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ORIGINAL ARTICLE

Luteolin Co-treatment abates Polystyrene Microplastics (PS-MPs) Induced Spermatotoxicity and Dysgonadogenesis in Rats Via up-regulation of Gonadotropin, Enhanced Spermatogenesis, Downregulation of Caspases, and Oxido-inflammation

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KEYWORDS

Luteolin;
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Oxido-inflammation;
Apoptosis;
Spermtotoxicity;
Dysgonadogenesis

ABSTRACT: Polystyrene microplastics (PS-MPs) pose a significant scientific concern due to their chemical makeup. Luteolin (LUT), a phytochemical-biological substance with anti-oxidative and anti-inflammatory properties, was tested against polystyrene microplastics (PS-MPs)-induced reproductive toxicity in male Wistar rats. The rats were randomly assigned to 4 groups and treated as follows: Group I received 0.01 mL kg^{-1} of DMSO alone, Group II received 100 mg kg^{-1} of LUT dissolved in DMSO, Group III received 0.01 mg kg^{-1} of PS-MPs, and Group IV received 0.01 mg kg^{-1} of PS-MPs and 100 mg kg^{-1} of LUT. PS-MPs alone dramatically increased sperm morphological defects, xanthine oxidase, and lipid peroxidation while decreasing testicular enzymes, luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, antioxidants, and interleukin-10 (IL-10). The study demonstrates that PS-MPs can alter male rat testicular function and androgenicity. However, as a result of LUT co-treatment, sperm count, motility, and viability were increased whereas MDA and XO, IL-1β and TNF-α, and caspase-3 activities in the testis were reduced. Supplementing with LUT might act as a cytoprotective agent to lessen the spermatotoxicity and dysgonadogenesis brought on by PS-MPs.

INTRODUCTION

Polystyrene microplastics (PS-MPs), a small environmental contaminant with a diameter of less than 5 mm, are a significant scientific concern because of their hazardous chemical composition, including undesirable additives and residual monomers [1, 2]. MPs can harm human health by contaminating food, packaging, taps, and water bottles. Polystyrene microplastics (PS-MPs) are widely used because of their affordability and usefulness [3, 4]. However, MPs are absorbed through the skin, inhaled, and accidentally eaten [5]. Research has linked MPs to testicular oxidative damage, poor sperm quality, altered hormone levels, and reproductive system damage in mice [6, 7]. Exposure to MPs also affects copepod lipid metabolism, oxidative stress, reproductive production, and immobility, and triggers moulting [8]. Therefore, MPs are a significant concern for environmental health.

This study investigated the protective role of oral Luteolin (LUT) co-treatment on oxidative and inflammatory reactions in male Wistar rats treated with

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microplastics (MPs). LUT is found in fruits, leaves, vegetables, and herbs and has anti-oxidative, anti-inflammatory, autophagic regulatory, apoptotic, and anti-neoplastic effects [9, 10]. This research focuses on LUT's role in modulating survivability, testicular function biomarkers, and reproductive hormones in Mps-treated rats. This study quantifies markers of antioxidant enzymes, oxidative stress, inflammatory, and apoptotic responses in the rats' testicles. These results should provide insights into the link between MPs-mediated oxido-inflammatory stress and apoptotic responses in testicular functions.

MATERIALS AND METHODS

Chemicals

The study employed various chemicals and kits to evaluate various hormones and cytokines, including, glutathione, thiobarbituric acid, hydrogen peroxide, alkaline phosphatase (ALP), acid phosphatase (ACP), and glucose 6-phosphate dehydrogenase (G6PD). The chemicals were purchased from Sigma, BDH Ltd., William Hopkins Ltd., and Randox Laboratories Ltd. The enzyme-linked immunosorbent assay (ELISA) kits used to assess TNF-alpha, caspase-3, IL-1, IL-10, and IL-10 were bought from Elabscience Biotechnology Company in Beijing, China.

Animal model and experimental design

Male Wistar rats that were 9 weeks old, 160-220 g, and sexually mature were used for the study. They lived in a well-ventilated facility and had access to water, rat pellet food, and a natural photoperiod. The animals were kept in an environment with consistent light and dark cycles, following the ARRIVE standards approach and National Institutes of Health in Animal Experimentation and Care recommendations as stated in the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines. The DMSO, LUT [22], and PS-MPs [41] doses and techniques were chosen based on previous dose-response effects and exploratory research.

Experimental protocol

PS-MPs (0.01 mg kg^{-1}) and LUT (100 mg kg^{-1}) doses were utilized in the investigation of laboratory rats, using freshly made stock solutions every day. Each of the four treatment groups got DMSO, LUT, PS-MPs and PS-MPs plus LUT therapies respectively for a total of 28 days: Group I received 0.01 mL kg^{-1} of DMSO alone, Group II received 100 mg kg^{-1} of LUT dissolved in DMSO, Group III received 0.01 mg kg^{-1} of PS-MPs, and Group IV received 0.01 mg kg^{-1} of PS-MPs and 100 mg kg-1 of LUT. On day 29, animals were euthanized under a low level ether anesthesia, and blood was taken from the retro-orbital venous plexus. Fifteen (15) minutes was spent centrifuging blood at the revolution of 3000 rpm. The plasma was kept for hormonal analysis after separation at 20°C. The testicles were taken out, weighed, and ready for biochemical analysis.

Sperm assay

Rats' sperm motility was measured using a method modified by Oyovwi et al. [11]. A coverslip was placed on top of the diluted sperm after it had been removed from the cauda epididymis and heated sodium citrates dehydrate solution. The next step was to evaluate sperm motility using a phase-contrast microscope to calculate the progression proportion of mobile, immobile, and non-mobile sperm in the same field. The Oyovwi et al. [12] method was used to measure the epididymal sperm number (ESN) in rats given PS-MPs and LUT. A nylon mesh filter was used to capture the sperm, which was then combined with a diluent and put in a hemocytometer. Using an improved Neubauer chamber and a 400x light microscope, sperm were counted. The sperm were then given five minutes to settle before being counted. For morphological analysis, the study used sperm suspension from control, PS-MPs, and LUT-treated rats. Sperm viability was evaluated using a staining process after the suspension was stained with eosin and a fast green solution. For the investigation, 400 sperm cells from each rat were employed according to Oyovwi et al. [13].

The evaluation of serum hormones

Rats treated with PS-MPs and LUT had their serum levels of LH, FSH, and testosterone measured using ELISA plates from Elabscience Biotechnology as

adopted by Asiwe et al. [14].

Evaluation of marker enzymes of testicular function

The testicular activities of acid phosphatase (ACP) [13] and alkaline phosphatase (ALP) [13] as well as glucose 6-phosphate dehydrogenase (G6PD) activity [15] using NADP+ and glucose 6-phosphate as substrates were examined in rats treated with PS-MPs and LUT.

Evaluation of the testicular oxidative stress level

Testes were centrifuged after being homogenized in phosphate buffer for the investigation. The supernatant were used to measure the activities of SOD, CAT, GSH, MDA, and xanthine oxidase (XO) [16-19]. SOD activity, catalase activity at 240 nm, and GSH levels were measured at 480 nm, 240 nm and 412 nm respectively as an indicative of rat testes' susceptibility to varied oxidative stress situations.

Assay for pro-inflammatory and anti-inflammatory biomarkers

According to the manufacturer instructions, testicular IL-1β, IL-10, and TNF-α level [16, 17, and 19] were assessed using Spectra Max TM plate readers and commercially available ELISA kits from Elabscience.

Assay for biomarkers of apoptosis

In accordance with the manufacturer's recommendations, testicular caspase-3 activity [16, 17] was assessed in rats given PS-MPs and LUT treatment using ELISA kits from Elabscience.

Statistical analysis

The study used GraphPad Prism 8 Software for data analysis, identifying significant differences in P values less than 0.05 across treatment groups and displaying the mean standard error of the mean using ANOVA and post hoc Tukey test.

RESULTS

Effect of Luteolin (LUT) on the total decrease in FSH, LH and testosterone serum levels provoked by polystyrene microplastics (PS-MPs) treatment

In Figure 1, the study found that rats treated with PS-MPs alone showed a significant drop in FSH, LH, and testosterone levels, while those treated with LUT showed significantly higher serum levels of these hormones and prolactin compared to the control group.

Effect of Luteolin (LUT) on the decrease in testicular activities of ALP, ACP, and G6PD provoked by polystyrene microplastics (PS-MPs) treatment

LUT significantly restored changes in testicular functioning indicators in rats treated with PS-MPs, despite a significant reduction in activity compared to the control group (Figure 2).

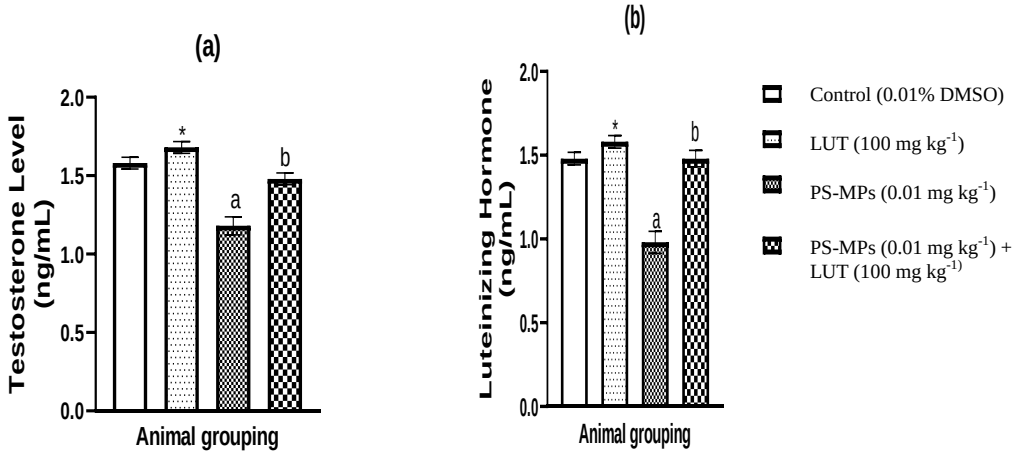

Figure 1. The effect of Luteolin on serum reproductive hormones of PS-MPs-treated rats. Each bar represents the mean ± SEM of 5 rats. *$p < 0.05$ versus control, [a]$p < 0.05$ versus LUT and [b]$p < 0.05$ versus PS-MPs. Follicle-stimulating hormone: FSH; luteinizing hormone: LH; standard error of the mean: SEM.

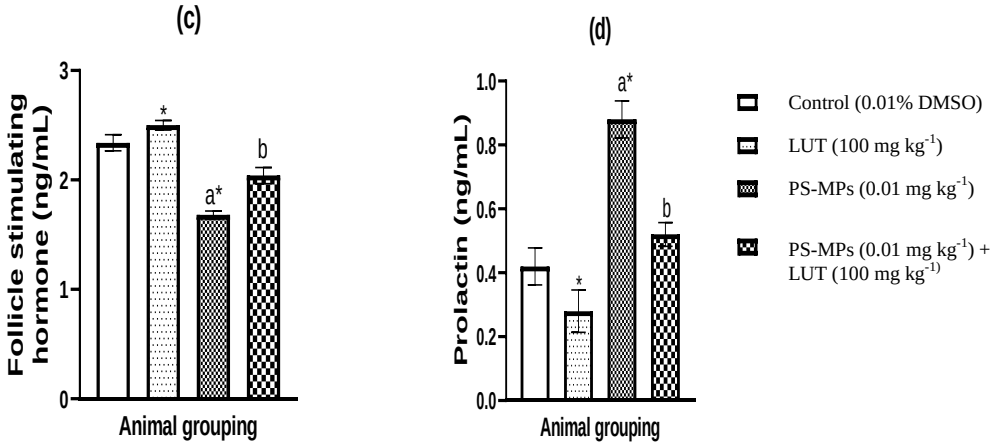

Figure 1. Continued.

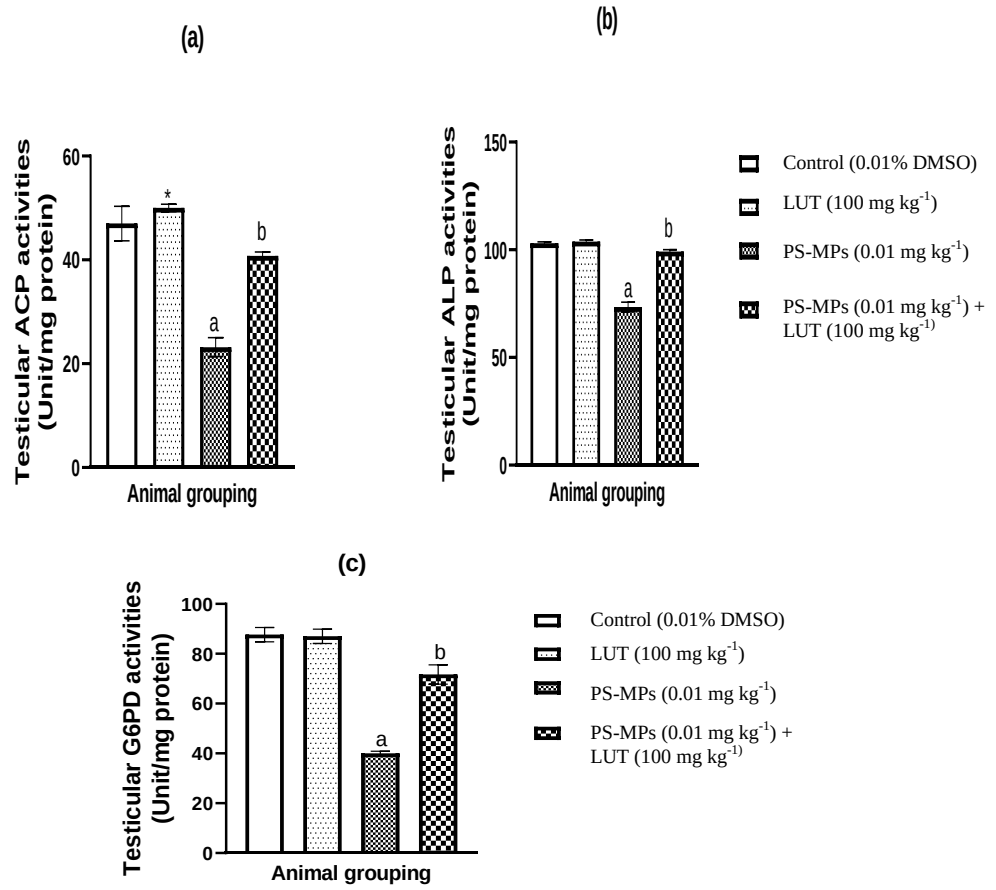

Figure 2. The effect of Luteolin on serum reproductive hormones of PS-MPs-treated rats. Each bar represents the mean ± SEM of 5 rats. *$p < 0.05$ versus control, [a]$p < 0.05$ versus LUT and [b]$p < 0.05$ versus PS-MPs. Acid phosphatase: ACP; alkaline phosphatase: ALP; glucose 6-phosphate dehydrogenase: G6PD; standard error of the mean: SEM.

Effect of Luteolin (LUT) on sperm morphological characteristics in polystyrene microplastics (PS-MPs) treated rats

In Figure 3, the study reveals that rats treated with PS-MPs showed lower sperm motility, sperm number, and viability, but increased overall sperm abnormalities. However, LUT administration reduced impairments in sperm functional characteristics and epididymal sperm quantity caused by PS-MPs.

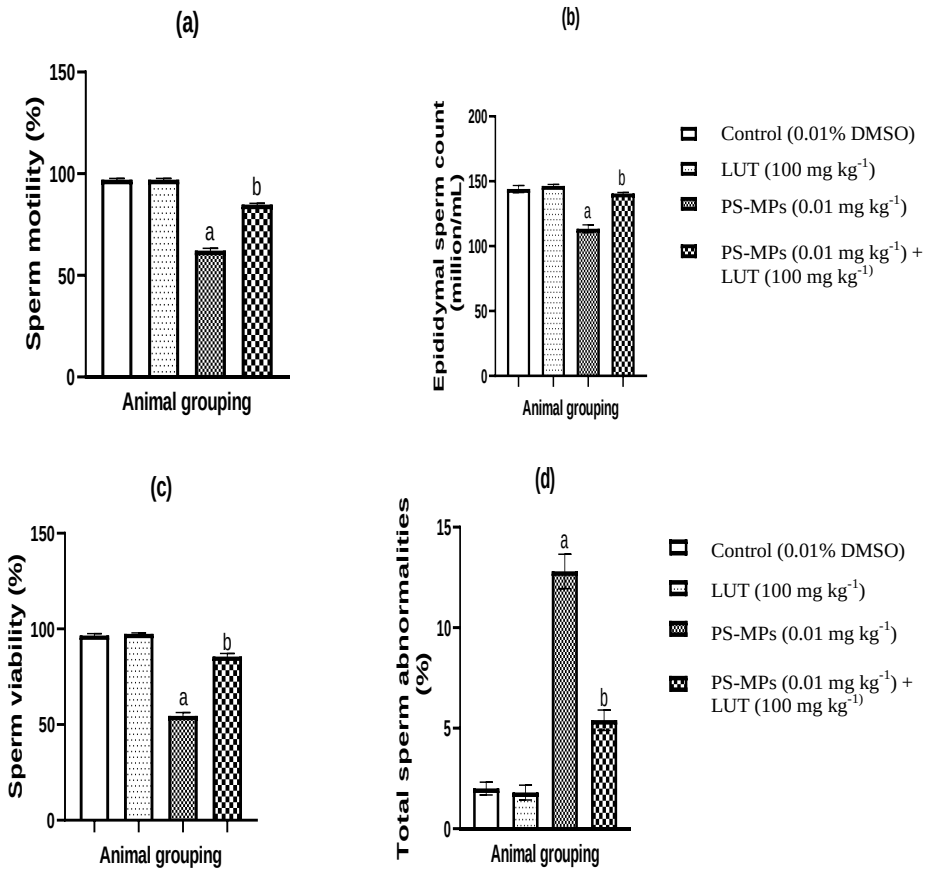

Figure 3. The effect of Luteolin on sperm morphological characteristics of PS-MPs-treated rats. Each bar represents mean ± SEM of 10 rats. $^{a}p < 0.05$ versus LUT and $^{b}p < 0.05$ versus PS-MPs. Standard error of mean: SEM.

Effect of LUT on oxidative damage in polystyrene microplastics (PS-MPs)-treated rats

The study reveals that rats treated with PS-MPs had significantly higher levels of MDA and XO in their testicles, while those treated with LUT and PS-MPs had significantly lower levels as indicated in Figure 4.

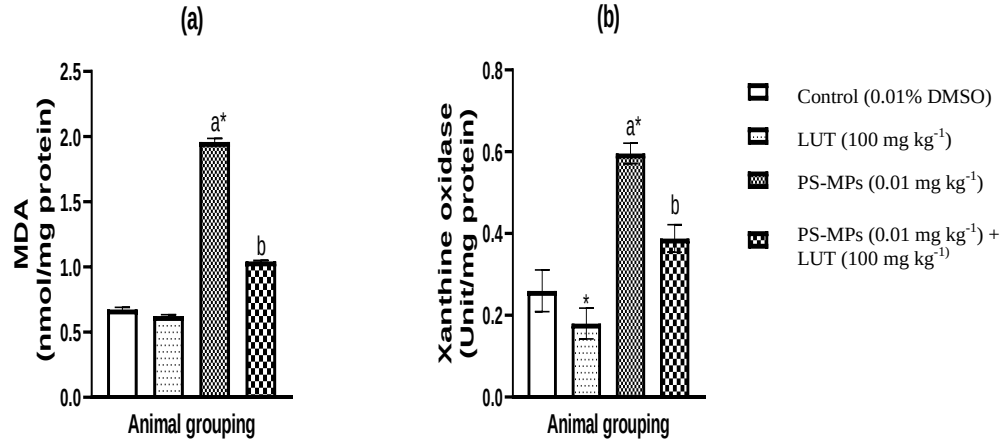

Figure 4. The effect of Luteolin on oxidative damage in PS-MPs-treated rats. Each bar represents mean ± SEM of 10 rats. $^{*}p < 0.05$ versus control, $^{a}p < 0.05$ versus LUT and $^{b}p < 0.05$ versus PS-MPs. Standard error of mean: SEM.

Effect of LUT on the non-enzymatic antioxidant status in

polystyrene microplastics (PS-MPs)-treated rats

In Figure 5, the study demonstrates that LUT therapy significantly increased the levels and activity of GSH and TSH antioxidants in rats given PS-MPs, despite significant reductions in their combined specific antioxidant activity alone.

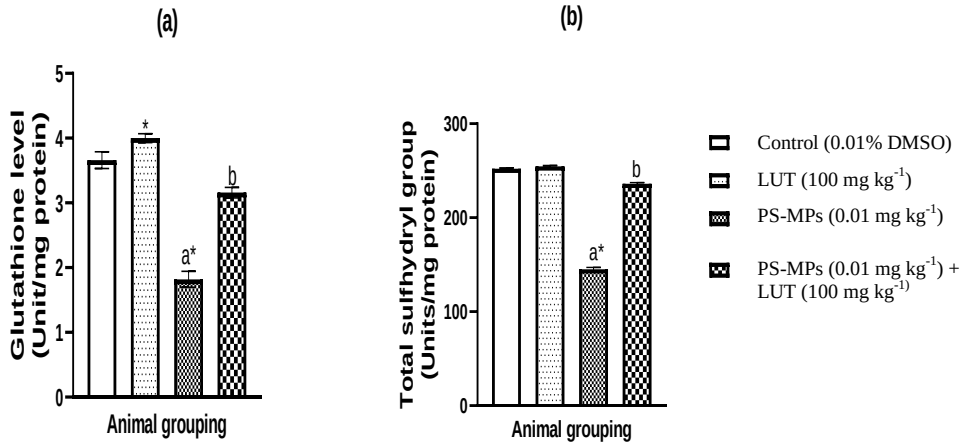

Figure 5. The effect of Luteolin on **non-enzymatic antioxidant status in** PS-MPs-treated rats. Each bar represents mean ± SEM of 10 rats. *$p < 0.05$ versus control, [a]$p < 0.05$ versus LUT and [b]$p < 0.05$ versus PS-MPs. Standard error of mean: SEM.

Effect of LUT on enzymatic antioxidant status in

polystyrene microplastics (PS-MPs)-treated rat's testes

The study reveals that LUT therapy significantly increased the levels and activity of SOD and CAT antioxidants in the testis of rats treated with PS-MPs, despite significant reductions observed in the control group (Figure 6).

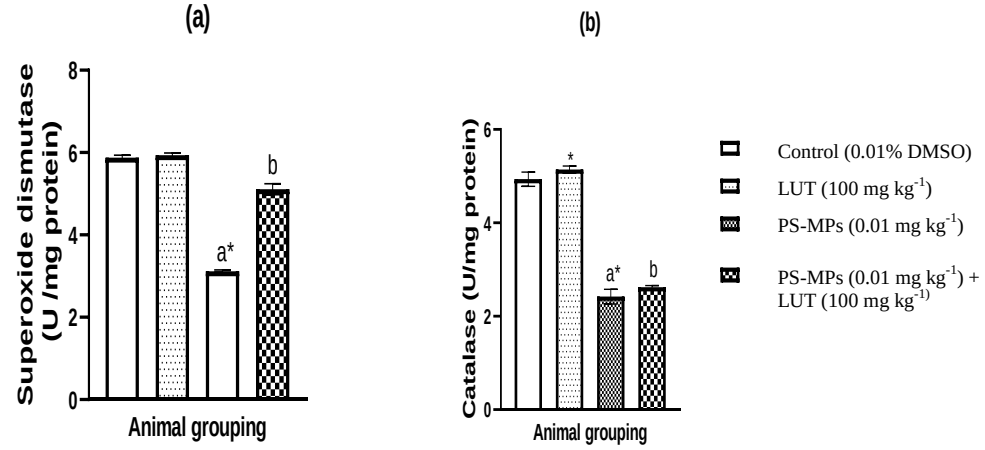

Figure 6. The effect of Luteolin on **enzymatic antioxidant status in** PS-MPs-treated rats. Each bar represents mean ± SEM of 10 rats. *$p < 0.05$ versus control, [a]$p < 0.05$ versus LUT and [b]$p < 0.05$ versus PS-MPs. Standard error of mean: SEM.

Effect of LUT on the inflammatory response in polystyrene

microplastics (PS-MPs)-treated rat's testes

In Figure 7, the study demonstrates that LUT therapy significantly impacts the inflammatory response in rats treated with PS-MPs. The control group had higher levels of IL-1β and TNF-α, while LUT reduced TNF-α

activity and increased IL-10 levels in the testis. However, the treatment with PS-MPs alone resulted in lower anti-inflammatory cytokine levels (In Figure 7).

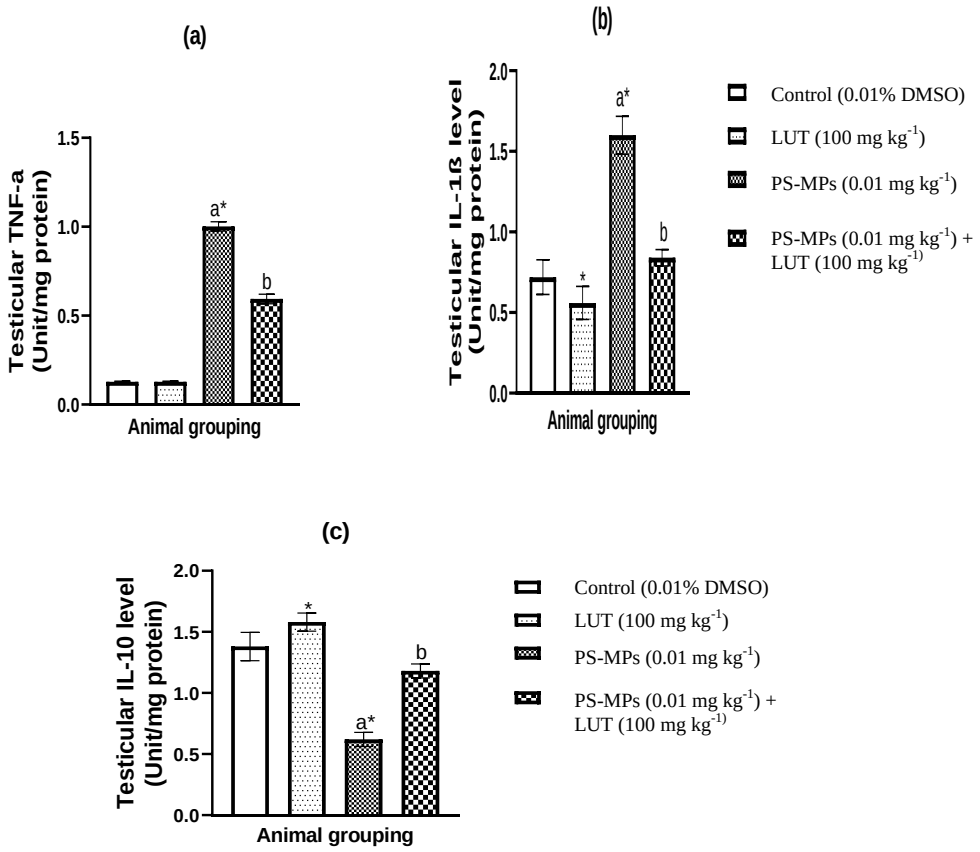

Figure 7. The effect of Luteolin on **inflammatory markers in** PS-MPs-treated rats. Each bar represents mean ± SEM of 10 rats. $^{*}p < 0.05$ versus control, $^{a}p < 0.05$ versus LUT and $^{b}p < 0.05$ versus PS-MPs. Standard error of mean: SEM.

Effect of LUT on apoptotic marker in polystyrene microplastics (PS-MPs)-treated rat's testes

Figure 8 shows the effect of LUT on apoptotic marker in PS-MPs-treated rat's testes. The study demonstrates that the administration of LUT to rats exposed to PS-MPs significantly increased the activity of caspase-3 in the testis, while the administration of LUT to rats exposed to PS-MPs resulted in a significant decrease in caspase-3 activity.

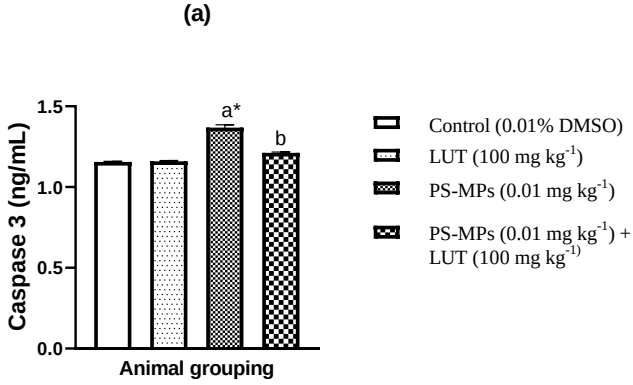

Figure 8. The effect of Luteolin on **caspase-3 activities in** PS-MPs-treated rats. Each bar represents the mean ± SEM of 10 rats. $^{*}p < 0.05$ versus control, $^{a}p < 0.05$ versus LUT and $^{b}p < 0.05$ versus PS-MPs. Standard error of the mean: SEM.

DISCUSSION

An ingredient in medicinal plants called luteolin (LUT) has anti-inflammatory, anti-oxidative, and anti-neoplastic properties [20, 21]. According to a study, LUT protects male Wistar rats from the oxido-inflammatory reactions brought on by chemotoxicants [22]. Due to the fact that PS-MPs drastically reduced serum levels of testosterone, LH, and FSH [23], it is probable that they had a gonadotoxic effect on the Leydig and Sertoli cells. Apoptosis in germ cells and Leydig cell degeneration may be responsible for the drop in testosterone levels [11, 16, and 17]. Following PS-MPs treatment, the observed increased prolactin levels disrupt the hypothalamic-pituitary-gonadal axis and block the release of gonadotropin-releasing hormone. In experimental rats, LUT also showed a protective effect against a hormonal imbalance related to reproduction that was mediated by PS-MPs.

This study aimed to determine the protective impact of LUT against PS-MPs-induced toxicity by assessing the activities of ALP, and G6PD in the testes of rats. These enzymes are critical for the stabilization of the testes, which is necessary for the development and maturation of sperm, and the metabolism of energy [13, 24-26]. Reduced testicular steroidogenesis, possibly because of less gonadotrophin production, is shown by the drop in ACP and ALP activity in rats given PS-MP treatment. ALP plays a role in producing phospholipids, nuclear proteins, and nucleic acids, and in mobilizing carbohydrate and lipid metabolites for spermatozoa [13, 27]. The decrease in ALP activity may indicate a deficiency in Sertoli and germ cell supplies of vital biosynthetic components.

According to the study, rats administered PS-MPs had much less G6PD activity than control rats, which is crucial for producing nicotinamide adenine dinucleotide (NADPH) [15]. NADPH availability and steroid hydroxylation may have been hampered by this restriction of G6PD action [28]. In addition to affecting DNA replication and GSH production, this decreased G6PD activity may also have an impact on spermatogenesis [29]. However, the fact that these testicular function markers' activities were greatly restored after receiving LUT, however, demonstrates the drug's favorable effects in reducing testicular harm brought on by PS-MPs.

Rats treated with PS-MPs demonstrated a significant decrease in sperm functional characteristics, such as motility, viability, and epididymal sperm count, which resulted in overall defective sperm. This shows that PS-MPs are harmful to the epididymis, which is responsible for moving, preserving, and maturing sperm cells [30-32]. However, LUT treatment restored spermatogenic activity and sperm characteristics in rats, supporting the protective role of LUT against PS-MPs-induced testicular and epididymal damage.

Testicular steroidogenesis, spermatogenesis, and the epididymal epithelium have antioxidant defense mechanisms that guard against oxidative stress [33, 34]. SOD, CAT, GSH, and TSH are components of this system that dismutate superoxide anions into hydrogen peroxide and water [6-18], blocking the participation of O_2 and H_2O_2 in Haber-Weiss and Fenton processes. Dysgonadogenesis can be caused by insufficient antioxidant systems [22]. Animals administered PS-MPs demonstrated a reduction in the activity of antioxidant enzymes as well as non-enzymatic antioxidants such as GSH and TSH. Furthermore, rats administered PS-MPs displayed an increase in LPO and XO activity. ROS-mediated damage to macromolecules and important enzymes involved in testicular steroidogenesis and spermatogenesis may result from increased oxidative stress. According to the study, testicular XO activity significantly increased in rats administered PS-MP treatment. This might be caused by a rise in Ca2+ levels, which activate the protease Calpain and cause it to change xanthine dehydrogenase (XDH) into XO, a form of xanthine oxidoreductase. When xanthine oxidoreductase (XO) catalyzes the conversion of hypoxanthine to xanthine, it produces reactive oxygen species such as superoxide radicals and hydrogen peroxide [35]. The anti-oxidative and peroxidation capabilities of LUT, which inhibit oxidative damage, maybe the cause of the decline in LPO levels and XO activity as well as the improvement in antioxidant status.

During spermatogenesis, inflammatory mediators control Sertoli cell activity and the mitosis and meiosis of

spermatogenic cells [36-38]. However, they may interfere with regular processes and impair spermatogenesis [36]. According to a study, rats administered PS-MPs had higher levels of pro-inflammatory indicators including IL-1β and TNF-α, which may indicate that PS-MPs cause Sertoli cells to recognize particular motifs like the Toll-like receptor.

Inflammation is significantly regulated by the anti-inflammatory cytokine IL-10 via autocrine and paracrine pathways [19]. A drop in IL-10 levels suggests inflammation, which could lead to tissue damage [19]. Nevertheless, a reduction in pro-inflammatory mediators and an increase in anti-inflammatory cytokines after LUT treatment indicate that the anti-inflammatory mechanism of LUT helps reduce the testicular toxicity brought on by PS-MPs.

A family of cysteine proteases known caspases, specifically caspase-8 and caspase-9, play a crucial role in PS-induced testicular toxicity by initiating the activation of effector caspases and inducing apoptosis in testicular cells [16, 39-41]. However, the potential protective effect of compounds, such as LUT, was demonstrated through its ability to suppress caspase-3 activation and reduce testicular cell apoptosis [42].

CONCLUSIONS

The study demonstrates that PS-MPs have the ability to modify the androgenicity and function of the male rat testes. However, LUT treatment has the potential to mitigate the damage by decreasing oxidative stress, apoptosis, and inflammation. The possible mechanism for this includes LUT's ability to scavenge, decrease inflammatory markers, and block proapoptotic proteins.

CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest for the research, authorship, and publication of this article.

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