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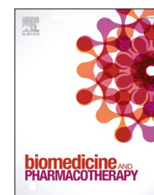

Pinostrobin alleviates testicular and spermatological damage induced by polystyrene microplastics in adult albino rats

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ABSTRACT

Background: Polystyrene microplastics (PS-MPs) have become major environmental pollutants that adversely effects multiple organs specifically testicles. Pinostrobin (PN) is an important flavonoid which, shows several pharmacological potentials.
Purpose: The current study was designed to elucidate the mitigative effects of PN against PS-MPs induced testicular toxicities in rats.
Methods: 48 male albino rats were randomly distributed into 4 groups, control, PS-MPs group (0.01 mg/kg), PS-MPs + PN group (0.01 mg/kg of PS-MPs; 40 mg/kg of PN) and PN group (40 mg/kg).
Results: PS-MPs intoxication substantially lessened the activities of glutathione peroxidase (GPx), glutathione reductase (GSR), superoxide dismutase (SOD) along with catalase (CAT) while, raised the level of malondialdehyde (MDA) as well as reactive oxygen species (ROS). Additionally, PS-MPs reduced luteinizing hormone (LH), plasma testosterone, follicle-stimulating hormone (FSH) concentration, sperm motility, sperm count, expression of steroidogenic enzymes and Bcl-2 (anti-apoptotic protein) along with the count of spermatogenic cells. While, dead sperm count, sperm abnormalities (tail, neck and head), Bax and caspase-3 (apoptotic proteins) expression along with histopathological anomalies were elevated. Moreover, PS-MPs exposure increased the level of inflammatory markers. However, PN treatment considerably decreased oxidative stress (OS) by reducing ROS as well as increased sperm motility and alleviated all the damages induced by the PS-MPs.
Conclusion: Therefore, it is concluded that PN may prove a potential therapeutic candidate to restore all the PS-MPs-induced testicular toxicities.

1. Introduction

The use of plastic items has extensively expanded resulting in considerable pollution from plastic waste [1]. Many physical, chemical along with biological processes are involved in degradation of large plastic fragments into smaller particles (less than 5 mm in size) to form Microplastics (MPs) [2]. One of the most common type of MPs is Polystyrene microplastics (PS-MPs). PS-MPs are generated when styrene polymers degrade. Due to its thermoplasticity, low cost, PS is mostly used to manufacture foam insulation materials, disposable cups, lunch boxes, foam boxes and cleaning supplies [3,4]. Exposure to PS-MPs occurs via inhalation, ingestion, dermal contact and their existence in food packaging [5]. Due to their smaller size and low density, they might be easily consumed by fauna so, they build up in the biological food chain [6] and instigate severe injury to different organs such as liver, kidney, brain and gastrointestinal tract [7,8,9,10]. PS-MPs induce intestinal microbial growth and metabolic disorders in mice [11]. PS-MPs exposure also stimulates inflammatory reactions and oxidative stress [12].

One of the most crucial systems in the organism is reproductive system. Oxidative stress causes partial or complete dysfunction of the male reproductive system [13]. Excess ROS generation, i.e., superoxide (O_2), hydrogen peroxide (H_2O_2) along with hydroxyl radical (OH^-) causes oxidative stress. PS-MPs has been reported to provoke ROS

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generation and instigate oxidative stress [14]. According to previous literature PS-MPs intoxication causes testicular damage which leads to infertility [15]. According to Jin et al. [16] PS-MPs exposure resulted in abscission and irregular organization of germ cells as well as multi nucleation in gonocytes of seminiferous tubules. Another study revealed that PS-MPs administration augmented the level of inflammatory indices i.e., IL-6, TNFα as well as IL-1β in mouse testicular tissues through activation of the p38 MAPK pathway [17]. In PS-MPs-intoxicated animals, the plasma and intra-testicular testosterone levels were reduced significantly [18]. Moreover, PS-MPs exposure also results in an increased rate of sperm deformity [4].

Flavonoids are polyphenolic compounds, present in plants as secondary metabolites [19]. Flavonoids are widely used as drugs and dietary supplement due to their potential pharmaceutical properties and low toxicity [20]. PN is an important dietary bioflavonoid, is a major constituent of heartwood of *Pinus strobus* L [21]. PN is reported to show anti-inflammatory, [22] gastroprotective, antioxidant [23] as well as anti-fungal properties [24]. As PS-MPs has the tendency to induce OS, which leads to testicular dysfunction as well as it adversely affects spermatogenesis that results in male infertility. Therefore, the therapeutic potential of PN in the amelioration of reproductive damage still remains unascertained. Hence, the recent investigation was intended to assess the therapeutic potential of PN in mitigating the PS-MPs-prompted testicular damage in rats.

2. Materials and methods

2.1. Chemicals

PS-MPs (size 100 nm, monodispersed shape and 1 ± 2 mV zeta potential), PN (appearance powder form, density 1.284 ± 0.06 g/cm3, boiling point 494.9 ± 45.0 °C, melting point 100 °C) formalin, sodium bicarbonate, eosin, nigrosin, fructose, formaldehyde, alcohol, paraffin, hematoxylin, NADH, phenazine methosulfate, guaicol, hydrogen peroxide, EDTA and ferric chloride were purchased from Germany (Sigma-Aldrich).

2.2. Animals

48 adult male albino rats (250 ± 20 g) were used for the current experiment. They were restrained in steel enclosure in the research station of University of Agriculture, Faisalabad (UAF). Rats were housed in 12-hour cycles of light and darkness each, at a consistent temperature (22–25 ^{o}C). Moreover, food chaw along with tap water was given to rats. All the experiments were performed in line with the ethical guideline of UAF Animal Protection and Handling Committee.

2.3. Experimental design

Adult male albino rats (n = 48) were equally distributed into 4 groups, each group containing 12 rats: control group, PS-MPs administrated (received 0.01 mg/kg of PS-MPs), PS-MPs + PN co-treated (received both 0.01 mg/kg of PS-MPs + 40 mg/kg of PN orally), and PN administrated (40 mg/kg of PN by oral gavage) group. The mentioned doses were given on daily basis. PS-MPs at a dose of 0.01 mg/kg was given according to the earlier investigation [25], whereas a 40 mg/kg dose of PN was provided in compliance with the study of Tonum et al. [26]. After 56 days, rats were slaughtered by decapitation, blood was stored in sterile tubes and centrifuged at 3000 rpm for 10 min. Testes were removed and the right testis was stored at − 80^{0} C till further analysis. For histological analysis, the left testis was fixed in 10% solution of formalin.

2.4. Biochemical markers evaluation

CAT activity was accessed by using the technique explained by Chance and Maehly [27], whereas, the activity of SOD was evaluated by employing the protocol reported by Sun et al. [28]. GPx activity was assessed in line with the methodology of Lawrence and Burk [29]. The activity of GRS was quantified by using the technique described by Carlberg and Mannevirk [30]. Hayashi et al. [31] methodology was used to ascertain the concentration of ROS. Placer et al. [32] protocol was applied to evaluate the level of malondialdehyde (MDA).

2.5. Assessment of motility, viability, count, and the structural abnormalities of sperm

According to the procedure outlined by Yokoi et al., hemocytometer was used to measure the epididymal sperm count [33]. The semen samples were taken from caudal part of the epididymis. Initially, a part of the epididymis was finely minced in 5 mL of physiological saline solution and incubated at 37 °C for about 30 min to permit the sperms to abscond from the epididymis. A solution of 25 mg eosin/100 mL of distilled H_2O, 1 mL of formalin (35%) and 5 g of sodium bicarbonate ($NaHCO_3$) was added to dilute the supernatant. A 10 μL droplet of this solution was added in sperm counting chamber and examined under the light microscope (400X). Sperm motility was evaluated by using phase contrast microscope (400X) [34]. While, the viability of sperm was quantified by eosin as well as nigrosin staining, followed by microscopic inspection [35]. Moreover, sperm morphological (tail, neck/mid piece and head) irregularities were determined by using the approach of Cao et al. [36]. The measurements were expressed in percentage (%).

2.6. Hypo osmotic swelling (HOS) test

The integrity of the sperm plasma membrane was determined using HOS test, according to Correa and Zavos method [37]. Initially, 20 μL of sperm sample was added in 180 μL of fructose solution at 80 mOsm/L for twenty minutes. Then sperms were stained with eosin as well as nigrosin, following further incubation and processing. Finally, a light microscope set at 400X was used to observe 200 sperms with and without swollen tails.

2.7. Hormonal assay

The concentrations of LH, FSH as well as plasma testosterone were quantified by Enzyme-linked immunosorbent assay (ELISA) kits (Los Angeles, CA USA). Hormonal values were expressed as ng/mL.

2.8. Ribonucleic acid (RNA) extraction and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The expressions of 17β-HSD and StAR, 3β-HSD as well as the anti and pro-apoptotic markers such as, Bc1–2, Bax as well as Caspase-3 were assessed by using qRT-PCR. TRIzol reagents were used to separate total RNA, then the total RNA was transformed into complementary deoxyribonucleic acid (cDNA) through reverse transcription. Variations in the expression of these parameters were examined with the help of $2^{-\Delta\Delta CT}$ considering β-actin as an internal control [38]. The primer sequences of target genes are shown in Table 1, as reported earlier [13].

2.9. Inflammatory markers assessment

To ascertain the inflammatory indices of testicles, commercially available kits were used. The activity of TNF-α, IL-1β, NF-κB p65, IL-6 as well as COX-2 was quantified by using ELISA kit (Shanghai, China). Analyses were carried out in accordance with the manufacturer's instructions using an ELISA Plate-Reader (BioTek, Winooski-VT, USA).

2.10. Histopathology

For the histopathological observation, testicular samples were fixed

Table 1
Primers sequences for the real-time quantitative reverse transcription-polymerase (qRT-PCR).

Gene	Primers 5'— 3'	Accession number
3β-HSD	Forward: GCATCCTGAAAAATGGTGGC Reverse: GCCACATTGCCTACATACAC	NM_001007719
17β-HSD	Forward: CAGCTTCCAAGGCTTTTGTG Reverse: CAGGTTTCAGCTCCAATCGT	NM_054007
StAR	Forward: AAAAGGCCTTGGGCATACTC Reverse: CATAGAGTCTGTCCATGGGC	NM_031558
Bax	Forward: GGCCTTTTTGCTACAGGGTT Reverse: AGCTCCATGTTGTTGTCCAG	NM_017059.2
Bcl-2	Forward: ACAACATCGCTCTGTGGAT Reverse: TCAGAGACAGCCAGGAGAA	NM_016993.1
Caspase-3	Forward: ATCCATGGAAGCAAGTCGAT Reverse: CCTTTTGCTGTGATCTTCCT	NM_012922.2
β-actin	Forward: TACAGCTTCACCACCACAGC Reverse: GGAACCGCTCATTGCCGATA	NM_031144

RT-qPCR: reverse transcription-polymerase chain reaction; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; StAR: steroidogenic acute regulatory protein.

in 10% formaldehyde for about 48 h, dehydrated in ascending grades of alcohol which were later inserted in paraffin wax. Then, 5 μm thick pieces were cut, stained with eosin & hematoxylin and observed under the microscope (Nikon, Japan). Finally, image-J2X software was employed to examine the photographs of the specimens.

2.11. Statistical analysis

The values were presented as Mean ± SEM. Tukey's test was applied after one-way analysis of variance (ANOVA) to compare different groups. The level of significant was $p < 0.05$.

3. Results

3.1. Effect of PS-MPs and PN on biochemical parameters

Mean values of biochemical parameters are shown in Table 2. PS-MPs treatment considerably ($p < 0.05$) lowered the activities of GSR, SOD, GPx along with CAT whereas, noticeably ($p < 0.05$) augmented the levels of MDA as well as ROS in PS-MPs administrated rats relative to control rats. However, co-administration of PS-MPs with PN resulted in a profound ($p < 0.05$) upsurge in GSR, SOD, GPx as well as CAT activities, besides a noticeable ($p < 0.05$) reduction was exhibited in ROS as well as MDA levels in comparison to PS-MPs-intoxicated group. Furthermore, the mean values of PN only supplemented group were comparable to control group.

Table 2
Effect of PS-MPs and PN on biochemical parameters.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
CAT (U/mg protein)	8.47 ± 0.16^{a}	4.63 ± 0.14^{b}	8.12 ± 0.15^{a}	8.49 ± 0.17^{a}
SOD (U/mg protein)	7.73 ± 0.16^{a}	2.36 ± 0.13^{c}	6.19 ± 0.18^{b}	7.77 ± 0.21^{a}
GPx (U/mg protein)	17.51 ± 0.08^{a}	7.36 ± 0.08^{c}	12.54 ± 0.09^{b}	17.55 ± 0.74^{a}
GSR (nM NADPH oxidized/min/mg tissue)	4.61 ± 0.16^{a}	1.57 ± 0.11^{c}	3.58 ± 0.19^{b}	4.64 ± 0.18^{a}
MDA (nmol/g)	0.61 ± 0.09^{b}	2.23 ± 0.17^{a}	1.07 ± 0.09^{b}	0.59 ± 0.09^{b}
ROS (nmol/g)	0.74 ± 0.07^{c}	7.04 ± 0.19^{a}	1.86 ± 0.16^{b}	0.71 ± 0.06^{c}

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

3.2. Effect of PS-MPs and PN on spermatogenic parameters

Effects of PS-MPs and PN on spermatogenic parameters are shown in Table 3. PS-MPs induced a considerable ($p < 0.05$) decrease in epididymal sperm count along with sperm motility, while dead sperm as well as morphological abnormalities of sperms (tail, mid sperm/neck and head), were augmented in comparison to control group. Nonetheless, in PS-MPs + PN co-treated group, these abnormalities were considerably ($p < 0.05$) lessened in comparison to the PS-MPs administrated rats. Additionally, PN only treated group showed these values near to the control group.

3.3. Effect of PS-MPs and PN on hormonal levels

Alterations in hormonal level are presented in Table 4. Hormonal levels were substantially ($p < 0.05$) reduced in PS-MPs administrated rats as compared to control rats. Nonetheless, the co-treatment of PS-MPs with PN considerably ($p < 0.05$) increased the hormonal levels in contrast to PS-MPs administrated group. Additionally, the hormonal levels in PN only administrated group were near to control group.

3.4. Effect of PS-MPs and PN on the expression of testicular steroidogenic enzymes

The alterations in the expression of steroidogenic enzymes are shown in Fig. 1. A substantial ($p < 0.05$) reduction was observed in the expression of these enzymes in the testicles of rats in PS-MPs treated rats relative to control rats. While, the supplementation of PN upsurged the expression of these enzymes in co-treated (PS-MPs + PN) group in comparison to PS-MPs intoxicated group. Moreover, expression of these steroidogenic enzymes in PN only administrated group was near to control rats.

3.5. Effect of PS-MPs and PN on testicular apoptotic markers

Variations in apoptotic markers due to the administration of PS-MPs and PN are presented in Fig. 2. PS-MPs intoxicated rats presented a considerable ($p < 0.05$) upsurge in apoptotic markers (Bax and caspasee-3) in comparison to the control rats. While, the levels of anti-apoptotic marker Bcl-2 were remarkably ($p < 0.05$) lowered in PS-MPs administrated rats. PN supplementation resulted in considerable ($p < 0.05$) decrease in Bax and Caspase-3 level along with remarkable augmentation in the expression of Bcl-2 in PS-MPs + PN administrated group in contrast to PS-MPs treated group. Additionally, these values in PN only treated group were near to control group.

Table 3
Effect of PS-MPs and PN on spermatogenic parameters.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
Epididymal sperm count (10^{6}/mL)	26.78 ± 1.03^{a}	11.44 ± 0.73^{c}	21.45 ± 0.78^{b}	27.46 ± 1.00^{a}
Sperm Motility (%)	85.89 ± 0.97^{a}	29.57 ± 1.07^{c}	68.64 ± 1.97^{b}	86.25 ± 1.66^{a}
Dead sperms (%)	16.20 ± 0.58^{c}	81.17 ± 1.34^{a}	32.85 ± 0.97^{b}	16.77 ± 1.11^{c}
Head abnormality (%)	3.18 ± 0.16^{c}	19.49 ± 0.714^{a}	6.51 ± 0.13^{b}	3.07 ± 0.17^{c}
Mid sperm abnormality (%)	0.74 ± 0.05^{c}	9.25 ± 0.29^{a}	1.85 ± 0.14^{b}	0.68 ± 0.06^{c}
Tail abnormality (%)	3.86 ± 0.14^{c}	16.85 ± 0.93^{a}	6.92 ± 0.18^{b}	3.77 ± 0.13^{c}

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

Table 4
Effect of PS-MPs and PN on hormonal levels.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
LH (ng/mL)	2.36 ± 0.15^{a}	1.08 ± 0.10^{b}	2.15 ± 0.10^{a}	2.38 ± 0.14^{a}
FSH (ng/mL)	3.68 ± 0.11^{a}	1.32 ± 0.08^{c}	2.85 ± 0.14^{b}	3.71 ± 0.13^{a}
Plasma testosterone (ng/mL)	4.55 ± 0.14^{a}	2.29 ± 0.09^{c}	3.76 ± 0.11^{b}	4.61 ± 0.15^{a}

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

3.6. Effect of PS-MPs and PN on inflammatory markers

The effects of PS-MPs and PN on inflammatory markers is displayed in Table 5. Levels of inflammatory indices were considerably ($p < 0.05$) augmented in PS-MPs administrated rats in comparison to control rats. However, the administration of PS-MPs and PN resulted in remarkable ($p < 0.05$) decline in inflammatory markers in co-administrated group in comparison to PS-MPs administrated group. Moreover, PN only administrated group showed the level inflammatory markers near to control group.

3.7. Effect of PS-MPs and PN on histomorphometry

Variation in morphometry of testicular tissues is shown in Table 6. PS-MPs administration prompted a considerable ($p < 0.05$) reduction in seminiferous epithelial tubule as well as tunica propria height and tunica propria width. Moreover, a considerable ($p < 0.05$) elevation was exhibited in luminal diameter and interstitial space in PS-MPs administrated rats relative to control group. Nonetheless, in the PS-MPs + PN co-treated group a considerable ($p < 0.05$) increase was observed in epithelial and tunica propria height along with the tunica propria width, while interstitial spaces and luminal diameter were reduced in comparison to PS-MPs intoxicated group. Moreover, no significant damage was noticed in PN only supplemented rats relative to control rats (Fig. 3).

Effects of PS-MPs and PN on different stages of germ cells are displayed in Table 7. A considerable ($p < 0.05$) reduction in the count of germ cells was recorded in PS-MPs administrated rats in contrast to control rats. However, PN treatment remarkably ($p < 0.05$) augmented the count of all the germ cells in PS-MPs + PN co-administrated group as compared to PS-MPs intoxicated group. Moreover, PN only administrated group exhibited a normal count of germ cells as in control group.

4. Discussion

In the current study, ameliorative potential of PN was assessed

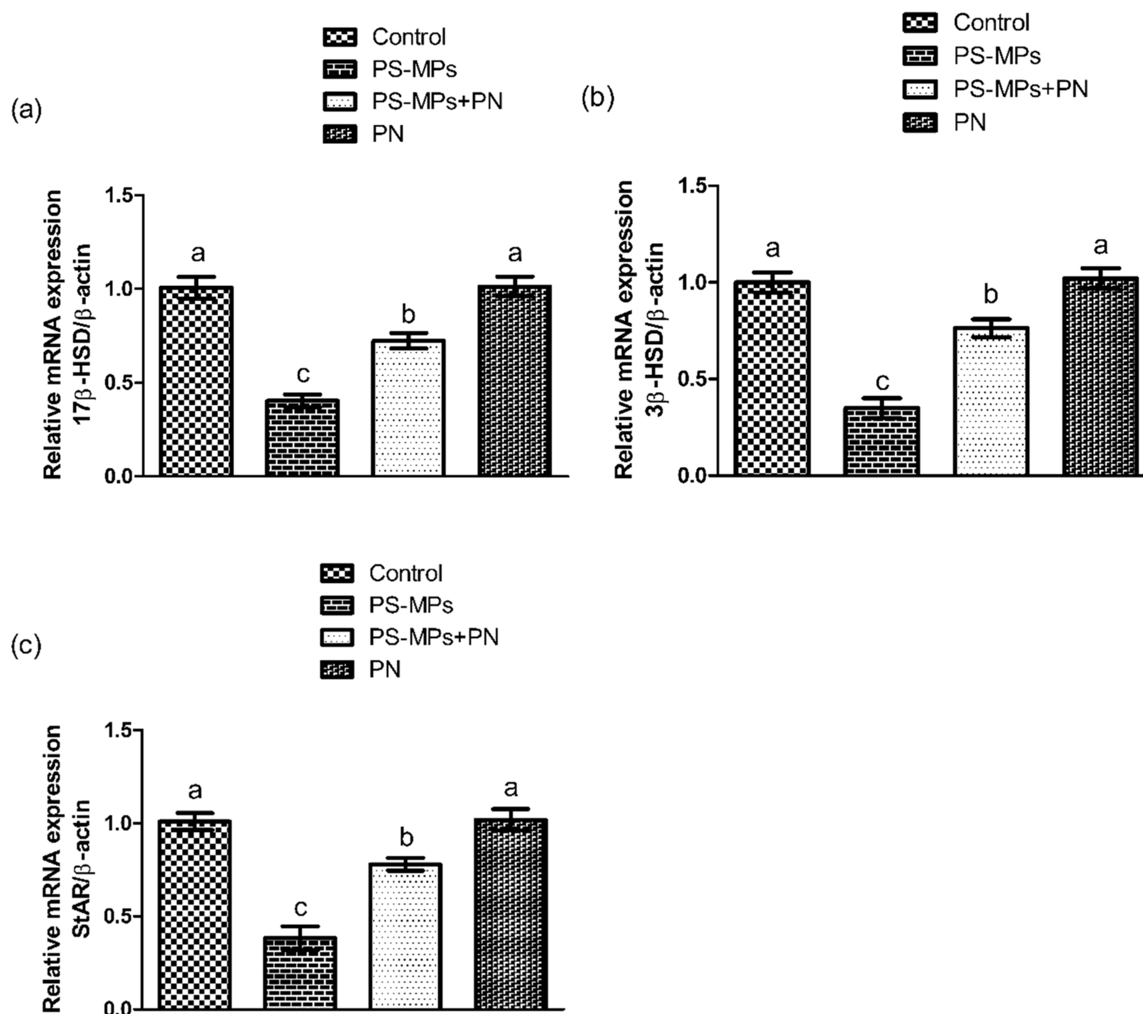

Fig. 1. Effect of PS-MPs and PN on expression of steroidogenic enzymes. Bars are presented on basis of Mean + SD values. Different subscripts on bars displaying substantial difference at $p < 0.05$. SEM: standard error of mean.

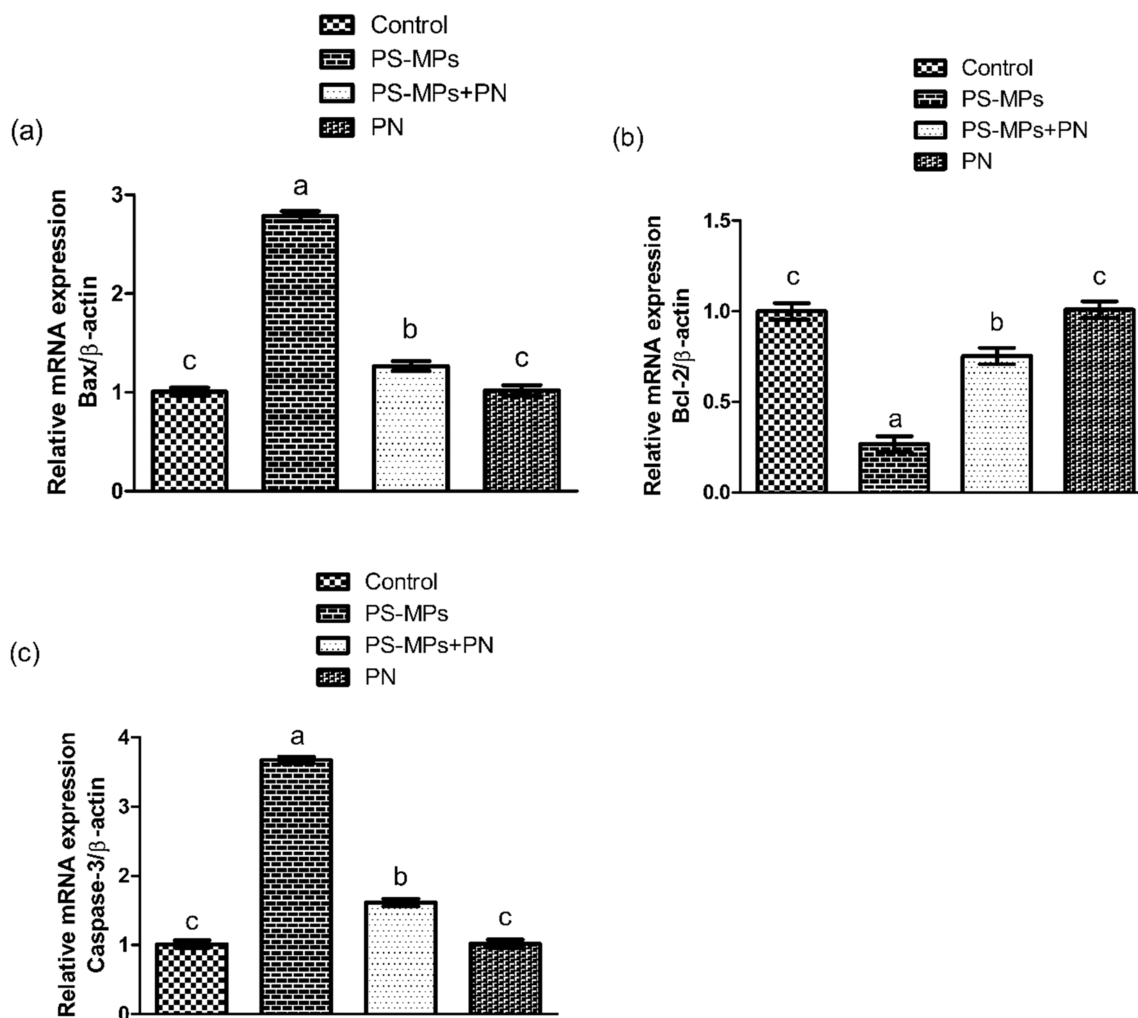

Fig. 2. Effect of PS-MPs and PN on expression of apoptotic markers. Bars are presented on basis of Mean + SD values. Different subscripts on bars expressing considerable difference at $p < 0.05$. SEM: standard error of mean.

Table 5
Effect PS-MPs and PN on inflammatory indices.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
NF-kB p65 (ng/g tissue)	18.47 ± 0.76^{b}	65.93 ± 1.91^{a}	22.65 ± 0.56^{b}	18.11 ± 0.41^{b}
TNF-α (ng/g tissue)	7.14 ± 0.16^{c}	19.97 ± 0.16^{a}	10.79 ± 0.48^{b}	7.09 ± 0.16^{c}
IL-1β (ng/g tissue)	23.55 ± 1.17^{c}	81.79 ± 2.00^{a}	30.96 ± 1.84^{b}	22.52 ± 0.88^{c}
IL-6 (ng/g tissue)	6.98 ± 0.14^{b}	25.38 ± 1.14^{a}	8.93 ± 0.60^{b}	6.81 ± 0.21^{b}
COX-2 (ng/g tissue)	24.36 ± 1.33^{c}	75.77 ± 2.20^{a}	34.24 ± 1.58^{b}	23.65 ± 1.61^{c}

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

Table 6
Effect of PS-MPs and PN on morphometry of rat testes.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
Interstitial space (μm)	7.12 ± 0.23^{c}	26.05 ± 0.87^{a}	11.23 ± 0.90^{b}	7.03 ± 0.21^{c}
Tunica propria (μm)	56.81 ± 1.65^{a}	13.44 ± 0.78^{c}	38.27 ± 1.88^{b}	57.97 ± 1.79^{a}
Tunica albuginea height (μm)	363.78 ± 7.51^{a}	104.9 ± 8.06^{c}	287.69 ± 14.66^{b}	368.11 ± 9.95^{a}
Epithelial height of seminiferous tubule (μm)	87.50 ± 3.59^{ab}	28.91 ± 2.28^{c}	74.68 ± 2.08^{b}	89.59 ± 3.51^{a}
Tubular lumen (μm)	37.45 ± 2.69^{b}	96.18 ± 4.70^{a}	47.76 ± 2.62^{b}	36.13 ± 2.63^{b}

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

against PS-MPs-induced impairment in the testicles of rat. PS is one of the aromatic polymers that is often used in plastics [18]. PS-MPs have become an emerging environmental pollutant that is reported to induce considerable damage to organisms [39]. It is reported that PS-MPs administration lead to an upsurge in the inflammatory response and oxidative stress [40]. Small size of PS-MPs facilitates its incorporation into the biota [41]. According to Hwang et al. [42] human exposure to PS-MPs occurs through the ingestion of seafood, water as well as contact with packaged food. PS-MPs can reduce sperm quantity and quality, consequently disrupt the male reproductive system. PS-MPs intoxication in rats is reported to disturb anti-oxidant enzymes, pro-apoptotic proteins, anti-apoptotic proteins as well as sperm parameters, hormonal level, steroidogenic enzymes, testicular morphology along with spermatogenic cells [4]. PN is a novel dietary flavonoid that is a major

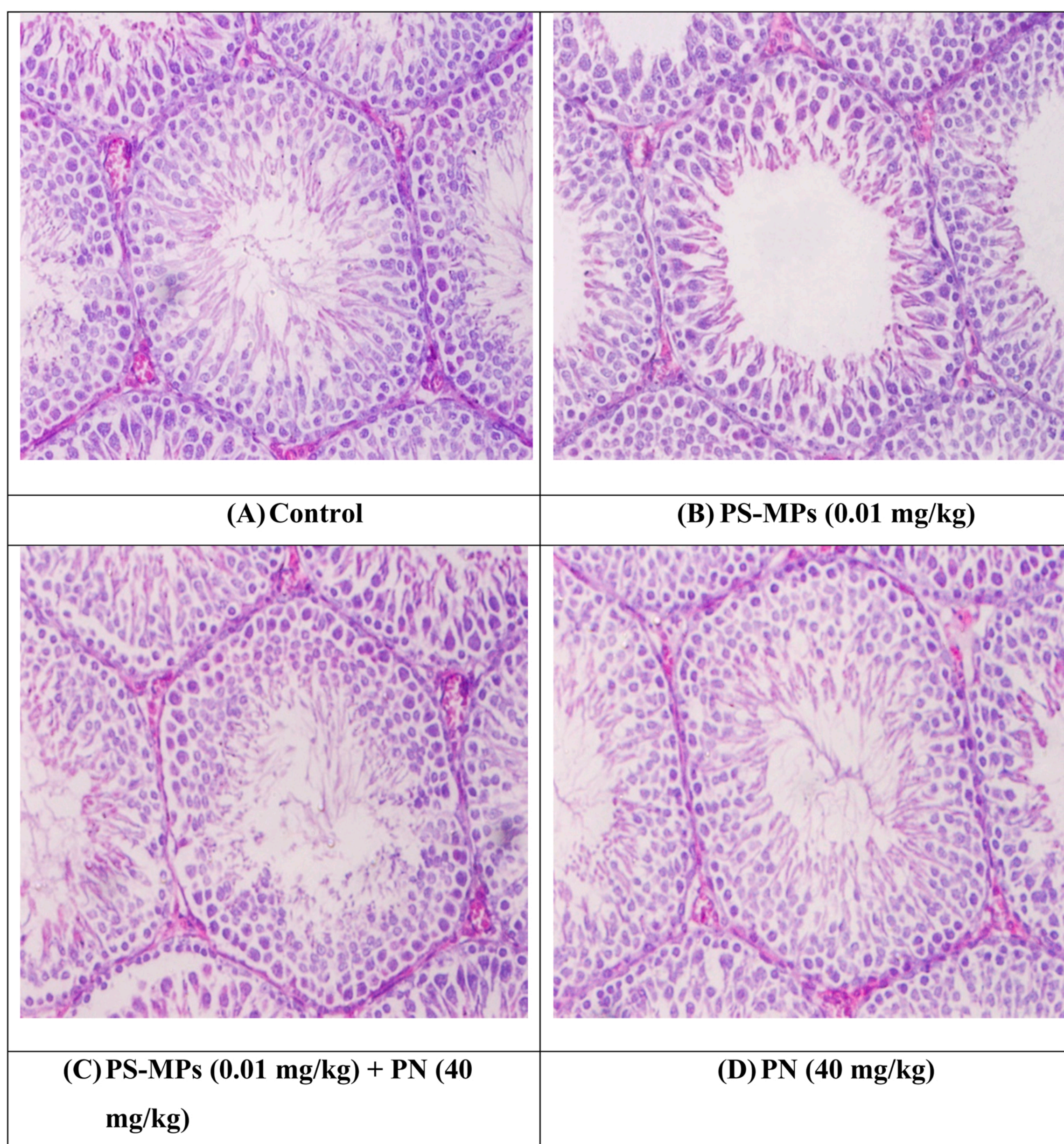

Fig. 3. Micro-images of the adult Albion rat testes (H&E, 400X): (A) Control group displaying impenetrable germinal epithelium as well as germ cells along with tapered luminal area containing spermatozoa; (B) PS-MPs-induced group showing sloughing of the epithelial layer, vacant lumen as well as degenerated area of IS; (C) PS-MPs+PN co-treated group demonstrating restoration in epithelial part and TL filled with germ cells as well as recovered the deteriorated IS; (D) PN supplemented group expressing compacted seminiferous tubules with less IS as well as luminal part filled with germ cells thus improved spermatogenesis. PS-MPs: Polystyrene microplastics; PN: Pinostrobin; IS: Interstitial spaces; SHE: Seminiferous Epithelial height; TL: Tubular lumen.

constituent of heartwood of *Pinus strobus* L [21]. Previous study conducted by González et al. [43] demonstrated that PN displayed anti-inflammatory, analgesic as well as free radical scavenging potential, followed by cyclodextrins provoked toxicity in mice skin. Therefore, it is hypothesized that PN might be used as a pharmacological agent to mitigate the harmful potential of PS-MPs on testicular tissues of male rats.

PS-MPs intoxication lessened the activities of anti-oxidant enzymes, while augmented the level of MDA as well as ROS. ROS include OH^-, O_2^- and H_2O_2, which are byproducts of cellular metabolism that harm cells in the body. The amount of ROS production is proportional to OS that eventually damages spermatozoa and semen quality [44],[45]. Increased ROS production results in a damaged anti-oxidant defense system of spermatozoa [46]. One of the key antioxidant enzymes is CAT which has a crucial role in H_2O_2 catabolism [47]. It also separates superoxide radicals produced by NADPH oxidase and protects sperm from oxidative damage [48]. SOD participates in the breakdown of superoxide to H_2O_2 and oxygen [49]. GSR assists in the conversion of reduced form of GSH into the oxidized form [50]. Accumulation of H_2O_2 results in escalated oxidative stress (OS) and gonadal toxicity in males. GSH plays a pivotal role in the suppression of H_2O_2 [51]. GPx participates in the reduction of H_2O_2 and lipid peroxide levels to decrease oxidative stress [8]. MDA is a stable product of ROS-induced lipid peroxidation as well as it is commonly used as indicator of ROS. It is one of several indices used to assess oxidative stress, lipid peroxidation, and cell viability. It reflects the damage caused by oxygen free radicals indirectly [52]. Anti-oxidant enzymes i.e., GPx, SOD, GST and CAT are crucial to minimize ROS and OS and ultimately avert damage [53]. Plant sources can be used to complement these antioxidants to reduce oxidative stress [54]. Thus, co-administration of PN with PS-MPs

Table 7
Effect of PS-MPs and PN on various stages of germ cells.

Parameters	Groups			
	Control	PS-MPs	PS-MPs + PN	PN
Spermatogonia (n)	54.90 ± 1.62[a]	21.75 ± 1.10[c]	44.27 ± 1.18[b]	55.65 ± 2.03[a]
Primary spermatocytes (n)	46.07 ± 1.61[a]	19.78 ± 0.57[c]	36.20 ± 1.92[b]	46.98 ± 1.75[a]
Secondary spermatocytes (n	37.27 ± 1.38[a]	11.78 ± 1.28[c]	27.86 ± 1.47[b]	38.12 ± 1.47[a]
Spermatids (n)	56.11 ± 1.90[a]	16.06 ± 1.90[c]	44.87 ± 1.59[b]	57.90 ± 1.91[a]

Values having different subscripts are substantially ($p < 0.05$) different from the other groups.

mitigated the damaging effects of PS-MPs by minimizing the OS in testes of rats. Our study demonstrated that PN administration escalated anti-oxidant enzymes activities, whereas reduced the level of MDA and ROS. This ameliorative potential of PN may be credited to its anti-oxidant and free radical scavenging ability. Moreover, PN has benzo-ɣ-pyrone in its structure that possibly imparted anti-oxidant potential to PN [23].

PS-MPs exposure significantly decreased sperm's number, motility as well as viability. While dead sperm's count and abnormalities of head, midpiece and tail of sperms were increased in PS-MPs treated group. Sperm quality is an important index for determining male fertility, with the main parameters being sperm number and mortality index. A decrease in sperm count is often caused by slower development and growth of germ cells [55]. A decrease in testosterone is considered as another important factor in reducing the sperm number [56]. Male gametes are more susceptible to ROS because they divide continuously. OS is reported to have effects on plasma membrane's fluidity and spermatogenic motility [57]. As previously stated, high amounts of polyunsaturated fatty acids (PUFAs) makes testicles chiefly vulnerable to OS. PUFAs are essential for sperm function and membrane integrity [58]. However, excessive ROS production disrupts the permeability and membrane's viscosity of spermatozoa, which prompts peroxidation of PUFAs [59]. OS also reduces ATP production by directly affecting sperm's mitochondria. A decrease in ATPs affects flagellar function which results in sperm immobility as well as apoptotic death [60]. Contrarily, these damaging effects were potentially lowered by the exposure of PN possibly owing to its ROS scavenging properties.

PS-MPs exposure decrease the expression of 3β-HSD, 17β-HSD and StAR. These enzymes perform a critical role in steroid hormones production [61]. StAR act as a regulatory protein in testosterone production that is responsible for the transportation of cholesterol (a testosterone precursor) within the mitochondrial membrane in Leydig cells (LCs) [62,63], while 3β-HSD and 17β-HSD accelerate the conversion of this cholesterol to testosterone [64]. Due to reduced expression of these enzymes the concentration of testosterone decreases [65]. However, PN treatment potentially mitigated the repression of testosterone by increasing the expression of these enzymes. Furthermore, it was also reported that flavonoids have cholesterol like chemical structure that could affect the synthesis of androgens in LCs [66]. This may be a possible reason behind the therapeutic potential of PN.

Our study revealed that PS-MPs exposed rats exhibited remarkable reduction in the level of plasma testosterone, LH as well as FSH that play an important role in spermatogenesis. Pituitary gland produces LH hormone that activates LCs to generate testosterone on the other hand, FSH promotes the proliferation of Sertoli cells [67,68]. The decrease in LH and FSH levels adversely affects the plasma level of testosterone [69]. Moreover, testosterone and FSH regulates the growth and release of spermatids [70]. PS-MPs disrupts the hypothalamus-pituitary-gonadal (HPG) axis, which controls the function of LCs and Sertoli cells through hormone production, thus severely disturbs the process of sperm production [39]. Hence, appropriate concentration of these hormones is required for spermatogenesis. The outcomes of our research demonstrated that PN administration reversed the effects of PS-MPs as well as elevated the hormonal levels probably by stabilizing the HPG axis. [71].

PS-MPs-induced testicular damages along with the ameliorative effect of PN were observed by analyzing the expression of apoptotic indices using qRT-PCR. Exposure to PS-MPs led to an upsurge in apoptotic proteins' expression i.e., Caspase-3 and Bax, whereas a remarkable downregulation in expression of anti-apoptotic protein (Bcl-2) was exhibited. Caspase-3 proteins plays a central role in apoptosis [72]. Bcl-2 (anti-apoptotic protein) protects the cells from death as a result of apoptosis, whereas Bax promotes apoptosis [73]. Decrease in Bcl-2 as well as augmentation in Bax severely change mitochondrial membrane's permeability and consequently results in increased liberation of cytochrome C [74]. This augmentation of cytochrome C activates Caspase-3 that results in apoptosis and cell death [75]. Following the PS-MPs exposure, apoptosis was induced by the overexpression of Caspase-3 and Bax as well as downregulation of Bcl-2. However, PN administration resulted in the increased expression of Bcl-2 as well as lowered Bax and Caspase-3 expression. The findings of our investigation showed that PN possesses a significant anti-apoptotic potential.

To evaluate the effect of PS-MPs and PN on inflammatory indices in testes, inflammatory marker profile was evaluated. Intoxication of PS-MPs significantly escalated the levels of inflammatory markers (NF-kB p65, IL-1β, TNF-α, IL-6 and COX-2). Inflammation is among the main causes of male infertility [76]. NF-kB is a key regulator of inflammation besides, NF-kB activation is crucial in expression of inflammatory markers that are linked with acute inflammatory reactions and other ROS associated disorders [77,78]. COX-2 is another important inflammatory marker that has a central role in inflammation [79]. The results of our investigation are further endorsed by the study of Xie et al. [17] who demonstrated that PS-MPs intoxication escalated the level of inflammatory indices in testicular tissue of rats. However, the co-treatment of PN with PS-MPs noticeably reduced the level of these markers to the normal level. This may be attributed to the anti-inflammatory potential of PN.

In the current study, PS-MPs administration provoked severe structural damages in testicles by persuading a reduction in seminiferous tubules epithelial height and diameter along with tunica propria height, on the other hand the diameter of the interstitial spaces as well as tubular lumen were augmented. Furthermore, PS-MPs exposure also reduced the count of all the germ cells. According to Adejuwon et al. [80] pro-oxidants and anti-oxidants imbalance leads to excessive ROS production, which causes histological damage. Moreover, the outcomes of Chouhan et al. [81] described that reduction in the level of testosterone could disturb the normal histology. However, PN treatment significantly raised the count of all the germ cells and restored all the testicular damages possibly because of its anti-oxidative, anti-inflammatory, anti-apoptotic as well as androgenic nature.

5. Conclusion

The current study indicated that PS-MPs intoxication induced OS in male reproductive system and damaged the biochemical, hormonal, spermatogenic as well as histopathological profiles of testicles. Moreover, PS-MPs administration lowered the expression of steroidogenic enzymes, anti-apoptotic protein and the expression of apoptotic protein as well as the level of inflammatory indices. However, PN administration showed alleviating effects against PS-MPs induced testicular toxicities and averted all these damages owing to its anti-oxidative, anti-inflammatory, anti-apoptotic as well as androgenic nature.

Animal ethics approval

All experimental protocols for animal handling were monitored by the University of Agriculture, Faisalabad's ethical committee in accordance with the European Union's approved (CEE Council 86/ 609) protocol for animal care and experimentation.

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CRediT authorship contribution statement

Muhammad Umar Ijaz: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Supervision, Writing – review & editing. **Saira Najam:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization. **Ali Hamza:** Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization. **Rabia Azmat:** Conceptualization, Methodology, Formal analysis, Visualization. **Asma Ashraf:** Formal analysis, Investigation, Visualization. **Jeremiah Oshiomame Unuofin:** Conceptualization, Methodology, Formal analysis, Visualization. **Sogolo Lucky Lebelo:** Conceptualization, Methodology, Formal analysis, Visualization. **Jesus Simal-Gandara:** Formal analysis, Investigation, Visualization, Writing – review & editing.

Conflict of interest statement

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

Data availability

Data will be made available on request.

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