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Impact of Polystyrene Microplastics and Bisphenol A Co-Exposure on Epididymal Sperm Maturation, Fertility, and Apoptotic Alterations in Rats

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Running Title: PS-MPs and BPA Co-exposure Impairs Epididymal Function and Fertility

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Abstract

Exposure to polystyrene microplastics (PS-MPs) and Bisphenol A (BPA) has emerged as a major concern for male reproductive health; however, their combined effects on epididymal function remain poorly understood. The present study investigated the impact of PS-MPs and BPA, individually and in combination, on epididymal structure, biochemistry, sperm maturation, and fertility in adult Wistar rats. Animals were exposed orally to PS-MPs (10 or 40 mg/kg/day) with or without BPA (2 μg/kg/day) for 45 consecutive days. Co-exposure significantly reduced epididymal weight and fertility compared with individual exposures. Marked declines in sperm count, motility, and viability, accompanied by increased sperm abnormalities, were observed particularly in co-exposed groups. Histological analysis revealed degeneration of the pseudostratified epididymal epithelium, basal cell damage, epithelial sloughing, and disruption of smooth muscle and connective tissue integrity. Biochemical analysis demonstrated significant depletion of sialic acid, L-carnitine, and neutral α-glucosidase, indicating severe impairment of epididymal secretory and maturational functions. Enhanced Annexin V and Caspase-3 expression further suggested activation of apoptotic pathways in spermatozoa and epididymal epithelial cells. Pearson's correlation analysis revealed strong associations between epididymal biochemical alterations and sperm dysfunction. Collectively, these findings demonstrate that BPA potentiates PS-MP-induced epididymal toxicity, resulting in impaired sperm maturation and compromised male fertility.

Keywords: Polystyrene microplastics, Bisphenol A, Epididymal toxicity, Sperm maturation

Introduction

The testis has traditionally received greater research attention than the epididymis, largely because spermatogenesis and endocrine regulation are central to sperm production. However, the epididymis is increasingly being recognized as indispensable for post-testicular sperm maturation and the acquisition of fertilizing capacity. Despite this, the epididymis has remained comparatively underexplored, and several mechanisms underlying the acquisition of sperm fertilizing ability during post-testicular transit remain inadequately understood (Nie et al. 2024). Functions such as sperm membrane remodelling, maintenance of sperm quiescence in the cauda epididymis, and epithelial regeneration and plasticity under toxicant and/or stress exposure have received comparatively limited attention. Although spermatogenesis is considered a major target of environmental contaminants, resulting in disruption and abnormalities in newly formed spermatozoa, functional impairment during epididymal maturation may also profoundly affect sperm motility, membrane integrity, and fertilizing potential. Therefore, important environmental toxicants should be investigated specifically for their effects on sperm maturation during epididymal transit.

Bisphenol A (BPA) is a widely used plasticizer employed in the manufacture of polycarbonate plastics and epoxy resins. It functions as an endocrine disruptor by interfering with hormonal signalling and reproductive hormone homeostasis and has been associated with declining sperm counts and reproductive dysfunction in both humans and experimental animals, thereby adversely affecting overall fertility (Reyaz et al. 2025). Primarily acting as a synthetic oestrogen agonist and androgen antagonist, BPA interferes with spermatogenic processes (Jambor et al. 2021). Similarly, microplastics have recently been implicated in the induction of oxidative stress and hormonal dysregulation in the male reproductive organs of experimental models (Kumar & Mangla, 2025). Notably, microplastics have also been detected

in human semen, prompting increasing concern regarding their possible contribution to the rising global incidence of male infertility (Guo et al. 2025).

Co-exposure to microplastics and associated environmental contaminants has been reported to severely impair the male reproductive system. Previous studies have suggested that microplastics may act as vectors for toxic substances, thereby amplifying cellular damage through enhanced oxidative stress, inflammation, and testicular apoptosis, often accompanied by reduced testosterone levels (Liu et al. 2023; Xiang et al. 2025). Similarly, combined exposure to BPA and microplastics poses a substantial threat to male reproductive health and frequently results in greater toxicity than exposure to either agent alone. Verzola et al. (2024) reported that co-exposure to BPA and microplastics exacerbated damage to renal tubular cells. Likewise, Xue et al. (2024) demonstrated that combined exposure to BPA and microplastics produced additive reproductive toxicity in adult zebrafish. The authors further observed significant transgenerational effects in the offspring of co-exposed groups, which differed markedly from those observed following exposure to BPA or microplastics alone.

There are multiple studies that have shown individual adversity caused by BPA and MPs in spermatogenesis and steroidogenesis (Jin et al. 2012; Celar Šturm et al. 2025; Jin et al. 2022; Jin et al. 2025). However, the role in epididymal maturation of sperm is limited and there is no study on effect of co-exposure on epididymal function based on the available resources and to the best of our knowledge. The present study an attempt was made to understand exclusive role of co-exposure of BPA and PS-MPs in epididymal function and structural integrity. Apoptosis in cauda epididymal sperm and epididymal epithelium was also evaluated to explore exclusive damages and how it deviates from BPA alone and PS-MPs alone exposure. The current study also attempted to related potential alterations in biochemical milieu of epididymis and its potential role in altering the sperm quality post-testicular maturation.

Materials and methods

Test material

Polystyrene microparticles (PS-MPs; 0% cross-linked; density: 1.05 g/cm^3) with a particle size of 5 µm (SD < 0.1 µm; CV < 2%) were procured from Sigma-Aldrich, Merck. The particles were stored at 4 °C throughout the experimental period. Similarly, Bisphenol A [2,2-bis(4-hydroxyphenyl)propane; $C_{15}H_{16}O_2$; 99% purity] was purchased from Sigma-Aldrich (Missouri, USA).

Experimental animal model

Male Wistar rat of proven fertility, weighing 250-280 g and aged 3-4 months, were used in the present study. The animals were maintained under standard laboratory conditions in an advanced animal facility. A controlled photoperiod of 12 h light:12 h dark was maintained throughout the experimental period, and the animals were housed in polypropylene cages (43 × 27 × 15 cm). The animal room temperature was maintained in accordance with standard laboratory guidelines for animal care and handling. All animals were provided with commercially available pellet diet and drinking water ad libitum.

Ethical approval

Prior approval for the experimental protocol was obtained from the Institutional Animal Ethics Committee (IAEC). The study was conducted in strict accordance with the guidelines of the Indian National Science Academy (INSA) for the care and use of laboratory animals. All experimental procedures were performed under the supervision and regulatory framework of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2010).

Experimental design

To evaluate the effects of microplastics alone and in combination with BPA on reproductive functions, two doses of 5 µm PS-MPs were selected based on previously

established reproductive toxicity studies. Doses of 10 and 40 mg/kg/day, previously used by Lu et al. (2023), were considered as low- and high-dose groups, respectively, due to their ability to induce measurable reproductive alterations without causing severe systemic toxicity. These doses also enabled assessment of dose-dependent responses. Both PS-MP doses were further combined with 2 µg/kg/day of BPA, a dose previously reported to impair spermatogenesis and reproductive functions in male rats (Jin et al. 2012). Importantly, this BPA dose does not induce complete infertility, thereby allowing evaluation of subtle alterations in sperm maturation and epididymal function under chronic exposure conditions. The selected exposure design was also considered relevant for simulating environmentally realistic low-dose exposure scenarios. However, certain limitations should be acknowledged, including the use of only two PS-MP concentrations and a single BPA dose, which may limit broader dose–response interpretation. In addition, the study focused exclusively on 5 µm PS-MPs and may not fully represent the effects of other microplastic sizes, polymer types, or environmentally aged particles.

To evaluate the effects of microplastics on reproductive function, healthy fertile male rats were randomly divided into six experimental groups, each comprising five animals. A group size of five animals was selected in accordance with standard practices followed in preliminary reproductive toxicology studies to ensure adequate statistical comparison while minimizing unnecessary animal use in compliance with ethical guidelines. Group I (control) received olive oil orally as the vehicle in volumes equivalent to those administered in the treatment groups. Groups II and IV were orally administered microplastics alone at doses of 10 and 40 mg/kg/day, respectively, for 45 consecutive days. Groups III and V received combined oral administration of microplastics (10 and 40 mg/kg/day, respectively) together with BPA at a dose of 2 µg/kg/day for the same duration. In the co-exposure groups, both agents were administered in the same volume of olive oil used for the other experimental groups to

maintain dosing consistency. Group VI received BPA alone at 2 µg/kg/day orally for 45 consecutive days and served as the positive control group in the present study (Table 1).

Parameters

Body weight and epididymal weight

Body weights of all animals were recorded at the initiation of the experiment and again at the end of the experimental period. Following completion of the treatment schedule, the primary and accessory reproductive organs were carefully dissected, cleared of adhering tissues, and weighed individually.

Fertility record

Periodic fertility assessments were performed following the commencement of the experimental schedule by cohabitating treated male rats with fertile female rats at a ratio of 1:2. Successful mating was confirmed by the presence of a vaginal plug and/or detection of spermatozoa in vaginal smear examinations.

Cauda epididymal sperm characteristics

Sperm parameters, such as count, motility, viability, and abnormalities, were evaluated according to the guidelines specified in the WHO manual (1999). Samples were obtained by excising a segment of the cauda epididymis and gently disintegrating it in 1 ml of normal saline (pH 7.0).

Cauda epididymal sperm morphology

A portion of cauda epididymal tissue was dissected and agitated gently in normal saline. The sample was washed in distilled water and examined using Papanicolaou staining for morphological observation, as described in the WHO (1999) guidelines.

Epididymal histology

A small segment of the epididymis and seminal vesicle was fixed in 4% paraformaldehyde overnight. The tissues were then dehydrated using ethanol, cleared with xylene, and embedded in paraffin wax. Thin sections of 5 µm were prepared and stained with Harris's hematoxylin and eosin. The stained specimens were subsequently examined under a light microscope at different magnifications.

Epididymal biochemistry

Estimation of sialic acid

Sialic acid levels in the epididymis were estimated using method explained in Svennerholm et al. (1957). Briefly, 2 mL of samples containing 10–30 µg of N-acetylsialic acid or equivalent sialic acids were prepared. Two test tubes received 2 mL of resorcinol reagent, while a third (sample blank) received blank reagent. Standard solutions of 0, 15, and 30 µg of N-acetylsialic acid were also prepared. The tubes were heated at 110°C for 15 minutes, cooled, mixed with 5 mL of amyl alcohol, shaken, and chilled in ice water. After centrifugation, the amyl alcohol phase was transferred to microcells and analyzed at 450 nm and 580 nm using a spectrophotometer. Absorbance values from the blank were subtracted, and N-acetylsialic acid concentrations were calculated using standard curves.

Estimation of L-carnitine

L-Carnitine was determined using the spectrophotometric method described by Marquis and Fritz (1964). A 0.1 mL aliquot of supernatant was added to a reaction medium containing 0.1 mol/L Tris-HCl, 0.05 µmol/L AcCoA, 0.1 µmol/L DTNB, and 0.05 mL CAT (19 U/mL). The mixture was incubated at 37°C for 30 minutes, after which 2 mL of Tris buffer was added. The absorbance was measured at 412 nm using a spectrophotometer. The L-carnitine content in caudal epididymal fluid is expressed as mmol/mg protein.

Estimation of α-glucosidase activity

Neutral α-glucosidase activity in the epididymis was measured using the method described by Wang et al. (1999). The reaction system included 1.2 mL of buffer (69 mmol/L citric acid, pH 6.8), 0.2 mL of p-nitrophenyl-α-D-glucopyranoside (PNPG, 23 mmol/L), and 0.2 mL of supernatant. The reaction mixture was incubated at 37°C for 4 hours, and the reaction was stopped by adding 0.25 mL of Na_2CO_3 (0.1 mol/L). Absorbance was measured at 405 nm using a UV/VIS spectrophotometer (Systronics 108, Ahmedabad, India). PNPG content was quantified using a standard curve, and neutral α-glucosidase activity was expressed as mg PNP/mg protein of tissue.

Assessment of membrane phospholipid flip-flop in cauda epididymal sperms

Membrane phospholipid flip-flop was assessed following the method of Anto et al. (2003) using annexin V–FITC staining according to the manufacturer's protocol. Briefly, cauda epididymal spermatozoa were washed twice with 1× phosphate-buffered saline (PBS) at 1200 rpm for 5 min and resuspended in 1× assay buffer. The sperm suspension was aliquoted into 100 µl samples and incubated with annexin V–FITC conjugate under dark conditions at room temperature. Stained spermatozoa were examined under a non-confocal fluorescence microscope using excitation wavelengths of 480 and 525 nm and emission barrier filters of 535 and 615 nm. Unstained sperm suspensions served as negative controls. Fluorescence images were captured using DigiPro 4.0 (DigiPro, USA) and analysed using ImageJ software (ImageJ, USA).

Immunofluorescence analysis of Caspase-3 expression

HEK-293T cells (1×10^4 cells/well) were seeded on sterile coverslips and allowed to reach 50–60% confluency before treatment with different concentrations of compound 22 for 48 h. Following treatment, cells were washed with 1× PBS and fixed in chilled acetone:methanol (1:1, v/v) for 15 min at -20 °C. Non-specific binding was blocked using 2%

BSA prepared in 0.02% Triton X-100 in 1× PBS for 30 min at 37 °C. Cells were then incubated with primary Caspase-3 antibody (Cell Signalling Technology, MA, USA) for 3 h, followed by DAPI counterstaining. Coverslips were mounted on glass slides and visualized under a fluorescence microscope using excitation/emission wavelengths of 550/570 nm for Caspase-3 and 358/461 nm for DAPI.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons between control and experimental groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's and Dunnett's multiple comparison tests wherever appropriate (MINITAB, Pennsylvania, USA). Statistical significance was evaluated at confidence intervals of 95%, 99%, and 99.99%. Pearson's correlation was applied to evaluate strength of association between biochemical parameters and sperm characteristics using MS Excel (Microsoft, USA).

Results

Body weight gain and epididymal weight

Exposure to PS-MPs, BPA, and their combination produced distinct alterations in body and epididymal weights. Body weight gain across treatment groups ranged from 7-19%, compared with a 16.5% increase in the control group. The highest gain was observed in Group VI following BPA-only exposure (18.91%), followed by Group III treated with low-dose PS-MPs in combination with BPA (17.29%). In contrast, Group V exhibited a significant reduction in body weight gain ($p < 0.001$), showing only a 7.65% increase relative to initial body weight (Table 2).

Similarly, epididymal weight showed a significant decline ($p < 0.05$) in Groups III-VI compared with the control group (74.17 ± 1.24 mg). The greatest reduction was observed in

Group V (66.05 ± 1.28 mg), followed by Group III (68.20 ± 1.34 mg), whereas Groups IV and VI showed comparatively moderate declines (Table 2).

Fertility record

Fertility assessment revealed a decline in the number of conceived females mated with male rats exposed to PS-MPs, BPA, and their combination (Table 3). Among the treatment groups, Group V exhibited the earliest reduction in fertility, with only 90% conception recorded on the 30th day of mating, whereas all other groups maintained 100% fertility at this stage. By the 45^{th} day, all treatment groups showed a 10-30% decline in conception rate compared with the control group, which maintained 100% fertility throughout the experimental period. The greatest reduction was observed in Group V, followed by Group III, while Groups II, IV, and VI each exhibited a comparatively lower decline of 10% (Table 3).

Cauda epididymal sperm characteristics

Cauda epididymal sperm characteristics were significantly affected following exposure to PS-MPs, BPA, and their combination. Sperm count declined in all treatment groups; however, significant reductions ($p < 0.01$) were observed in Groups III, V, and VI, which recorded 35.02 ± 1.29, 18.40 ± 2.19, and 33.40 ± 2.36 million/ml, respectively, compared with 45.17 ± 1.47 million/ml in the control group. The greatest decline (>60%) was observed in Group V, whereas Groups II and IV showed only marginal reductions (Figure 1).

Sperm motility also declined significantly in Groups III-VI, with values of 50.72 ± 3.17%, 58.94 ± 1.96%, 40.04 ± 3.34%, and 48.04 ± 2.41%, respectively, compared with 67.58 ± 2.33% in the control group. Group V exhibited the lowest motility, showing an approximately 40% reduction relative to the control group ($p < 0.01$), whereas Group II remained comparable to controls (Figure 1).

Similarly, sperm viability showed significant reduction in Groups IV-VI, recording 69.98 ± 1.03%, 53.43 ± 4.27%, and 61.43 ± 3.49%, respectively, compared with 82.99 ± 1.69% in controls ($p < 0.01$). Group II remained within the control range, while Group III showed only a moderate reduction in viability despite marked declines in sperm count and motility (Figure 1).

Sperm morphology

Cauda epididymal spermatozoa from PS-MPs- and/or BPA-treated groups exhibited distinct morphological abnormalities. Prominent alterations included retention of cytoplasmic droplets along the tail, coiled tails, defects in the neck and midpiece regions, and broken heads and tails (Figure 2). Among these, cytoplasmic droplet retention and neck-region defects were the most consistently observed abnormalities in stressed groups (Figure 2 B-C, E-F).

The percentage of abnormal spermatozoa increased significantly in all treatment groups irrespective of PS-MP and/or BPA dose. Although the control group (Group I) also exhibited baseline abnormalities (22.95 ± 0.90%), treatment groups showed an additional 4-10% increase in sperm abnormalities. The abnormality percentages recorded in Groups II-VI were 26.16 ± 0.99%, 34.15 ± 4.24%, 31.78 ± 1.14%, 41.43 ± 2.53%, and 33.43 ± 1.37%, respectively. The highest incidence of abnormal spermatozoa was observed in Group V (Figure 2).

Epididymal histology

Histological examination of the epididymis in the control group (Group I; Figure 3) revealed well-organized epididymal tubules lined by characteristic pseudostratified epithelium composed of basal and principal cells. Basal cells appeared cuboidal with elongated nuclei, whereas principal cells were tall and columnar with granular cytoplasm, indicative of active secretory function. The tubular lumen was densely packed with mature spermatozoa, reflecting normal sperm maturation and storage.

Group II exhibited histological features largely comparable to the control group, although mild damage to basal cells and stereocilia was evident (Figure 3). More pronounced alterations were observed in Groups III-V, including shortening and flattening of basal cells, reduced cytoplasmic granularity, and epithelial disorganization (Figure 3 C-E). Group V showed the most severe damage, characterized by focal epithelial sloughing, compromised epithelial integrity, and reduced luminal sperm density, indicating impaired sperm maturation and transport (Figure 3 E). Although Group VI largely retained normal epididymal architecture, subtle reductions in smooth muscle and connective tissue integrity, along with mild alterations in luminal sperm density, were observed (Figure 3 F).

Biochemical evaluation of epididymal tissues

Sialic acid

Biochemical analysis of epididymal tissue revealed significant alterations in sialic acid concentration following exposure to PS-MPs and/or BPA. The control group (Group I) exhibited a sialic acid level of 1.18 ± 0.05 mg/g tissue, whereas Groups II–VI showed reduced concentrations of 0.86 ± 0.04, 0.73 ± 0.10, 0.71 ± 0.12, 0.58 ± 0.12, and 0.82 ± 0.06 mg/g tissue, respectively (Table 4). The greatest decline was observed in Group V ($p < 0.01$). Although intergroup differences among the remaining treatment groups were comparatively less pronounced ($p > 0.05$), all exposed groups exhibited significantly lower sialic acid levels than the control group ($p < 0.05$). Comparable trends were observed between Groups II and VI, as well as between Groups III and IV (Table 4).

L-carnitine

The control group exhibited an L-carnitine concentration of 1.20 ± 0.14 mM/mg protein. Significant reductions ($p < 0.05$) were observed in Groups III and V, with levels declining to 0.77 ± 0.06 and 0.52 ± 0.11 mM/mg protein, respectively. In contrast, Groups II,

IV, and VI showed comparatively moderate reductions, recording concentrations of 0.87 ± 0.05, 1.06 ± 0.06, and 1.02 ± 0.08 mM/mg protein, respectively, which did not differ significantly from the control group (Table 4).

Neutral-α-glucosidase (NAG)

Neutral α-glucosidase (NAG) activity in epididymal tissue showed a significant decline in the experimental groups, particularly in Groups III-VI, compared with the control group (33.60 ± 2.18 mg PNP/mg protein). The greatest reduction was observed in Group V, where NAG activity declined to 17.38 ± 2.13 mg PNP/mg protein ($p < 0.01$). Groups III, IV, and VI also exhibited significantly reduced activities of 20.86 ± 3.28, 23.82 ± 3.39, and 21.18 ± 1.59 mg PNP/mg protein, respectively ($p < 0.05$). Although Group II showed a moderate decrease in NAG activity (26.57 ± 1.67 mg PNP/mg protein), the change was not statistically significant (Table 4).

Annexin V-FITC staining of cauda epididymal sperm

Although Annexin V-positive spermatozoa were observed in all experimental groups, differences in the abundance and distribution of fluorescence were considered for representative group-wise imaging (Figure 4). Sperm samples collected from the cauda epididymis exhibited positive green fluorescence predominantly in the ventral (Figure 4 B), dorsal (Figure 4 F), and apical regions of the sperm head (Figure 4 D-E). In addition, intense Annexin V positivity was also observed in the connecting and mid-piece regions of several spermatozoa (Figure 4 D, F). These expression patterns were evident across all treatment groups but were comparatively more abundant in animals co-exposed to 40 mg PS-MPs and 2 µg BPA (Figure 4 E).

Expression of cleaved caspase-3 in epididymal tissues

Caspase-3 expression in epididymal tissue indicated apoptotic activation characterized by chromatin condensation and structural disruption following exposure to PS-MPs and/or BPA. A marked increase in apoptotic cells was observed in all treatment groups compared with the control group. Co-exposure groups particularly exhibited a higher frequency of Caspase-3-positive principal cells. Notably, exposure to 40 mg PS-MPs alone also induced substantial apoptosis in epididymal tissue, with effects comparatively greater than those observed in the low-dose co-exposure group (10 mg PS-MPs + 2 µg BPA) (Figure 5 A-B). The highest number of Caspase-3-positive basal and principal cells was observed in animals co-exposed to 40 mg PS-MPs and 2 µg BPA (Figure 5 C). In contrast, BPA-alone exposure induced comparatively fewer apoptotic cells, although positive Caspase-3 expression was still evident within the pseudostratified epididymal epithelium (Figure 5 A).

Pearson's correlation tests between sperm characteristics and epididymal biochemistry

Pearson's correlation analysis revealed strong associations between sperm characteristics and epididymal biochemical parameters (Table 4). Among the biochemical markers evaluated, NAG exhibited the strongest correlations with sperm count, motility, viability, and abnormality ($r > 0.800$). Sialic acid and L-carnitine also demonstrated strong correlations with sperm parameters ($0.700 < r < 0.800$), although these associations were comparatively weaker than those observed for NAG. Sperm abnormality showed a strong negative correlation with all three biochemical parameters (Table 4). Overall, these findings suggest that polystyrene microplastic (PS-MP)-induced alterations in epididymal biochemistry may substantially contribute to deterioration in sperm quality.

Discussion

BPA and MPs are independently associated with adverse effects on the male reproductive system. BPA primarily acts as an endocrine disruptor, whereas MPs have been

reported to accumulate within reproductive tissues and semen, leading to deterioration in sperm quality (Kumar & Mangla, 2025). Previous studies have further suggested that BPA may impair epididymal sperm maturation by altering epithelial secretions, thereby affecting sperm motility and membrane stability (Zhang et al. 2023). In contrast, MPs are known to induce oxidative stress and inflammatory responses in epididymal tissue, resulting in disruption of the luminal microenvironment and compromised post-testicular sperm maturation (da Cunha et al. 2025).

Epididymal maturation is essential for the acquisition of sperm motility, membrane stability, and fertilizing ability. During epididymal transit, spermatozoa undergo extensive biochemical and structural modifications mediated by epididymal secretions, proteins, and small regulatory molecules (Cornwall, 2009; Sullivan & Mieusset, 2016). Although previous studies have indicated that both BPA and MPs may alter the epididymal milieu through hormonal dysregulation and tissue damage, studies specifically evaluating the combined effects of MPs and low-dose BPA on sperm characteristics, morphology, fertility, epididymal biochemistry, and histological integrity remain limited. This is particularly important because available evidence suggests that BPA and MPs may impair epididymal function through distinct yet potentially interacting mechanisms.

The present study demonstrated reduced body weight gain in rats co-exposed to 40 mg PS-MPs and 2 μg BPA, whereas the remaining treatment groups largely remained within the control range. Interestingly, the group receiving 10 mg PS-MPs in combination with BPA did not show significant alterations in overall body weight gain, suggesting that lower doses of microplastics may exert differential biological effects. Similar non-linear dose-dependent responses to microplastic exposure have also been reported previously (Boga Pekmezekmek et al. 2025; Li et al. 2025). In contrast, several studies have suggested that low-dose exposure to BPA and microplastics individually may promote weight gain and exhibit obesogenic properties (Huang et al. 2023; Chen et al. 2025).

A significant decline in epididymal weight was observed in rats co-exposed to 40 mg PS-MPs and 2 µg BPA, which corresponded with the reduced body weight gain observed in this group. Notably, the low-dose co-exposure group (10 mg PS-MPs + 2 µg BPA) also exhibited a significant reduction in epididymal weight despite maintaining body weight gain comparable to controls, suggesting a comparatively selective effect on epididymal integrity. Rats exposed to BPA alone also showed reduced epididymal weight, consistent with previous findings reported by Chitra et al. (2003). Similarly, Alsenousy et al. (2026) demonstrated that PS-MP exposure could independently reduce epididymal weight. In the present study, epididymal weight appeared comparatively less affected by individual exposure to PS-MPs but more sensitive to combined exposure with BPA. Both co-exposure groups showed significant reductions in epididymal weight, indicating that BPA may potentiate PS-MP-induced epididymal toxicity in a dose-dependent manner.

The mating study demonstrated an overall decline in fertility across all treatment groups following exposure to PS-MPs and BPA, either individually or in combination, indicating adverse effects on male reproductive competence. As all animals were confirmed to be fully fertile prior to the initiation of the experiment, even a 10% reduction in conception success suggests biologically relevant impairment of fertility potential. Previous studies have similarly reported independent adverse effects of PS-MPs and BPA on fertility in rodents. Ijaz et al. (2021) observed that PS-MP exposure at concentrations ranging from 2 to 2000 µg/l reduced germ cell numbers, sperm count, motility, and viability, thereby compromising overall fertility in rats. Likewise, BPA exposure at doses of 5, 25, and 100 µg/kg body weight has been associated with reduced fertility outcomes in male mice, accompanied by declines in sperm count and reproductive organ weights.

Thus, both PS-MPs and BPA, even when administered individually, exhibited the potential to impair fertility in exposed animals; however, co-exposure substantially intensified

these adverse effects. The present study further demonstrated a dose-dependent decline in fertility among co-exposed groups, with 40 mg/kg PS-MPs producing a greater reduction in fertility than 10 mg/kg PS-MPs when combined with 2 μg/kg BPA. Overall, combined exposure imposed a more pronounced reproductive burden, resulting in an additional 10-20% decline in fertility compared with individual exposures alone.

Analysis of cauda epididymal sperm characteristics further supports the observed decline in fertility and provides insight into the underlying reproductive impairments induced by PS-MPs and BPA. The present study demonstrated marked reductions in sperm count, motility, and viability following exposure to PS-MPs and BPA, indicating disruption of testicular and/or epididymal functions essential for sperm production and maturation.

Interestingly, rats exposed to 10 mg/kg PS-MPs for 45 consecutive days showed only marginal, non-significant reductions in sperm count, motility, and viability compared with controls. Nevertheless, this group exhibited a 10% decline in fertility. It is well recognized that moderate reductions in sperm quality may not immediately impair fertility because of the presence of a biological reserve in male reproductive capacity (Miller & DuTeaux, 2005). However, the significant increase in sperm abnormalities observed even in the low-dose PS-MP group suggests that structural defects in spermatozoa may have contributed more substantially to reduced fertility than declines in sperm number alone.

In contrast, the BPA-only group exhibited significant reductions in sperm count, motility, and viability, which were associated with a comparable decline in fertility. These findings suggest that PS-MPs and BPA may impair male reproductive function through partially distinct mechanisms. PS-MPs appeared to exert a greater influence on sperm morphology, whereas BPA affected both sperm quantity and functional competence. Importantly, combined exposure further aggravated these alterations, indicating a potential

synergistic interaction between PS-MPs and BPA that resulted in greater impairment of sperm quality than exposure to either compound alone.

Papanicolaou (PAP) staining revealed several characteristic sperm abnormalities in PS-MPs- and BPA-treated rats, including broken or malformed heads, abnormal midpieces, twisted or coiled tails, and bulging or disrupted plasma membranes. Previous studies have similarly demonstrated that BPA exposure induces structural abnormalities involving the sperm head, midpiece, and tail. Karnam et al. (2015) reported a high frequency of head and tail defects in spermatozoa following BPA exposure in male rats. Likewise, microplastic exposure has been associated with impaired sperm head and tail morphology, resulting in reduced sperm quality and motility (Ademola et al. 2020; Faisal & Akbarsha, 2021). Collectively, these findings suggest that combined exposure to PS-MPs and BPA may exacerbate sperm morphological defects, thereby contributing to impaired fertility in exposed animals.

The epididymis plays a critical role in sperm maturation, storage, and transport by facilitating the acquisition of motility, viability, and fertilizing capacity through a series of biochemical and structural modifications. Histological examination in the present study revealed consistent damage to basal cells and stereocilia in rats exposed to PS-MPs and BPA. Damage to these structures may compromise the specialized epididymal microenvironment required for sperm maturation and maintenance, thereby contributing to impaired fertility (Arrighi, 2014).

More severe alterations were observed in animals exposed to higher doses and combined PS-MP and BPA treatments. These changes included disruption of smooth muscle layers and reduced sperm density within the epididymal lumen. Co-exposed groups exhibited particularly pronounced basal cell degeneration along with marked smooth muscle disruption. Epididymal smooth muscle is essential for generating peristaltic contractions that facilitate sperm transport from the caput to the cauda epididymis and subsequently into the vas deferens

(Borges et al. 2021). This process is strongly regulated by androgenic signalling, particularly testosterone and dihydrotestosterone. Therefore, reduced testosterone production, likely resulting from previously observed Leydig cell impairment following co-exposure, may have contributed to altered epididymal contractility and sperm transport. Collectively, these findings indicate that combined exposure to PS-MPs and BPA may severely disrupt epididymal structure and function, thereby compromising post-testicular sperm maturation.

Epididymal biochemistry reflects the functional status of the epididymis and its capacity to support sperm maturation, protection, and storage. Alterations in enzymatic activity, protein composition, and the luminal microenvironment may compromise sperm viability and fertilizing potential. Following spermatogenesis, immature spermatozoa are transported from the seminiferous tubules through the rete testis to the epididymis, where they undergo maturation and acquire progressive motility and fertilizing competence. These processes involve extensive biochemical modifications to the sperm membrane and metabolism during epididymal transit, which are essential for successful fertilization (Fraser, 1992).

The present study evaluated three major epididymal biochemical markers; sialic acid, L-carnitine, and NAG, that are closely associated with sperm maturation. A significant decline in sialic acid concentration was observed across all treatment groups. Comparable reductions in the low-dose PS-MP and BPA-only groups suggest that both exposures exerted similar adverse effects on epididymal sialic acid levels. However, the most pronounced depletion occurred in rats co-exposed to 40 mg/kg PS-MPs and 2 µg/kg BPA, where sialic acid levels declined by nearly 50%. As sialic acid and sialoglycoproteins are synthesized and secreted primarily by epididymal epithelial cells (Ma et al. 2016), these findings indicate that combined exposure may impair epithelial secretory activity and disrupt the luminal environment necessary for sperm maturation.

L-carnitine, another critical epididymal metabolite, plays an essential role in mitochondrial fatty acid transport, sperm energy metabolism, motility, viability, and protection against oxidative stress. In the present study, significant reductions in L-carnitine concentration were observed only in co-exposed groups, indicating a likely synergistic interaction between PS-MPs and BPA. Moreover, the decline was dose dependent, with the higher PS-MP co-exposure group exhibiting greater depletion. Reduced L-carnitine availability may therefore contribute to the pronounced decline in sperm motility and viability observed in cauda epididymal spermatozoa. The increased retention of cytoplasmic droplets in sperm tails further supports the possibility of disrupted antioxidative defence mechanisms during epididymal maturation.

Neutral α-glucosidase (NAG), a recognized marker of epididymal function, also showed substantial reduction following PS-MP and BPA exposure, particularly in co-exposed animals. NAG is involved in carbohydrate metabolism and sperm maturation and reflects the functional integrity of epididymal epithelium. Therefore, reduced NAG activity strongly suggests epididymal dysfunction and impaired sperm maturation. The concurrent decline in NAG and L-carnitine levels indicates severe disruption of the epididymal biochemical milieu, which likely contributed to the deterioration in sperm quality and fertility observed in the present study.

Previous studies have reported that PS-MPs and BPA can induce premature sperm capacitation, oxidative stress, DNA damage, and alterations in the epididymal microenvironment (Santiago et al. 2021; da Cunha de Medeiros et al. 2025; Park et al. 2020). Consistent with these observations, the marked depletion of sialic acid, L-carnitine, and NAG in the present study indicates substantial impairment of epididymal sperm maturation, particularly under co-exposure conditions where the adverse effects appeared to be amplified.

The expression of Annexin V in cauda epididymal sperms indicated positive staining in ventral and dorsal section of head, while mid-piece, principal and end pieces also exhibited positive for Annexin V. The phenomenon observed indicated potential induction of early apoptosis. It is also important to note that despite being Annexin V positive sperms may still be motile however considered compromised in quality (Shen et al. 2002). Binding of Annexin V in acrosomal an head region may directly impact the acrosome reaction thus can compromise fertility (Glander & Schaller, 1999). While positive expression at the tail regions correlates with reduced motility which was affirmatively observed in the present study and discussed earlier.

Caspase-3 expression in the epididymis showed increased positive localization in rats exposed to PS-MPs and/or BPA, with co-exposed groups exhibiting markedly higher numbers of positive cells than individually exposed groups. These findings suggest that combined exposure may aggravate apoptotic activity within epididymal tissue. Previous studies have similarly reported that PS-MP exposure can enhance apoptotic signalling through increased Caspase-3 activity in reproductive tissues. For instance, Alsenousy et al. (2026) demonstrated elevated testicular Caspase-3 activity following PS-MP exposure, while Ijaz et al. (2021) associated epididymal histopathological damage with apoptotic activation.

In the present study, the most prominently affected cells were principal cells within the pseudostratified columnar epithelium. As principal cells are the major secretory and absorptive cells of the epididymis, increased apoptosis in these cells may lead to epithelial dysfunction and disruption of the epididymal microenvironment required for sperm maturation. Therefore, intensified Caspase-3 activity in co-exposed groups strongly suggests compromised epididymal function, which may contribute to impaired sperm maturation and reduced fertility (Kempinas & Klinefelter, 2015).

Pearson's correlation analysis demonstrated strong associations between alterations in epididymal biochemistry and sperm characteristics following exposure to PS-MPs and/or BPA. Among the biochemical markers evaluated, NAG exhibited the strongest association with reductions in sperm count, motility, and viability, and also showed a strong negative correlation with sperm abnormalities. This observation is particularly important because principal cells of the pseudostratified columnar epithelium are responsible for the synthesis and secretion of several epididymal proteins and enzymes, including NAG (Zhou et al. 2018). NAG plays a critical role in sperm maturation by participating in membrane glycoprotein remodelling, carbohydrate metabolism, and maintenance of fertilizing capacity (Zhang & Zhou, 2024). Therefore, the marked decline in NAG activity observed particularly in co-exposed groups may be attributed to toxicant-induced apoptosis and degeneration of principal cells, resulting in disruption of the epididymal luminal microenvironment essential for normal sperm maturation.

Conclusion

Co-exposure to PS-MPs and BPA induced pronounced epididymal toxicity and aggravated male reproductive dysfunction compared with individual exposures, with higher doses of PS-MPs producing more severe effects in the presence of a constant BPA dose. The selective reduction in epididymal weight observed in co-exposed groups, together with the marked decline in fertility, suggests a possible synergistic interaction between PS-MPs and BPA. Co-exposure significantly impaired sperm quality by reducing sperm count, motility, and viability while increasing morphological abnormalities. Histopathological alterations, including degeneration of the pseudostratified epididymal epithelium, disruption of smooth muscle and connective tissue integrity, and basal cell damage, were accompanied by substantial depletion of sialic acid, L-carnitine, and NAG, indicating severe impairment of epididymal secretory and maturational functions. In addition, enhanced apoptotic activity in spermatozoa and epididymal principal cells suggests activation of apoptosis as a key mechanism underlying

epididymal dysfunction. Collectively, these findings demonstrate that BPA may potentiate PS-MP-induced epididymal injury, thereby disrupting sperm maturation and compromising male fertility.

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Declaration of competing interest

The authors declare no competing financial interest or personal relationship that could have appeared to influence work reported in this paper.

Data availability

Data will be made available on request.

Author Contributions

Imtiyaz Alam: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft, Writing-review.

S. C. Srivastava: Supervision, Visualization, Review and Editing, Resources.

All authors reviewed and approved the final manuscript.

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Table 1: Body weight and absolute epididymal weight (mean of both left and right) are shown below in Mean±SEM (n=5). The statistical comparisons were made against control and expressed in *p<0.05, **p<0.01, and ***p<0.001.

	Control	*10 mg MPs*	*10 mg MPs+2 µg BPA*	*40 mg MPs*	*40 mg MPs+2 µg BPA*	*2 µg BPA*
Initial Body Weight (g)	261.20±1.16	257.80±2.67	252.20±1.20	262.20±2.78	251.00±1.18	248.00±1.58
Final Body Weight (g)	304.40±3.99	297.80±4.73	295.80±7.12	299.80±4.28	270.20±1.98***	292.80±4.93
Epididymal weight (mg)	74.17±1.24	73.02±1.16	68.20±1.34**	72.24±1.22	66.05±1.28*	70.07±0.49*

Table 2: Fertility record of male rats before and during investigation taken on 15 days interval until the 45th day. Significant reduction in fertility was represented in *10%, **20%, and ***30%.

%Fertility						
	Control	*10 mg MPs*	*10 mg MPs+2 µg BPA*	*40 mg MPs*	*40 mg MPs+2 µg BPA*	*2 µg BPA*
Pre-Treatment	100	100	100	100	100	100
Treatment Phase						
15 days	100	100	100	100	100	100
30 days	100	100	100	100	90*	100
45 days	100	90*	80**	90*	70***	90*

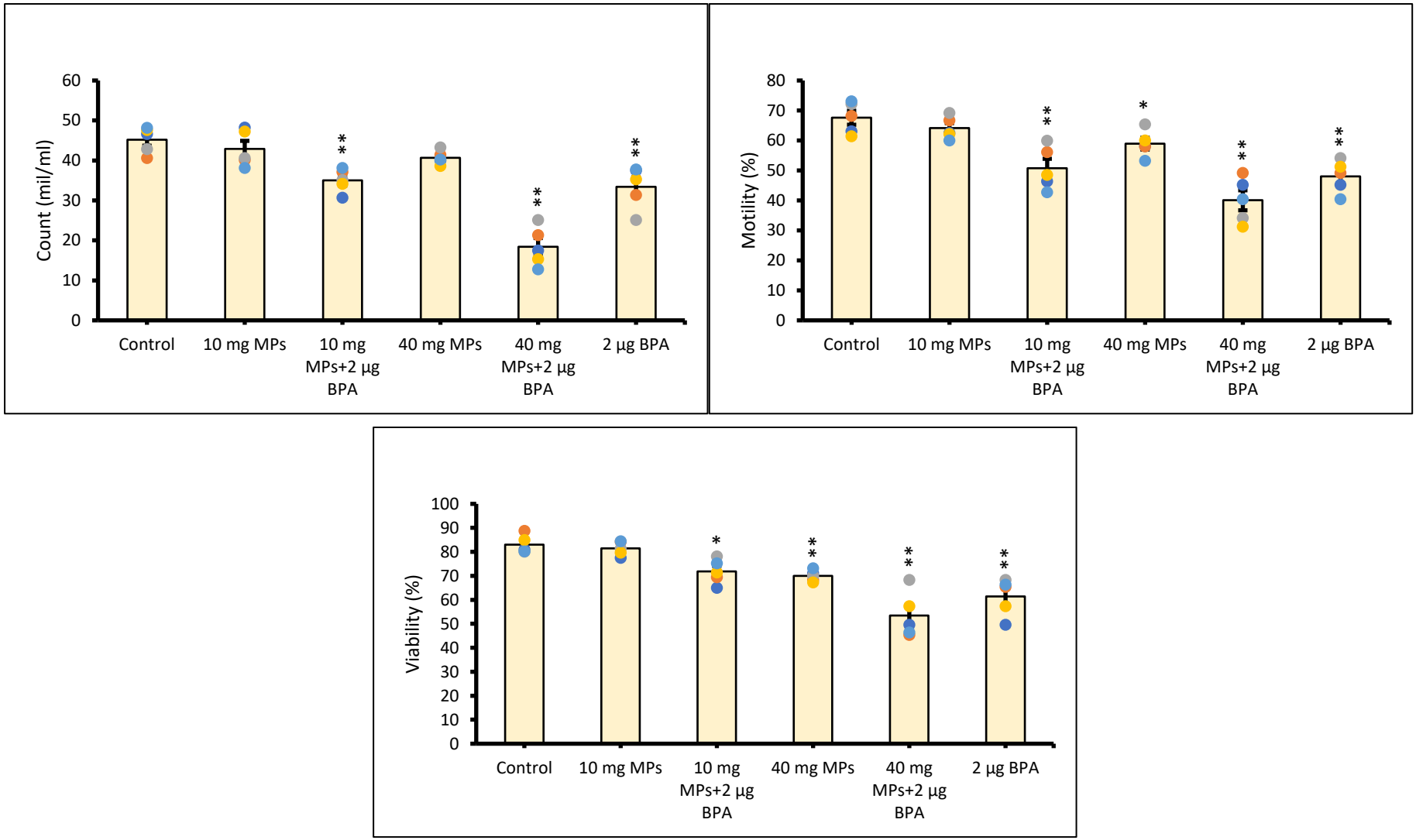


Figure 1: Cauda epididymal sperm count, motility and viability were measured and compared with control and individual exposures of PS-MPs and BPA against combined exposures. Level of significance were expressed in *p<0.05, **p<0.01, and ***p<0.001.

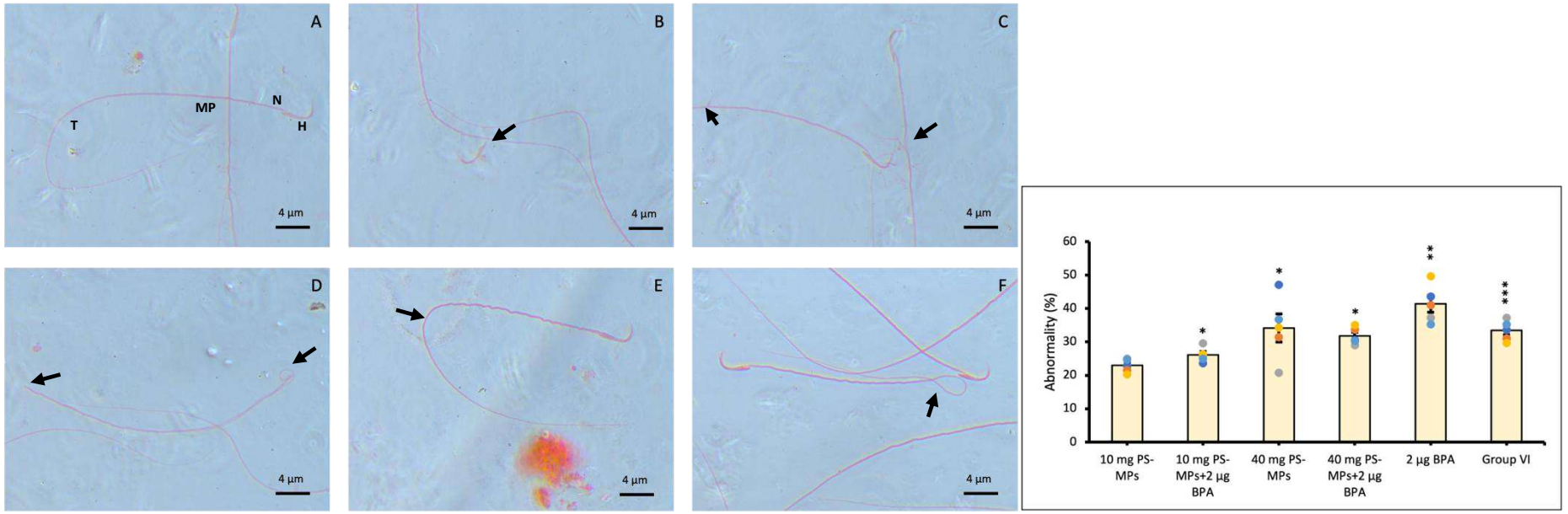


Figure 2: Papanicolaou (PAP) staining of cauda epididymal sperm showing typical damages observed in groups II-VI (PS-MPs/BPA treated). Comparing to control (A), plasma membrane leakage (C), broken neck (B), cytoplasmic droplet (C), abnormal head (D), abnormal and twisted tails (E and F) were more common in stress induced groups. Graph shows cauda epididymal sperm abnormalities in test groups and compared against control. N: Neck; H: Head; MP: Midpiece; T: Tail.

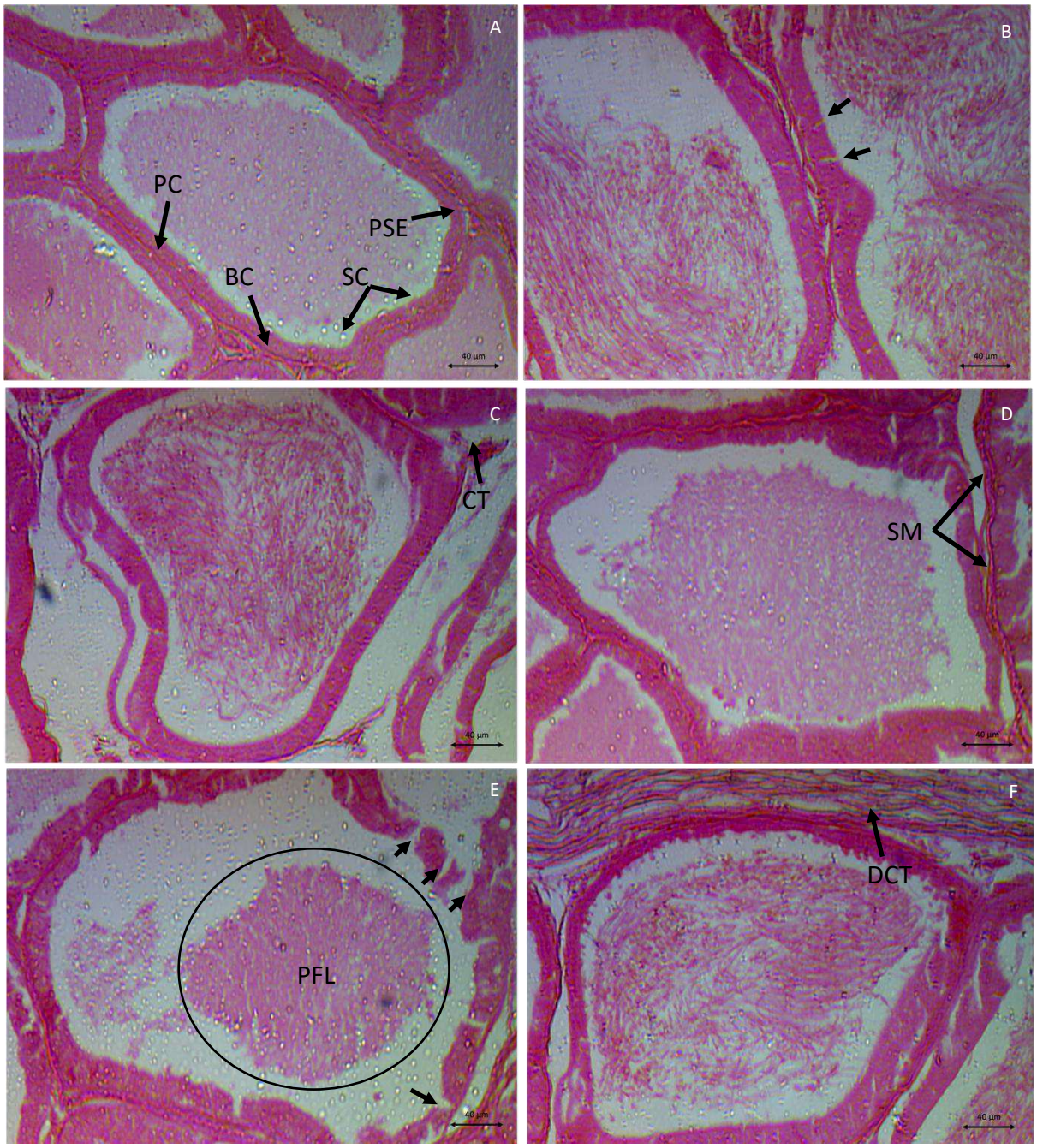


Figure 3: The histological observation of epididymis of control (Group I) (A) indicated round elongated tubules and abundant intertubular elements. The epithelium of epididymis showed pseudo-stratified configuration (PSC) with prominent basal cells (BC) and principal cells (PC). The basal cells were short cuboidal relatively less prominent, less granular and showed distinct elongated nuclei (EN). The principal cells were tall columnar and pseudo stratified; the nuclei

were located at the base and cytoplasm appeared granular. Lumen was tightly filled with sperms. The exposure of 10 mg MPs (Group II) (B) indicated damage in basal cells and stereocilia. Whereas animals exposed to 2 µg BPA (Group VI) (F) showed loss of smooth muscle and connective tissue structural integrity. Animals exposed to 40 mg MPs + 2 µg BPA (Group V) (E) indicated combination of disrupted smooth muscles and significantly low amount of sperm in lumen. Animals exposed to 10 mg MPs + 2 µg BPA (Group III) (C) showed distinctly low amount of sperm in lumen comparing to control. Rats exposed to 40 mg MPs (Group IV) (D) indicated distinctly higher damages in basal cells and stereocilia. S: Sperm; L: Lumen; PFL: Partially Filled Lumen; PSE: Pseudostratified epithelium; D-PSE: Disruption of PSE; CT: Connective tissue; SM: Smooth muscle.

Table 3: Biochemical evaluation of epididymal tissues in rats exposed to PS-MPs/BPA and control. All values are represented in Mean ± SEM (n=5). The level of significance was measured against control and considered significant when *p<0.05, and **p<0.01.

	Control	*10 mg MPs*	*10 mg MPs+2 µg BPA*	*40 mg MPs*	*40 mg MPs+2 µg BPA*	*2 µg BPA*
Sialic acid (mg/g tissue)	1.18±0.05	0.86±0.04**	0.73±0.10*	0.71±0.12*	0.58±0.12**	0.82±0.06*
L-carnitine (mM/mg protein)	1.20±0.14	0.87±0.05	0.77±0.06*	1.06±0.06	0.52±0.11*	1.02±0.08
Neutral-α-glucosidase (mg PNP/mg protein)	33.60±2.18	26.57±1.67	20.86±3.28*	23.82±3.39*	17.38±2.13**	21.18±1.59*

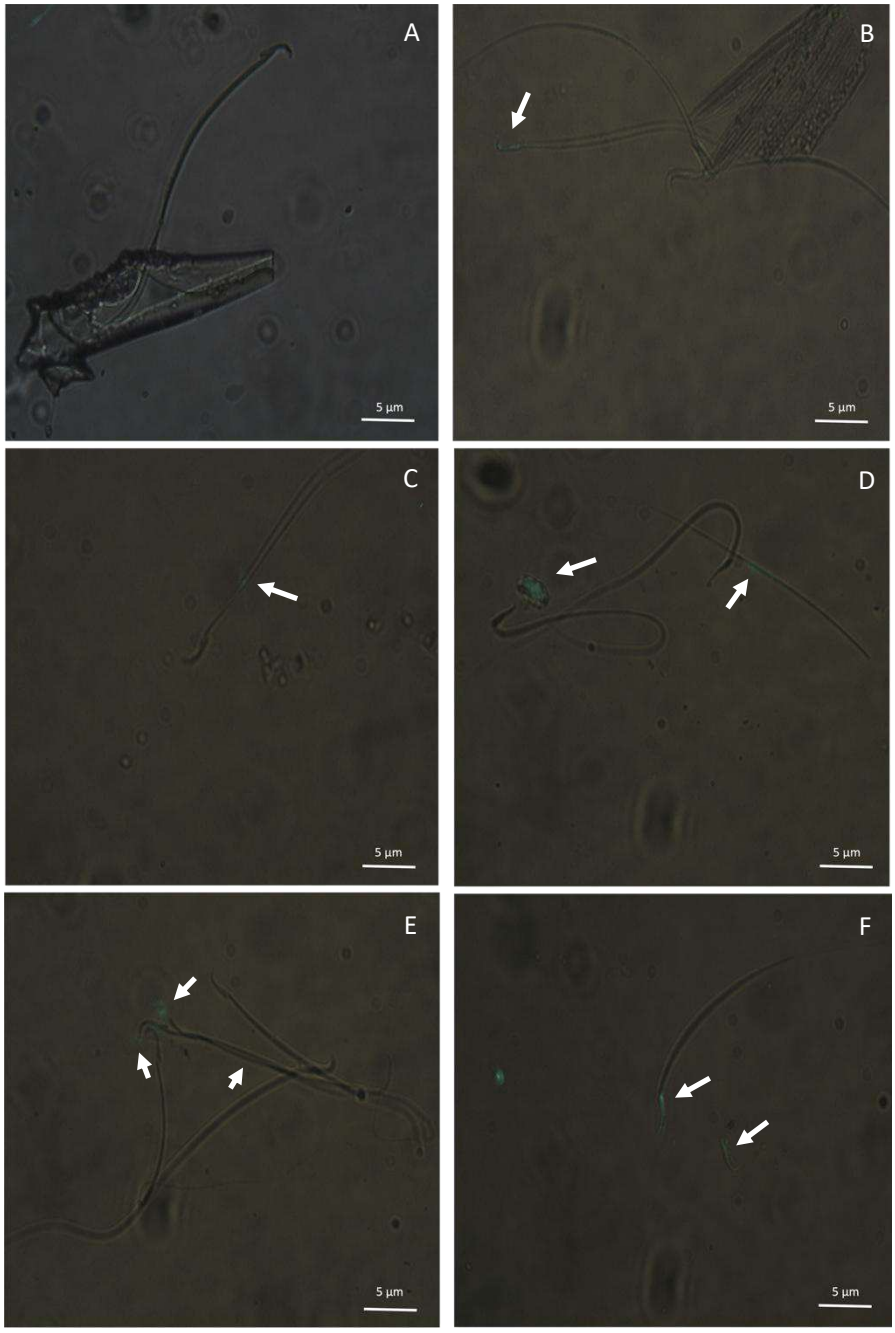


Figure 4: The above slides show representative group-wise images of sperms showing early signs of apoptosis in control (A), 10 mg PS-MPs (Group II) (B), 10 mg PS-MPs + 2 µg BPA (Group III) (C), 40 mg PS-MPs (Group IV) (D), 40 mg PS-MPs + 2 µg BPA (Group V) (E) and 2 µg BPA (Group VI) (F) groups. Phase contrast images at 25% transparency were superimposed on Annexin V-FITC stained DigiPro images showing specific positive localization at sperm heads (ventral section), mid-piece, neck (proximal area) and in and around damaged heads.

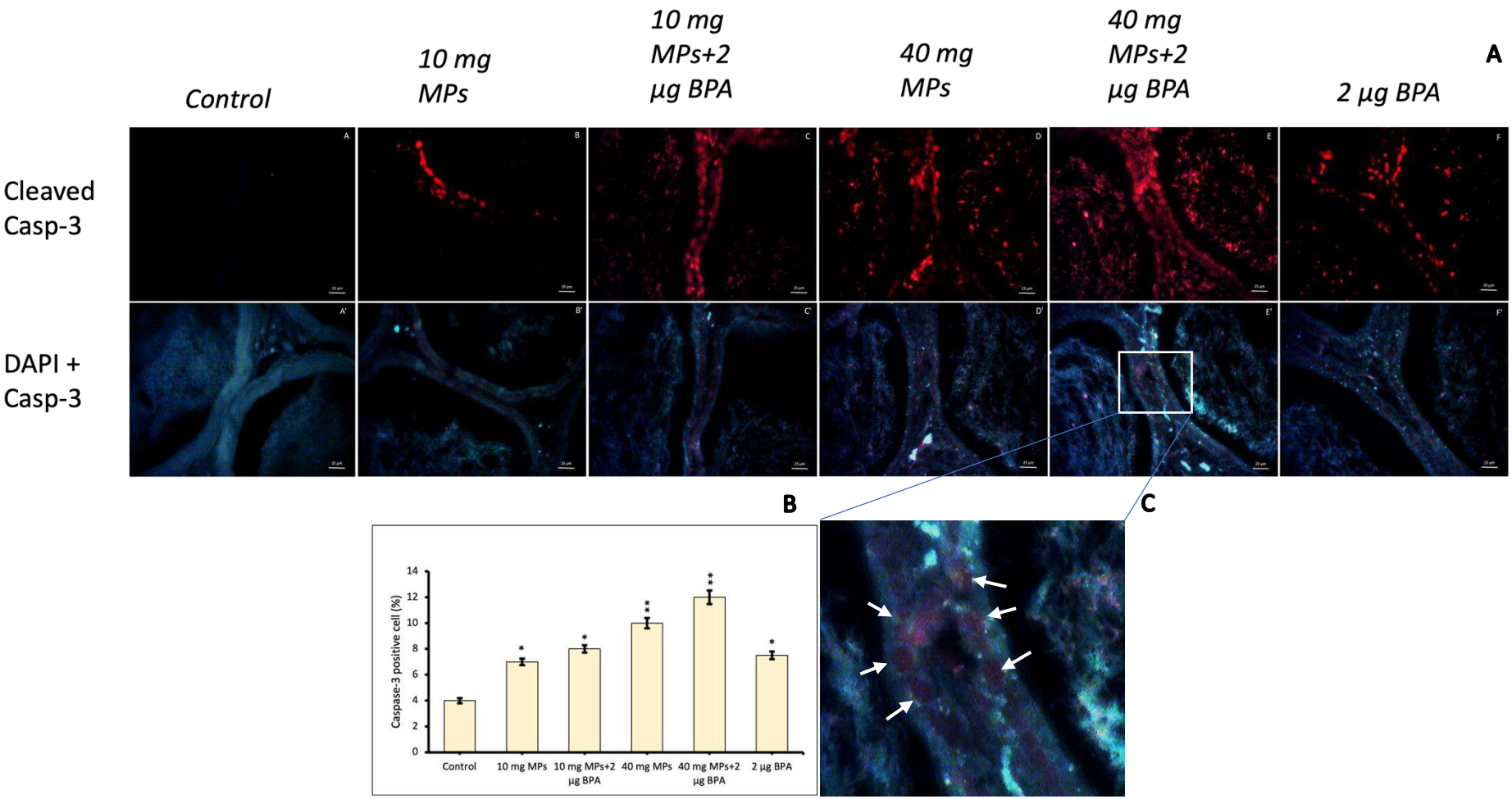


Figure 5: Expression of cleaved caspase-3, cell apoptosis marker in epididymis of PS-MPs/BPA exposed rats. The immunofluorescent staining of cleaved caspase-3 in rat epididymis is shown exclusively in upper panel (A-F), while lower panel shows same images merged with DAPI staining 4',6'-diamidino-2-phenylindole (A'-F'). Percentage caspase-3 positive cells were significantly high in treated groups (B). Increased caspase-3 immunoreactivity was predominantly observed in principal and basal cells (C-inset).

Table 4: Pearson's correlation test between variation in sperm characteristics and epididymal biochemistry in response to the PS-MP/BPA exposure.

	Count	*Motility*	*Viability*	*Abnormality*
Sialic acid	0.739	0.784	0.764	-0.892
L-carnitine	0.834	0.753	0.617	-0.794
Neutral-α-glucosidase	0.843	0.930	0.873	-0.953

	Extremely associated
	Highly associated
	Moderately associated