds light on the intricate and detrimental reproductive effects resulting from exposure to di-n-butyl phthalate (DBP) and polystyrene nanoplastics (PSNPs) in male Swiss albino mice. The findings underscore the urgency of understanding the combined impact of these environmental pollutants, given their

ubiquitous presence and potential interactive effects. The study reveals that while individual exposures to DBP and PSNP induce adverse effects on sperm quality, morphology, and testicular structure, their combined exposure leads to synergistic effects, exacerbating reproductive impairments. The examination of oxidative stress parameters further emphasizes the link between environmental exposures and compromised sperm function, aligning with established literature. Importantly, the histopathological and morphometric analyses provide valuable insights into the structural and functional alterations induced by DBP and PSNP, offering a quantitative understanding of testicular morphology and function. The emergence of extensive congestion in tunica albuginea along with both ST and Leydig cell damage following PSNP exposure raises significant concerns about the long-term impact of nanoplastic exposure on reproductive health. Overall, this study contributes to the growing body of evidence highlighting the complex interplay of environmental pollutants in compromising male reproductive health and emphasizes the need for targeted interventions and risk mitigation strategies.

Future recommendation

The future research entails prioritizing long-term and chronic exposure studies to uncover cumulative effects, along with mechanistic investigations to unravel the cellular and molecular pathways responsible for reproductive toxicity, establishing dose–response relationships, specific impact of DBP and PSNP on reproductive hormones, including testosterone and FSH, and investigating potential epigenetic effects on reproductive genes.

Abbreviations *NPs*: Nanoplastics; *MPs*: Microplastics; *DBP*: Di-n-butyl-phthalate; *PSNP*: Polystyrene nanoplastic; *PET*: Polyethylene terephthalate; *PS*: Polystyrene; *PE*: Polyethylene; *PVC*: Polyvinyl chloride; *EDC*: Endocrine-disrupting chemical; *ECHA*: European Chemical Agency; *CPCSEA*: Committee for the Purpose of Control and Supervision of Experiments on Animals; *IAEC*: Institutional Animal Ethics Committee; *dh*: Hydrodynamic diameter; *TBARS*: Thiobarbituric acid reactive substances; *MDA*: Malondialdehyde; *LPO*: Lipid peroxidation; *CAT*: Catalase; *SOD*: Superoxide dismutase; *GSH*: Glutathione peroxidase; *ANOVA*: One-way analysis of variance; *ROS*: Reactive oxygen species; *FSH*: Follicle-stimulating hormones; *MTBS*: Mean testicular biopsy score

Author contribution All the authors have contributed towards this paper. Material preparation, data collection, and analysis were conducted by Kirti Sharma. The first draft of the manuscript was written by Kirti Sharma and Anju Sharma and critically reviewed by Anju Sharma and Pradeep Bhatnagar. All authors read and approved the final manuscript.

Data availability The data used to support the findings of this study are included in this article.

Declarations

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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