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Evaluation of the beneficial impact of pomegranate seed extract against the testicular toxicity induced by mercuric chloride in male rats

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Abstract:

Mercury (Hg) is among the most hazardous heavy metals that adversely affect testicular tissue. Mercuric chloride ($HgCl_2$) is one of the most poisonous types of mercury. This study attempted to examine the potential ameliorative impact of pomegranate seed extract (PSE) against $HgCl_2$-induced testicular toxicity in adult rats. This study utilized a total of 40 male albino rats. Rats have been divided into four groups (10 per group): control, PSE, $HgCl_2$, and $HgCl_2$ plus PSE. PSE was provided at a dosage of 40 mg/kg bw, whilst $HgCl_2$ was given at a dosage of 1 mg/kg bw via gastric gavage. The dosage in the PSE and $HgCl_2$ groups was given for 28 consecutive days, blood samples were collected, and the animals were dissected to obtain the testes and epididymis. The findings revealed that $HgCl_2$ disrupted sexual hormones, by elevated serum FSH and testosterone levels and reduced LH levels. Additionally, rats given $HgCl_2$ exhibited significant impairment of sperm parameters (reduced sperm count and motility, along with heightened abnormalities) and elevated testicular oxidative stress, inflammation, and apoptosis. Treatment of $HgCl_2$-exposed rats with PSE demonstrated a significant restoration of male sexual hormones and sperm parameters to baseline levels. PSE effectively reduced testicular oxidative stress induced by $HgCl_2$, as seen by a significant reduction in testicular MDA levels, along with enhanced activities of SOD and CAT, and increased GSH content. Similarly, PSE modulated the testicular inflammation and histological characteristics generated by $HgCl_2$, as evidenced by reduced levels of TNF-α, IL-1β, and IL-6, along with the inhibition of COX-2 expression. The additional beneficial effect of PSE was demonstrated by the lowering of testicular apoptosis via decreased Bax expression.

Keywords: Mercury, Testis, Oxidative stress, Inflammation, Sperm parameters, Pomegranate.

INTRODUCTION

Male infertility is a complex clinical issue that considerably impacts global reproductive health. While global statistics on the exact number of individuals experiencing infertility are lacking, existing research suggests that male infertility may constitute approximately 50% of all human infertility cases (1). Although reports suggest that the aetiology of male infertility remains unclear, some scientific studies have identified the detrimental impact of oxidative stress on testicular functioning in the advancement of male factor-related pathologies (2, 3).

Heavy metals have emerged as harmful chemicals present in our environment (4). Numerous metals are recognized as highly hazardous (4, 5). Mercury (Hg) is a well-known hazardous heavy metal that has entered the environment as a result of extensive

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anthropogenic activity. Humans are exposed to mercury through contaminated food, water, air, and pharmaceutical items, including vaccines and dental amalgam fillings (6). Unintentional and occupational exposure to both inorganic mercury, such as mercuric chloride ($HgCl_2$), and organic mercury, such as methyl mercury, may cause diverse organ damage in both humans and animals (7).

$HgCl_2$ is the most hazardous form of mercury due to its propensity to form organomercury compounds with proteins (8). Exposure to $HgCl_2$ has been significantly linked to the pathophysiology of neurotoxicity (9), nephrotoxicity (10), hematotoxicity (11), and genotoxicity (12). Prior studies confirmed the testicular dysfunctions induced by $HgCl_2$ in male rats (13). Furthermore, $HgCl_2$ was observed to disrupt spermatogenesis, reduce sperm count and morphology, and produce androgen shortage (14). El-Desoky et al reported that oxidative damage, inflammation, and apoptosis have been proposed to be integral to mercury-induced testicular dysfunctions (15).

Medicinal plants constitute the primary raw materials for the global manufacturing and development of pharmaceutical goods **(16).** A variety of plants were proposed, including pomegranate. The pomegranate (*Punica granatum L.)* is a member of the *Punicaceae* family. Pomegranate is abundant in carbohydrates, vitamins, polyphenols, and minerals (17, 18). Additional reports indicate that pomegranate juice comprises various phenolic compounds, such as punicalagin isomers, ellagic acid, anthocyanins (3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin), and assorted flavanols **(19-22)**.

Research indicates that pomegranate seed extract can mitigate oxidative stress, cellular damage, and inflammation induced by specific toxins in diverse experimental models (22, 23). Pomegranate peel has been shown to enhance antioxidant capacity and mitigate liver and kidney damage in mice subjected to cadmium-induced toxicity **(24, 25)**. Pomegranate extract is extensively utilized in the treatment of several disorders, including cancer **(26)**, diabetes **(27)**, obesity **(28)**, vasculoprotection **(29)**, and hepatoprotection **(30).** Furthermore, the ingestion of pomegranate juice markedly enhances sperm quality and testosterone levels in male rats **(31)**. The ingestion of pomegranate extract markedly enhances antioxidant activity and testosterone levels in male rats **(31, 32).** Therefore, this study is planned to evaluate the ameliorative role of pomegranate seed extract (PSE) against $HgCl_2$-induced testicular oxidative stress, inflammation, and apoptosis in male rats.

MATERIAL AND METHODS

1. Mercuric chloride ($HgCl_2$)

$HgCl_2$ **(99**% purity) was supplied by Sigma Aldrich (Germany).

2. Preparation of pomegranate seed extract

Fresh pomegranate fruits were acquired at the local market in Mansoura, Egypt. Pomegranate seeds were extracted, desiccated, pulverized into a fine powder, and combined with 70% ethanol for 24 hours with continuous agitation. The ethanolic extract was acquired by performing the extraction technique three consecutive times. The resultant ethanol extracts were filtered and concentrated using a vacuum rotary evaporator (Heidolph®.VV2000) under decreased pressure at 55°C, after which the residues were lyophilized with a vacuum freeze dryer (Tilburg, Holland; 145Fm-RB). The lyophilized powder was reconstituted in distilled water on the day of administration.

3. Experimental design

This study employed forty male albino rats, each weighing between 170 and 180 g. The animals were kept in wire-bottom cages within a room featuring standard lighting; an equal light-dark cycle, a temperature of 25 ± 1°C, and 50% relative humidity. They also have access to potable water and a plentiful, nutritionally balanced diet. The rats were randomly categorized into four groups (n = 10) after a week of acclimatization as follows:

Group 1(Control**):** The rats were kept untreated and given distilled water.

Group 2 (pomegranate seed extract**):** the rats were orally supplemented with pomegranate seed extract (PSE) (40 mg/kg bw, daily, for 28 weeks) using gavage. The dose of PSE was chosen according to Setiadhi et al. (33), and then left without any treatment for 28 days.

Group 3 ($HgCl_2$**):** Rats were administered $HgCl_2$ via gavage (1 mg/kg bw, daily) for 28 days, and then left without treatment for 28 days (7).

Group 4 ($HgCl_2$+PSE**):** The rats were treated orally with $HgCl_2$ for 28 days, and followed by treatment with PSE for 28 days at the same doses as in groups 2 and 3.

Ethical approval:

All procedures were performed in accordance Faculty of Science, Kafrelsheikh University (**KFS-IACUC/252/2025).**

4. Sample collection and tissue preparation

At the finish of the experimental period, the rats had a 12-hour fast to eliminate variability in the assessed parameters. The rats were weighed and anaesthetized via an intramuscular injection of a ketamine hydrochloride (50 mg/kg body weight) and xylazine (5 mg/kg body weight) mixture. Blood was extracted from the retro-orbital venous plexus using a properly sterilized glass capillary tube. The blood sample was placed in a centrifuge tube, and the serum was isolated through centrifugation at 3000 rpm for 10 minutes, subsequently stored at −80°C for hormonal analysis. All rats were dissected, and the testes were excised and weighed. The epididymis was promptly excised and rinsed in normal saline. The right testes were preserved at −80°C for later biochemical analysis, while the left testes were fixed in 10% neutral buffered formalin for histological and immunohistochemical examination. The epididymis was utilized for the evaluation of sperm parameters.

5. Assessment of testicular weight and relative testicular weight

The absolute testis weight was measured with a precise weighing balance, while the relative testicular weight was calculated using the formula: (Testis weight/Body weight) x 100.

6. Assessment of sperm parameters (count, motility, and abnormalities)

The cauda epididymis was dissected in 5 mL of physiological saline within a petri dish and maintained on a preheated surface (35°C) for 5 minutes to facilitate sperm cell movement. The resultant liquid was deemed acceptable as a semen sample.

6.1. Sperm count

To measure the sperm count, semen was diluted with normal saline to the 101 mark. A single drop of diluted semen was placed in the Neubauer counting chamber and counted microscopically (Kruss MBL2000, A. Kruss Optronic, Germany) at a magnification of ×40, with results reported as 10^6 cells/mL (34).

6.2. Sperm motility

Sperm motility was assessed microscopically using the visual estimation technique. Approximately 20 µL of semen was deposited onto a microscope slide. The semen specimen was subsequently covered with a coverslip. The slide was positioned on the heated stage of a light microscope (Zeiss Primo Star, Carl Zeiss, Oberkochen, Germany). Sperm motility was assessed using ocular estimation for each rat. Two distinct locations were assessed, and the average motility score was documented as the final motility score (35).

6.3. Sperm morphology

To assess sperm morphology, roughly 10 µL of semen was placed on a slide, followed by the addition of 10 µL of 5% eosin dye, which was then mixed with a coverslip. The smear slide was made and thereafter rapidly dried at 50–55°C in front of the heater. All slides were examined using a light microscope at a magnification of ×400. Sperm cell abnormalities were quantified on each slide based on the morphological structures of the cells (head, mid-piece, and tail of sperm cells). Each slide contained a total of 200 sperm, and the rates of aberrant sperm were determined based on these findings (36).

7. Determination of serum testosterone, FSH, and LH levels

Serum samples were evaluated for hormone concentrations utilizing enzyme-linked immunosorbent assay (ELISA) kits designed for rats, following the manufacturer's guidelines. Cusabio Biotech Company supplied rat testosterone assay kits from Wuhan, China. Kamiya Biomedical Company supplied rat follicle-stimulating hormone (FSH) and luteinizing hormone (LH) ELISA kits (Seattle, WA, USA) following the method established by Zirkin and Chen (37)

8. Biochemical analysis in the testicular tissue

8.1. Preparation of testicular tissue homogenate

The testes were rinsed in sodium phosphate buffer (pH 7.2). The tissues were homogenized in a MagNA Lyser (Roche Diagnostics Corporation) for 30 seconds at a speed of 6000 RPM. Homogenates were centrifuged for 20 minutes at 10,000 rpm at 4°C, and the supernatants were utilized for the assessment of testicular antioxidants, lipid peroxidation, and inflammatory markers.

8.2. Assessment of antioxidants and oxidative stress

The activity of superoxide dismutase (SOD) was quantified using the method established by Marklund and Marklund (38), which involved assessing the auto-oxidation and illumination of pyrogallol at 440 nm for a duration of 3 minutes. One unit of SOD activity was defined as the quantity of protein that resulted in 50% inhibition of pyrogallol auto-oxidation. The SOD activity is quantified as U/mg of protein. Catalase (CAT) activity was assessed using the method outlined by Aebi (39), which involved measuring the hydrolysis of H2O2 and the corresponding reduction in absorbance at 240 nm over a 3-minute interval at 25 °C. CAT activity is quantified as nmol/mg protein. The glutathione (GSH) concentration in testis homogenate was assessed using the methodology outlined by Van Dooran et al. (40). GSH concentration is quantified as nmol/mg protein. Malondialdehyde (MDA) is the predominant aldehyde produced from the degradation of lipids by peroxidation in biological systems. The quantification of MDA in the testicular homogenate was conducted following the methodology established by Ohkawa et al. (41), which relies on its interaction with thiobarbituric acid (TBA) to provide a pink complex with an absorbance maximum at 535 nm. The MDA concentration is quantified in nmol/mg protein.

8.3. Determination of inflammatory markers

Inflammation in testicular tissue homogenate was assessed by measuring levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) using commercial ELISA kits (Sunred, Shanghai, China).

9. Histological examination

After dissection, the left testis specimens were promptly fixed in 10% phosphate-buffered formalin (pH 7.4) for 48 hours, followed by dehydration in escalating concentrations of ethyl alcohol and clearing in xylene. Tissues were subsequently treated according to standard procedures for paraffin embedding. Sections with a thickness of 5 µm were excised and affixed to glass slides (42). Following deparaffinization, the slides were stained with haematoxylin and eosin (H&E) and subsequently studied using bright field light microscopy (Hund Wetzlar H600/12, Germany, equipped with a Canon EOS 550D digital camera).

10. Immunohistochemical analysis of Bax and COX-2

The conventional horseradish-peroxidase immunohistochemistry method was utilized on positively charged slides of paraffin-embedded testicular tissue sections. Sections of testicular tissues, 4µm in thickness, were deparaffinized in xylene, rehydrated by falling concentrations of ethanol, and subjected to a pre-treatment with 3% H2O2 to inhibit endogenous peroxidase activity. Antigen retrieval was performed by microwaving slides for 10 minutes in a 10 mM sodium citrate solution at pH 6.0. Slides were incubated with the specific primary antibody against COX-2 (cat. no. BA0738) and polyclonal rabbit anti-Bax (Abcam, Cat: ab53154), diluted in 1% BSA/PBS pH 7.4 at a ratio of 1:100, and then rinsed with PBS. Subsequently, the sections were incubated with biotin-conjugated goat anti-rat IgG antiserum (Abcam, Cat: 182018) for 60 minutes, followed by rinsing with PBS and incubation with Streptavidin-peroxidase conjugate (Histofine Kit, Nichirei Corp) for 30 minutes. The sections were visualised with a 3,3′-diaminobenzidine tetrahydrochloride substrate chromogen solution, counterstained with haematoxylin, and subsequently examined and photographed using an Axioscop 2 plus microscope (Zeiss, Germany) equipped with a Leica DFC 320 digital camera (Leica, Germany). The frequency of cellular accumulations (dark brown) of Bax and COX-2 proteins in each group was quantified by image processing utilising an Intel® Core I7®-based computer.

11. Statistical analysis

The acquired values are presented as the mean ± standard error (SE). The data were analyzed using one-way ANOVA followed by Duncan's post hoc multiple comparisons test, utilizing the SPSS statistical software version 22.0 for Windows (IBM, Armonk, NY, USA). The significance threshold was established at $p \leq 0.05$.

RESULTS

1. Changes in body weight and testes weights

The effects of PSE and $HgCl_2$ on the body weight and testicular weight of male rats are shown in Figure 11. Rats administered $HgCl_2$ for 28 consecutive days showed a significant decrease in body weight, absolute testis weight, and relative testes weight ($P < 0.05$) compared to the control group. However, PSE treatment markedly restored the weight of the body and testes to the control's normalcy.

2. Changes in sperm parameters

Figure 2 displays the sperm count, sperm motility percentage, and sperm abnormalities of the various studied groups of rats. The obtained results showed a significant decrease in the sperm count and the percentage of sperm motility in $HgCl_2$-administered rats and a significant increase in the percentage of sperm abnormalities ($P < 0.05$) compared to the control. The reduction rate of sperm count and motility and the increased rate of sperm abnormality were 1.8, 2.4, and 2.8 times, respectively, in relation to the control. On the other hand, PSE successfully restored the sperm count and the percentage of sperm motility and abnormalities ($P > 0.05$) close to the control.

3. Changes in sexual hormones

Figure 3 illustrates the serum levels of testosterone, FSH, and LH in the examined groups of rats. In rats administered $HgCl_2$, testosterone and FSH levels were dramatically reduced, although LH levels were significantly elevated ($P < 0.05$) in comparison to the control group. Following the treatment of $HgCl_2$-administered rats with PSE, hormone levels were significantly normalized ($P > 0.05$) to those observed in the control group.

4. Changes in testicular antioxidants

Figure (4) illustrates that the activities of testicular SOD and CAT, as well as the levels of GSH and MDA, remained considerably unaltered ($P > 0.05$) in PSE-treated rats compared to the control group. In $HgCl_2$-administered rats, testicular SOD and CAT activities, as well as GSH content, were considerably reduced to nearly half, whereas MDA levels were significantly elevated, doubling compared to the control (*P < 0.05*). Conversely, following the treatment of $HgCl_2$-administered rats with PSE, the testicular antioxidants and MDA levels were considerably restored to normalcy (*P > 0.05*), comparable to the control group.

5. Changes in the testicular inflammatory markers

The concentration of testicular inflammatory markers, TNF-α, IL-1β, and IL-6 among the control and experimental groups of rats is indicated in Figure 5. The results showed non-significant changes in the concentration of these markers in PSE-supplemented rats (*P > 0.05*) when compared to the control. In $HgCl_2$-administered rats, the concentration of TNF-α, IL-1β, and IL-6 was significantly increased (by approximately 2.1, 1.5, and 1.8 times, respectively) (*P < 0.05*) compared to the control. After treatment of $HgCl_2$-administered rats with PSE, the concentration of all investigated inflammatory markers appeared significantly at the normal levels (*P > 0.05*) as in the control.

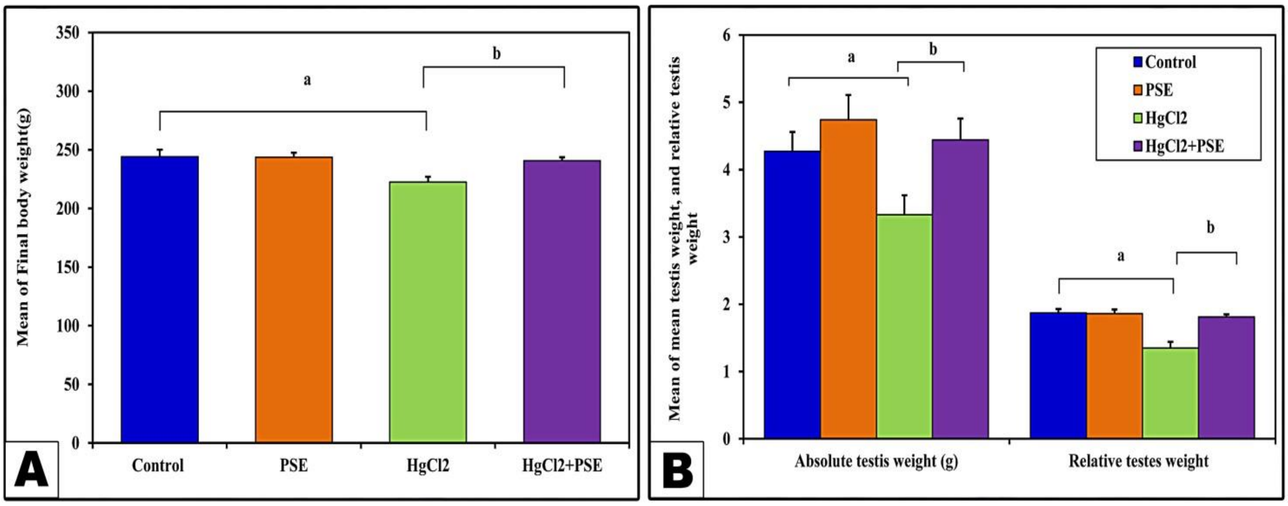

Figure (1): The mean body weights (Panel A), absolute testes weight, and relative testis weight (Panel B) among the studied groups of rats. [a] significant with the control, [b] significant with $HgCl_2$-administered rats, statistically significant at $P \leq 0.05$

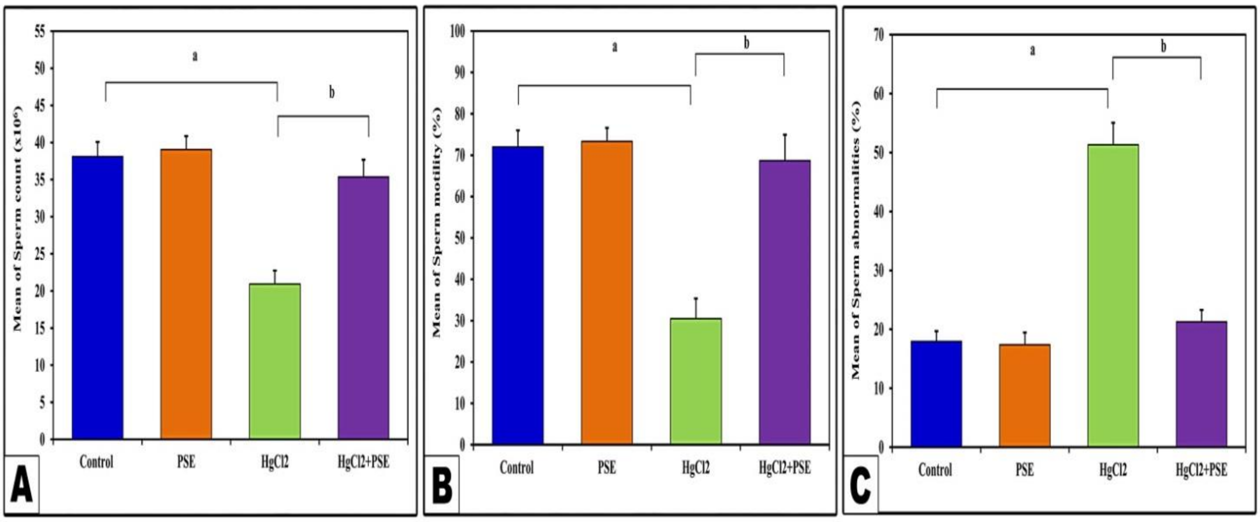

Figure (2): The mean of sperm count (Panel A), % of sperm motility (Panel B), and sperm abnormalities (Panel C) among the studied groups of rats. [a] significant difference with the control, [b] significant with $HgCl_2$-administered rats, statistically significant at $P \leq 0.05$.

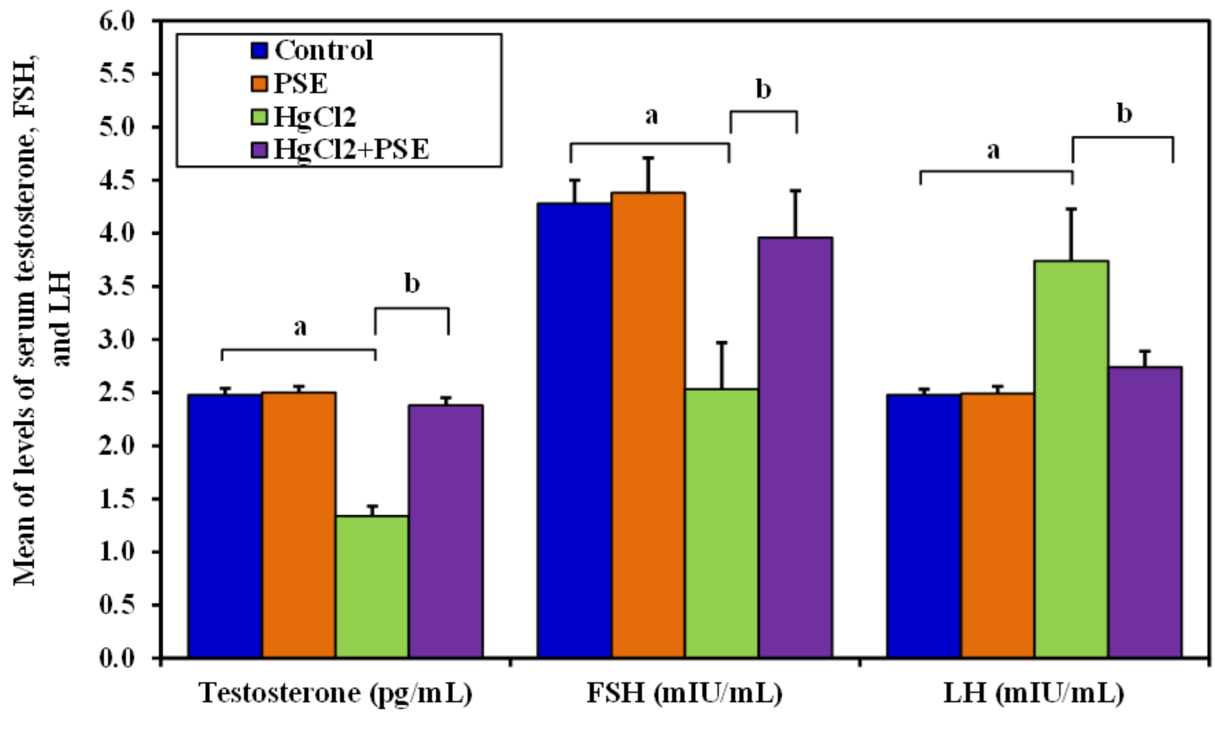

Figure (3): The mean level of serum sexual hormones, testosterone, FSH, and LH among the studied groups of rats. [a] significant with the control, [b] significant with $HgCl_2$-administered rats, statistically significant at $P \leq 0.05$

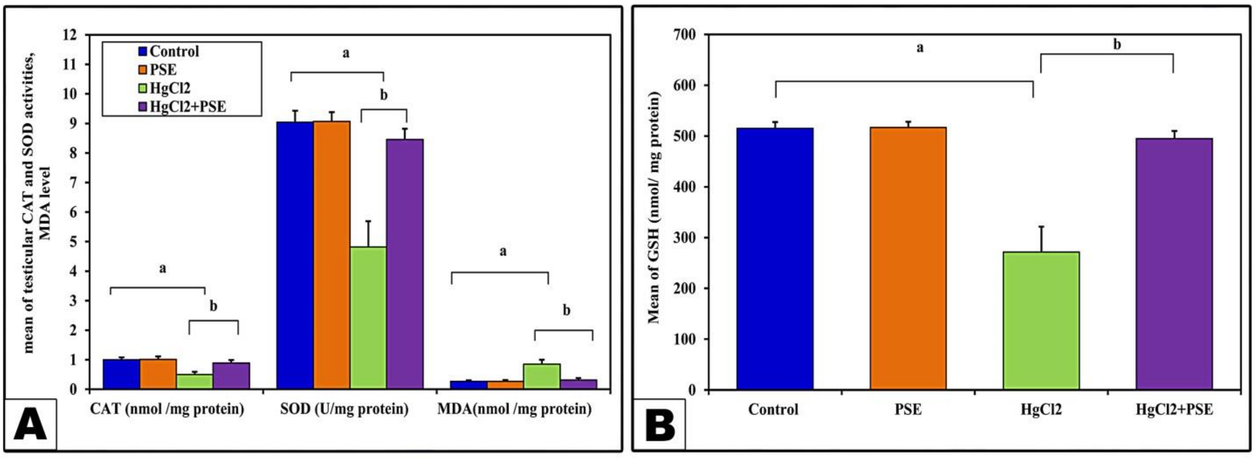

Figure (4): The mean activity of testicular SOD and CAT, MDA levels (Panel A), and GSH content (Panel B) among the studied rat groups. a: Statistically significant differences were seen with the control group, b: Statistically significant differences with $HgCl_2$-administered rats, with $P < 0.05$.

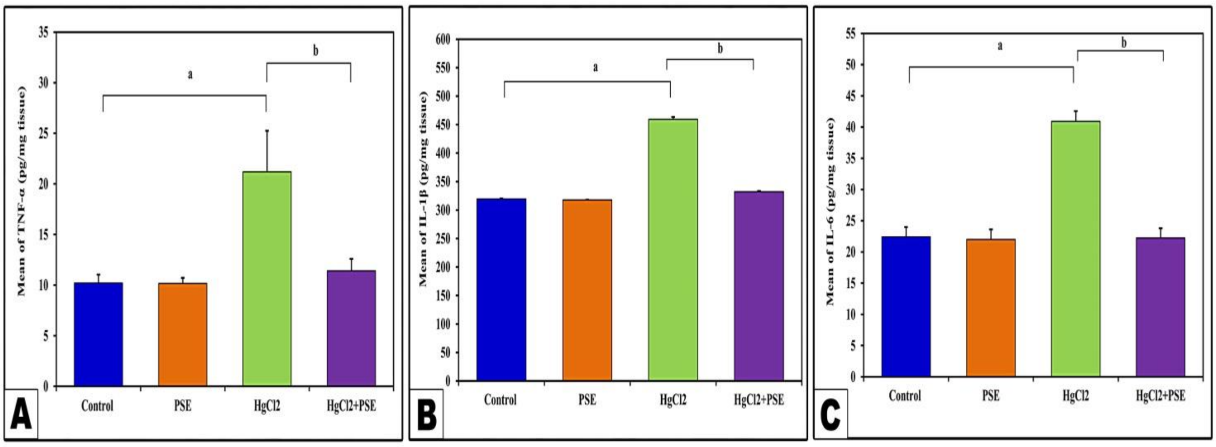

Figure (5): The mean of the testicular concentration of TNF-α, IL-1β, and IL-6 among the studied groups of rats. [a] significant with the control, [b] significant with $HgCl_2$-administered rats, statistically significant at $P \leq 0.05$

6. Histopathological findings

The testicular sections from the control and PSE-supplemented groups exhibited spherical, well-structured seminiferous tubules containing well-differentiated spermatogenic cells, characterized by a high density of spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa within the lumen. Sertoli cells looked to be situated on the basement membrane and extended towards the lumen. Leydig cells, characterized by their unique polygonal shape, eosinophilic cytoplasm, and prominent spherical nucleus, were also observed in the intertubular connective tissues (Figure 6A-D). In rats fed $HgCl_2$, the seminiferous tubules appeared dilated, while others exhibited congestion. Furthermore, the majority of the spermatogenic cells exhibited pyknosis, vacuolation, and displacement from the basement membrane. The spermatozoa were fragmented and either missing or sparse in the tubular lumen (Figure 6E-H). Nevertheless, following the administration of PSE post-$HgCl_2$, the histoarchitecture of the majority of seminiferous tubules was reinstated to the normal configuration observed in the control group, while a few tubules exhibited minor vacuolation and pyknosis in spermatogenic cells (Figure 6I-J).

7. Immunohistochemical changes in COX-2 and Bax

i. COX-2 expression (inflammatory marker)

The cells within the seminiferous tubules of both the control and PSE-supplemented groups exhibited negative expression of COX-2 protein, whereas the connective tissue between the tubules showed low expression. Conversely, the COX-2 protein exhibited a positive expression in the spermatogenic cells, approximately 8.6 times more than the control, in rats administered $HgCl_2$. This response appeared to be less pronounced during the spermatogenic phases and more pronounced in the intertubular connective tissues. PSE significantly diminished $HgCl_2$-induced testicular inflammation by suppressing COX-2 immunoreactivity, resulting in levels lower than those in $HgCl_2$-administered rats; however remained twice as high as the control group. The mean quantitative imaging analysis of COX-2-positive cells within the seminiferous tubule for the control, PSE, $HgCl_2$, and $HgCl_2$ + PSE groups was 0.739, 0.966, 6.367, and 1.445, respectively (Figure 7).

ii. Bax expression (apoptotic marker)

In the seminiferous tubules, cells from both the control and PSE-supplemented groups exhibited low immunohistochemical expression of the apoptotic marker, Bax protein. Quantitative image analysis revealed that the cell counts expressing Bax in the two groups were 0.294 and 0.301, respectively. The spermatogenic cells of rats administered $HgCl_2$ demonstrated elevated levels of Bax protein, assessed using image analysis, almost 6.5 times greater (1.889) than the control group. PSE supplementation in the $HgCl_2$-intoxicated group significantly diminished Bax protein expression, consequently lowering testicular cell apoptosis to levels comparable to the control (0.371 vs. 0.294) (Figure 8).

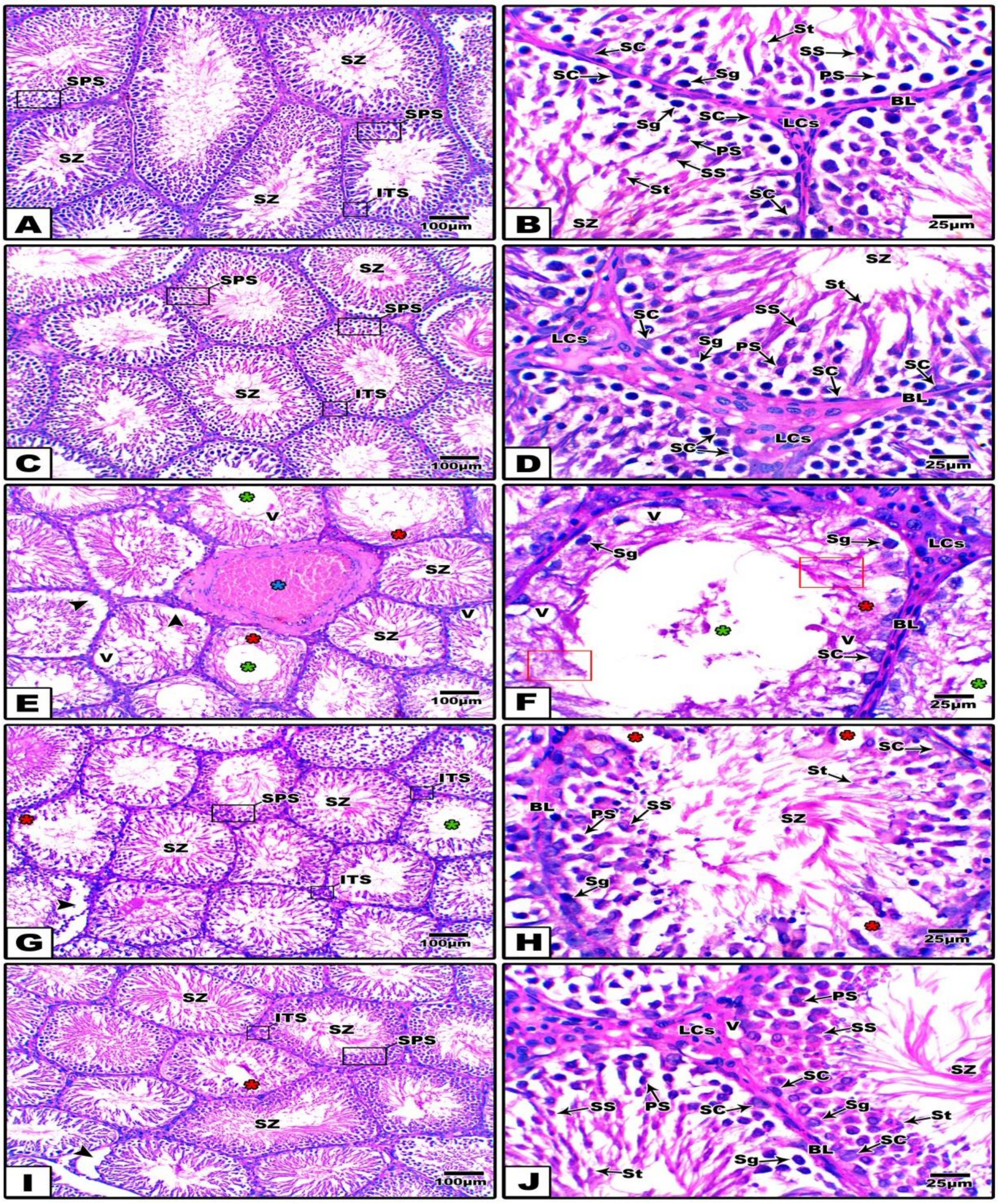

Figure 6: Images of the histological sections of testicular tissue from both the control and experimental groups. The testes sections from the control (Panels A&B) and PSE (Panels C&D) exhibit well-structured seminiferous tubules, developed spermatogenic cells (shown by black rectangles), Sertoli cells, and Ledge cells. In rats administered $HgCl_2$ (Panels E-H), certain seminiferous tubules exhibit atrophy and congestion (blue asterisk), with the majority of tubular lumens devoid of spermatozoa (green asterisks). Numerous spermatogenic cells (red rectangular) appear vacuolated (V), pyknotic (red asterisks), and dislocated (arrowheads) from the basement membrane. In rats given $HgCl_2$ and subsequently treated with PSE (Panels I-J), the histoarchitecture of the majority of seminiferous tubules is partially restored, resembling that of the control, while some tubules still exhibit minimal vacuolation and reduced sperm density. (Scale bar = 100μm for left-handed images, and 25 μm for right-handed images). **Abbreviations**: ITS: Inter-tubular space, SPS: Spermatogenic stages, LCs: Leydig cells, BL: Basal lamina, Sg: Spermatogonia, PS: Primary spermatocytes, SC: Sertoli cells, SS: Secondary spermatocytes, St: Spermatids, and SZ: Spermatozoa.

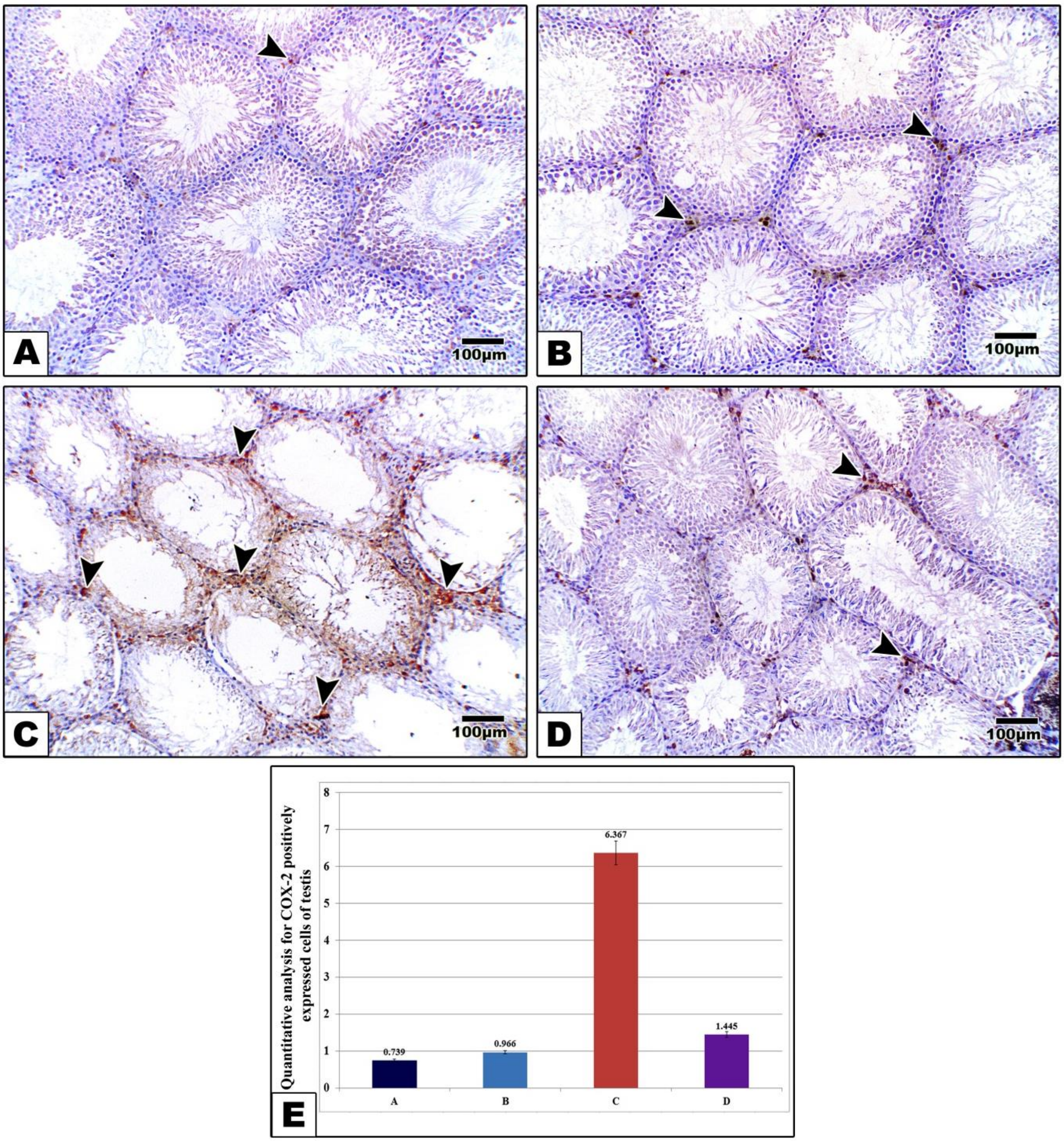

Figure 7: Immunohistochemical analysis of COX-2 in testicular tissues from both control and experimental groups. In the control group (Panel A) and the PSE-supplemented group (Panel B), COX-2 immunoreactivity (shown by a brown stain) is negative within the tubules and is only weakly expressed in the intertubular connective tissues. In contrast, the immunoreactivity of COX-2 is significantly elevated in the testicular sections of the $HgCl_2$-administered group (Panel C), whereas following treatment with PSE (Panel D), the immunoreactivity substantially decreased, demonstrating the potent anti-inflammatory impact of PSE. The quantitative analysis of COX-2-positivelly expressed cells of testis in the control (A), PSE (B), $HgCl_2$ (C), and $HgCl_2$+PSE (D) is indicated in panel E. (Arrows point to the immunolocalization of COX-2, Stain: COX-2 antibody, Scale bar = 100µm)

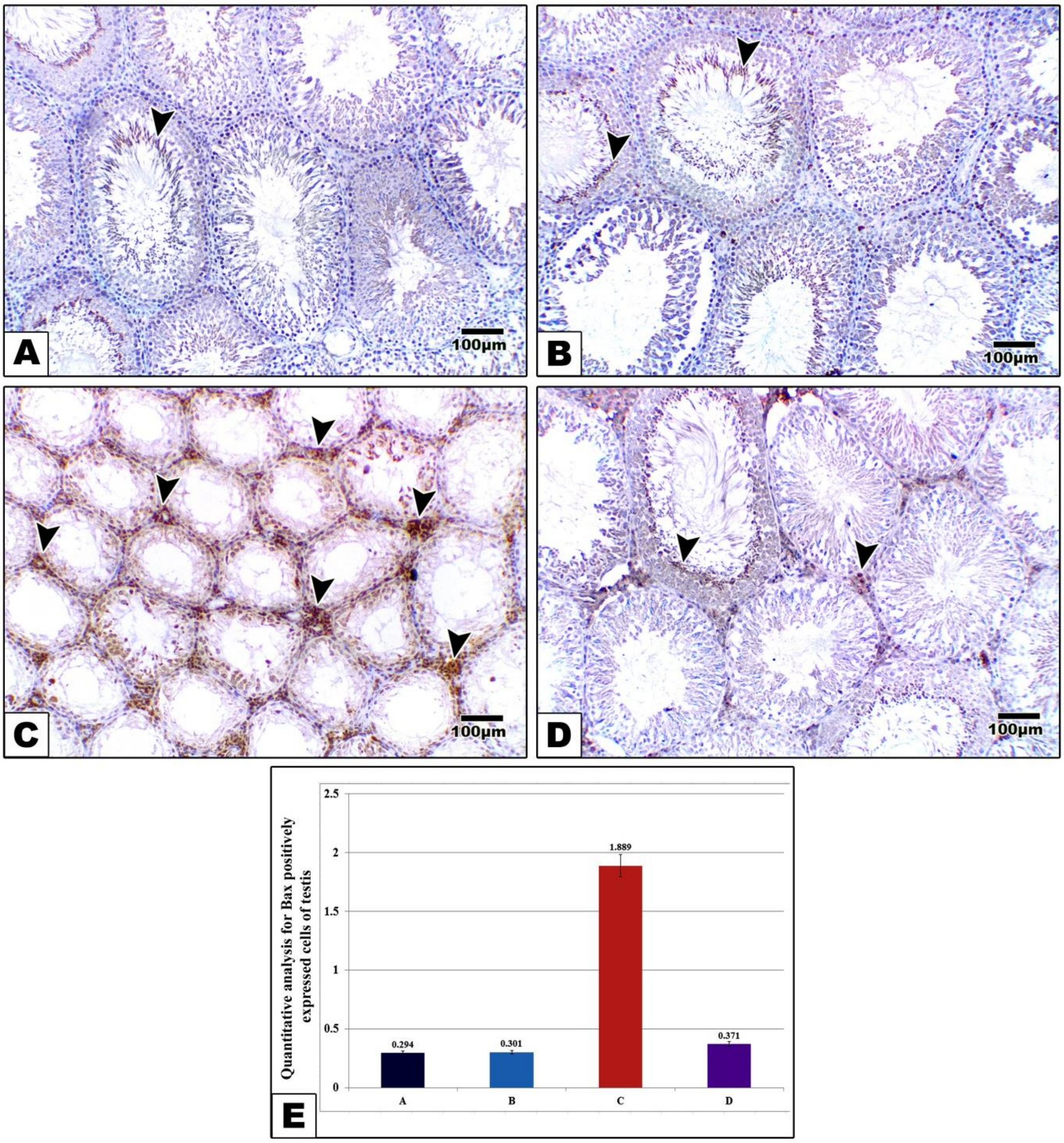

Figure 8: Immunohistochemical analysis of Bax protein in testicular tissues of both control and experimental groups. In the control group (Panel A) and PSE-supplemented group (Panel B), Bax-immunoreactivity (shown by a brown stain, quantitatively measured at 0.294 and 0.966, respectively) is notably weak within the tubules and is negatively expressed in the intertubular connective tissues. In contrast, the immunoreactivity of Bax is significantly elevated (quantitatively equal to 1.889; approximately 6.5 times greater than the control) in the testicular sections of the $HgCl_2$-treated group, whereas following treatment with PSE, the immunoreactivity substantially decreased (quantitatively equal to 0.371), indicating the potent antiapoptotic effect of PSE. The quantitative analysis of Bax-positivelly cells of the testis is indicated in panel E (A: control, B: PSE, C: $HgCl_2$, and D: $HgCl_2$+PSE). (Arrows point to the immunolocalization of Bax, Stain: Bax- antibody, Scale bar = 100µm).

DISCUSSION

Mercuric toxicity is documented to correlate with damage to the liver, kidneys, neurological system, reproductive system, renal system, and immunological system in living beings **(43).** Mercuric chloride ($HgCl_2$) is very toxic due to its capacity to permeate biological membranes and quickly create organo-mercury complexes with proteins **(44).** The mechanism by which $HgCl_2$ affects most body cells involves binding to sulphur. It substitutes the hydrogen ion in the body's sulfhydryl groups, resulting in cellular disruption **(45).** It also interacts with phosphoryl carboxyl and amide, resulting in modifications to cell membranes, enzymes, transport mechanisms, structural proteins, and nucleic acid production **(46).** This study aims to assess the protective benefits of pomegranate seed extract (PSE) against $HgCl_2$-induced testicular damage in male rats.

The results indicated a considerable reduction in both body weight and testicular weight in rats administered $HgCl_2$, consistent with prior studies **(47-49).** $HgCl_2$ was observed to diminish body weight by inhibiting the eating appetite centre, leading to decreased food intake **(50).** $HgCl_2$ has been documented to diminish testis weight by multiple mechanisms, including the disruption of spermatogenesis, damage to germ cells, impairment of steroidogenic synthesis, and the induction of oxidative stress **(15).**

The treatment of $HgCl_2$-administered rats with PSE resulted in notable enhancements in body weight and testicular weights, which were significantly different from the control group. Consistent with our findings, pomegranate consumption aids in body weight management due to its high fiber content, low caloric value, and powerful antioxidants **(28).** Pomegranate, especially its peel extracts and juice, demonstrates a protective impact on testicular weight in rats subjected to heavy metal exposure. Research has shown that pomegranate can mitigate the detrimental effects of heavy metals on testicular tissues. The protective effect is likely attributable to pomegranate's antioxidant characteristics, which may mitigate oxidative stress and inflammation induced by heavy metals **(51).**

Heavy metals induce testicular dysfunction, resulting in alterations in sperm parameters **(52).** Sperm quality is the initial criterion impacted by testicular toxicity **(53).** Prior research has indicated that $HgCl_2$ inhibits spermatogenesis, reduces sperm count, diminishes sperm motility, and elevates sperm abnormalities **(54).** The current investigation revealed a significant reduction in sperm count and percentage motility, alongside a notable increase in sperm abnormalities in rats administered with $HgCl_2$ compared to the control group. A study indicated that prolonged treatment of $HgCl_2$ influenced sperm density **(55, 56).** Mercury induces degradation of sperm shape and motility, as well as suppression of spermatogenesis **(57).** This suggests that $HgCl_2$ diminishes sperm motility and viability by inducing oxidative stress, as corroborated by Kandemir et al. **(54).** Reports indicate that mercury can traverse the blood-testis barrier and could cause testicular damage **(44).** The reduction in sperm motility due to mercury was believed to be associated with the inhibition of sperm microtubule structure **(58)**.

PSE dramatically enhanced sperm parameters towards normality in $HgCl_2$-administered rats. This indicates the beneficial effect of PSE on impaired sperm parameters induced by $HgCl_2$. Consistent with our findings, prior studies indicated that pomegranate juice and peel positively affect sperm parameters. Pomegranate juice consumption was seen to enhance sperm concentration and motility while decreasing sperm abnormalities relative to the standard reference **(31)**. PSE has been shown to enhance sperm count, motility, and reduce the rate of defective sperm in non-stressed, healthy experimental animals **(59)**. Moreover, in vitro supplementation of pomegranate juice to rooster semen enhanced sperm motility, viability, and acrosomal integrity during refrigerated storage **(60)**. Pomegranate extracts have been shown to improve

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sperm quality adversely affected by oxidative stress from lead acetate (61). Naik and Nangali observed that pomegranate juice significantly enhances sperm quality in rats subjected to mobile radioelectromagnetic radiation (62). The beneficial effect of PSE on sperm parameters may be ascribed to the presence of fatty acids and phenolic substances with antioxidant capabilities. Essential fatty acids and antioxidants are crucial for the generation of healthy sperm, since they enhance sperm membrane integrity and safeguard sperm against oxidative stress (63). Furthermore, it has been proposed that ellagic acid in pomegranate seeds exerts a protective influence on both the testes and spermatozoa. This impact may be linked to the potent action of ellagic acid against oxidative stress (64, 65). Furthermore, pomegranate has been found to possess androgenic qualities that are advantageous for mitigating male infertility concerns, including the enhancement of sperm quality (66).

Research indicates that mercury exposure results in reduced levels of FSH and LH, which may affect testosterone synthesis (67). Moreover, mercury can disrupt testicular steroidogenesis, hence exacerbating the decline in testosterone levels (68, 69). The data obtained indicated a considerable reduction in FSH and testosterone levels, accompanied by a notable increase in LH in rats treated with $HgCl_2$. Animal studies involving 30, 60, and 90 days of high-dose mercury exposure demonstrated reduced levels of testosterone, LH, and FSH (8,15). The results indicating a substantial reduction in FSH and testosterone align with the cited studies; however, the elevated LH contradicts certain recent research findings (41, 70). Consistent with our findings, several investigations identified a notable inverse correlation between chronic inorganic mercury exposure and LH levels (71, 72). The diminished serum LH levels underscore mercury's role as an endocrine disruptor, either by mimicking or obstructing hormonal effects at target receptors or by directly stimulating or inhibiting hormone production (73), particularly within the intricate hypothalamus-pituitary-gonad axis relevant to endocrine disruption (74).

PSE application mitigated the adverse effects of $HgCl_2$ on the concentrations of FSH, LH, and testosterone. This indicates that PSE may play a significant role in enhancing male fertility. Consistent with our findings, Al-Olayan et al. demonstrated significant enhancement in FSH, LH, and testosterone levels in rats administered CCl4 (75). Pomegranate peel extract was shown to enhance testosterone levels in rats subjected to experimental testicular torsion (76). Subsequent research by EL-Metwally Ibrahim demonstrated that pomegranate peels can enhance the levels of FSH, LH, and testosterone in diabetic rats (77). Pomegranate comprises tannins, phenols, and flavonoids that can directly or indirectly mitigate oxidative damage by inhibiting the excessive production of free radicals. The elevation of sex hormones in the current study attributed to the PSE may partially result from pomegranate's capacity to diminish stress hormones, including cortisol (78).

An excessive rise in free radicals impairs the antioxidant defense system, leading to oxidative stress (79, 80). Oxidative stress within physiological parameters is crucial for sperm functionality. Nonetheless, its excessive elevation is detrimental to spermatozoa (81). Elevated lipid peroxidation (LPO) is a contributing factor to reactive oxygen species (ROS), with malondialdehyde (MDA) serving as the primary marker of LPO (82). Prior research demonstrates that $HgCl_2$ significantly elevates MDA levels in testicular tissue (80). CAT and SOD are components of the enzymatic antioxidant defence system, while GSH is part of the nonenzymatic antioxidant defence system. Multiple investigations have demonstrated that $HgCl_2$ inhibits both enzymatic and nonenzymatic antioxidant components in testicular tissue (49, 83, 84). The present study associates mercury exposure with elevated oxidative stress biomarker (MDA) in testicular tissues, accompanied by a reduction in antioxidant components (SOD, CAT, and GSH). This study corroborates prior reports **(49, 84)**. The diminished antioxidant levels in testicular tissues rendered spermatogenic cells more vulnerable to oxidative stress, particularly during heightened free radical generation **(85)**.

PSE effectively mitigated the testicular oxidative stress induced by $HgCl_2$ by enhancing the levels of SOD, CAT, and GSH, while simultaneously reducing MDA levels. This aligns with prior studies that endorse the antioxidant capacity of pomegranate juice extract in alleviating oxidative stress, diminishing free radical damage, and activating the endogenous antioxidant system **(75, 86)**. Minisy et al. documented the protective effect of PSE against tramadol-induced testicular oxidative damage **(87)**. Moreover, the administration of pomegranate fruit and peel extracts has been shown to diminish lipid peroxidation, elevate GSH levels, and enhance CAT activity in the liver, kidney, and heart **(88, 89)**. Nasser et al. revealed that pomegranate juice extract mitigates cisplatin toxicity in peripheral blood mononuclear cells by scavenging free radicals **(90)**. The capacity of pomegranate fruit to elevate GSH levels and enhance SOD and catalase activities, while concurrently reducing MDA levels, signifies a restoration of testicular antioxidant capacity and integrity, thereby implying the fruit's beneficial effect against testicular oxidative damage and dysfunction. This observation may be ascribed to the reported bioactive chemicals and phenolic substances contained in PSE **(91)**. Recent reports indicate that the antioxidant properties of pomegranate are mostly due to its ascorbic acid, polyphenols, carotene, and vitamin E content, which offer a wide therapeutic range against various free radicals **(92)**.

Oxidative stress elevates cytokine production by inducing inflammation **(93, 94)**. Numerous studies have indicated that COX-2 elevates the production of tumor necrosis factor-α (TNF-α) **(93, 95-96)**. Pro-inflammatory cytokines, including interleukin-10 (IL-10), TNF-α, and interleukin-1β (IL-1β), are often produced in excess during the inflammatory response **(97-99)**. Excessive expression of these inflammatory cytokines can induce pathological situations in host defence **(100, 101)**. Research indicates that exposure to heavy metals may elevate the expression of pro-inflammatory cytokines **(102, 103)**. The present study demonstrated that $HgCl_2$ elevated the testicular concentrations of TNF-α, IL-1β, and IL-6, alongside an overexpression of COX-2 in testicular tissues, consistent with the findings of **(80)**. Reports indicate that reactive oxygen species (ROS) induce differential expression of some genes associated with certain inflammatory pathways **(104)**. The elevation in inflammation levels in the $HgCl_2$ group in the current study may be attributed to reactive oxygen species (ROS).

The treatment of $HgCl_2$-administered rats with PSE for 28 consecutive days resulted in a significant reduction in testicular inflammatory markers to levels comparable to the control group. This indicates the anti-inflammatory properties of PSE on testicular tissues. Our findings align with those of Jebur et al., who revealed that the antioxidant properties of pomegranate peel reduce inflammatory marker levels in the testes of rats intoxicated with fenpropathrin **(51)**. Furthermore, Xu et al. indicated that pomegranate extract can inhibit the synthesis of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and COX-2 in LPS-stimulated RAW264.7 macrophages **(105)**. Bousetta et al. suggest that punicic acid, a fatty acid found in pomegranate seed oil, exhibits an anti-inflammatory effect by inhibiting neutrophil activation, hence limiting lipid peroxidation **(106)**. Pomegranate seeds contain ellagic acid, a naturally occurring antioxidant with a polyphenolic structure. Ellagic acid has demonstrated anti-inflammatory properties in animal models **(107)**. Hydrolysable tannins, including punicalagin and punicalin, were extracted from pomegranate by fractionation, demonstrating significant anti-inflammatory effects **(108,109).**

Histological examinations of the testes revealed a disruption of the cytoarchitecture in rats given $HgCl_2$. The findings included atrophied and crowded seminiferous tubules, with the majority of the tubular lumens devoid of spermatozoa. Furthermore, the majority of the spermatogenic cells exhibited vacuolation, pyknosis, and dislocation from the basement membrane. Comparable histological characteristics were seen in rats given $HgCl_2$ **(110).** $HgCl_2$ was reported to induce oxidative damage in rat testicular tissues, alter testicular histology, and diminish sperm quality **(54).**

The testicular histological alterations induced by HgCl2 significantly diminished following treatment with PSE. The beneficial effect may be attributed to the bioactive compounds recognized for their diverse therapeutic properties, including tannins **(111)**, anthocyanins **(112),** alkaloids **(113),** phenolic acids **(114),** estrogenic flavonoids **(115),** and conjugated fatty acids **(116),** which are abundantly present in pomegranate **(117).**

Apoptosis is a cellular mechanism initiated by several environmental and chemical stimuli **(118).** $HgCl_2$ promotes apoptosis by facilitating the release of cytochrome c from mitochondria, leading to the activation of Caspase-3 and Caspase-9. Caspase-3 facilitates DNA fragmentation through the stimulation of cytochrome c release **(119).** Conversely, Bax is an apoptotic protein that is elevated under oxidative stress circumstances and translocates to the mitochondria of germ cells **(120).** Elevated Bax expression typically signifies heightened vulnerability to apoptosis **(121).** The current investigation revealed a significant rise in the immunohistochemistry expression of Bax in the testicular tissues of rats fed $HgCl_2$, quantitatively 6.5 times more than that of the control group. This implies that $HgCl_2$ can elicit apoptotic effects on testicular tissues. ROS is believed to effectively induce autophagy via $HgCl_2$ **(95)**. Furthermore, HgCl2 prompts testicular apoptosis by a synergy of oxidative stress and maybe endoplasmic reticulum stress, resulting in cellular damage and apoptosis. $HgCl_2$ can impair the antioxidant defence system, produce free radicals, and provoke lipid peroxidation, ultimately harming sperm cells and compromising the overall testicular architecture **(122)**.

Conversely, PSE mitigated the testicular apoptosis induced by $HgCl_2$ via the down-regulation of Bax. This indicates the ability of PSE to modulate apoptosis in testicular tissues induced by $HgCl_2$. Prior studies have investigated the anti-apoptotic efficacy of pomegranate on numerous organs, including the liver and kidney **(123,124)**, testes **(87,125)**, and spleen **(126)**. Punicalagin, a phytoconstituent of pomegranate, has been demonstrated to modulate cell death, specifically apoptosis and pyroptosis. Punicalagin treatment reduces Bax, resulting in the down-regulation of caspases associated with apoptotic cell death **(127)**.

Conclusion

Based on our findings, exposure of male rats to mercuric chloride (1 mg/kg bw, daily) for 28 consecutive days caused testicular weight loss, disruption of sperm parameters, testicular oxidative stress, inflammation, and apoptosis. In addition to this, supplementation of pomegranate seed extract could ameliorate the testicular toxicity caused by mercuric chloride through improvement of testicular antioxidants, inflammatory markers (COX-2, TNF-α, IL-1β, and IL-6), apoptotic markers (Bax and Bcl2), and sperm parameters (count, motility, and abnormalities). Pomegranate juice is a new approach for the treatment of male infertility.

Conflict of interest

The authors declare no conflicts of interest.

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