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Protective effects of *Withania somnifera* against cyclophosphamide-induced testicular damage in rats

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Objective: Cyclophosphamide (CP) is an alkylating agent commonly used in cancer treatment. It is known to have detrimental effects on the reproductive system, including the potential to cause infertility. Recently, herbal remedies have gained traction as a complementary approach to addressing these side effects. In this study, our goal was to investigate whether the aqueous-alcoholic extract of *Withania somnifera* (WS) could mitigate the adverse impacts of CP on testicular tissue.
Methods: Animals were randomly assigned to one of the following groups: control, WS (500 mg/kg), CP (100 mg/kg), CP+WS pre-treatment, and CP+WS post-treatment. WS was administered orally through gavage for 1 month. We assessed sperm parameters, testicular histopathology, and the expression of the *Bax* and *Bcl2* genes in the experimental groups.
Results: Sperm parameters (including count, viability, and motility), the number of spermatogonia, the seminiferous tubule diameter, and *Bcl2* gene expression, significantly decreased after CP injection ($p<0.05$). Conversely, the number of immotile sperm and *Bax* gene expression significantly increased ($p<0.05$). Treatment with WS, especially when administered as a pre-treatment, ameliorated the sperm parameters, histological alterations, and the expression of apoptosis-related genes ($p<0.05$).
Conclusion: The data suggest that WS may mitigate the detrimental effects of CP on testicular tissue by reducing apoptosis. Consequently, WS has the potential to be used as an adjunctive therapy to reduce the complications associated with CP treatment.

Keywords: Apoptosis; Ashwagandha; Cyclophosphamide; Sperm analyses; Testis

Introduction

Anti-cancer treatments such as chemotherapy and radiation therapy can damage germ cells, leading to infertility in cancer survivors. Consequently, the need for infertility treatment after cancer therapy is increasing due to the improved survival rates of cancer patients [1]. Cyclophosphamide (CP) is a chemotherapy agent commonly used in cancer treatment. It acts as an alkylating agent after being metabolized in the liver. It can cross the blood-testicular barrier and potentially harm the reproductive organs [2]. Animal studies have demonstrated that CP treatment can result in oligospermia, reduced testicular weight, and biochemical and histological alterations in the testis and epididymis [3]. Furthermore, variable levels of oligospermia or azoospermia have been observed in men who have received CP treatment [4]. Cell death, apoptosis, and necrosis are harmful consequences of CP administration [5,6].

The exact mechanism by which CP affects the testicles remains unclear; however, numerous studies in this area indicate that CP induces abnormalities and hormonal changes via DNA damage, protamine oxidation, lipid peroxidation of membranes, oxidative stress in

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mitochondria, adenosine triphosphate depletion, and the modulation of heat shock proteins [7].

The administration of antioxidants during chemotherapy may help to mitigate the side effects of CP on non-target tissues [8]. The potential of plants and other natural resources has long captivated researchers, especially in the treatment of diverse medical conditions. Consequently, herbal remedies and natural antioxidant supplements could play a beneficial role in decreasing the toxicity associated with CP [9].

Withania somnifera (WS), commonly known as ashwagandha, Indian ginseng, winter cherry, ajagandha, and kanaje hindi, is a member of the Solanaceae family and is indigenous to the Eastern Mediterranean and South Asia [10]. WS has antioxidant and anti-apoptosis properties [11], and there is also literature supporting the efficacy of WS in enhancing male fertility [12]. WS has been shown to improve semen quality by modulating reproductive hormone levels and reducing oxidative stress in the seminal plasma of infertile males. It enhances both the quantity and quality of sperm by preventing lipid peroxidation and regulating sex hormone levels [13]. Additionally, WS treatment has been found to increase sperm motility in individuals who smoke or are under physiological stress [14]. Furthermore, in rats subjected to arsenic treatment, WS has been shown to preserve testicular structure and maintain normal function [15]. Considering these beneficial effects of WS, the aim of this study was to investigate the potential of this substance to counteract the adverse effects of CP on testicular tissue and sperm parameters.

Methods

The research conducted adhered to the guidelines established by the National Research Council. Approval for this study, including the animal care methods, was granted by the Ethics Committee of Arak University of Medical Sciences (approval ID: IR.ARAKMU.REC.1400.091). The animals were accommodated in plastic cages situated within an environment that maintained a 12-hour light/dark cycle and a temperature controlled between 22 to 25 ℃. They had free access to food and water throughout the study. The data that support findings of this study are available from the corresponding author upon reasonable request.

1. Experimental design

In this study, 30 adult male Wistar rats (200 to 250 g, Pasteur) were randomly assigned to five groups (n=6): control; CP (100 mg/kg, single dose) [16]; WS (500 mg/kg) [17,18]; CP+WS post-treatment; CP+WS pre-treatment (Figure 1). WS was given orally for 1 month [19].

2. Extraction of WS

The roots of WS were acquired from a reputable store and authenticated by the herbarium of Arak University of Medical Sciences. The dried WS was ground into a powder, extracted through maceration with 70% ethanol, and then concentrated using a rotary evaporator to yield a viscous extract [15]. This extract was dissolved in saline, and each animal was administered a daily dose of 500 mg/kg WS for 1 month.

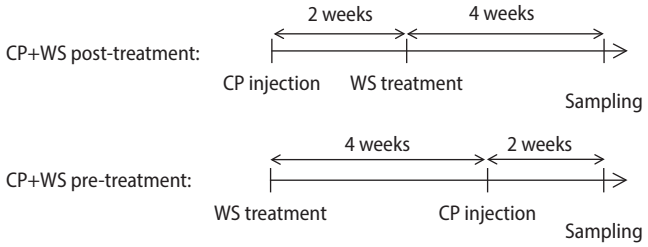

Figure 1. Timeline of the experiment. CP, cyclophosphamide; WS, *Withania somnifera*.

3. Sample collection

One month after treatment, the experimental animals were euthanized under deep anesthesia. The left cauda epididymis and testis were excised. The tail of the epididymis was utilized for the assessment of sperm parameters. For histological examination, one testis was fixed in Bouin's solution, while the other was flash-frozen in liquid nitrogen and stored at –80 ℃ for subsequent analysis.

4. Sperm parameter analysis

The cauda epididymis was finely minced and then incubated in Ham's/F12 medium (Gibco) for 20 minutes at 37 ℃. Subsequently, the proportion of motile sperm was determined by analyzing the pattern of sperm movement [20]. The sperm suspension was diluted at a 1:10 ratio with a fixative solution (1% formalin in phosphate buffered saline), and sperm concentration was quantified using a Neubauer counting chamber [21]. To assess sperm vitality, the Eosin-Nigrosin staining method (Merck) was employed. Through this staining process, non-viable sperm were stained red, while viable sperm remained unstained [22].

5. Testicular histomorphometry

After tissue fixation with Bouin's solution (Merck), the samples were dehydrated, embedded, sectioned, and subsequently stained with hematoxylin and eosin. For each section, at least 100 seminiferous tubules from each animal were analyzed [23], and the number of spermatogonia, as well as the seminiferous diameter, were determined using Image J Analysis software ver.1.33 (National Institutes of Health) [24]. Spermatogonial cells, identifiable by their dark nuclei, are located near the basement membrane.

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6. Quantitative reverse transcriptase polymerase chain reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was utilized to evaluate the expression levels of the *Bax* and *Bcl2* genes, as well as *CycloA* (serving as an internal control), across the various experimental groups. To this end, total RNA was first isolated from testicular tissue using the RNX-plus reagent (Yekta Tajhiz Azma), strictly following the manufacturer's protocol. The RevertAid First Strand cDNA Synthesis Kit (Aryatous) facilitated the synthesis of cDNA from 2 µg of the extracted RNA in a final reaction volume of 20 µL. The qRT-PCR reaction mixture included the following components: cDNA, 5 mmol/L of each forward and reverse primer, and SYBR green reagent (Yekta Tajhiz Azma), with primer sequences detailed in Table 1. Samples were run in duplicate and processed using the LightCycler 96 System (Roche). Relative gene expression was subsequently quantified employing the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).

7. Statistical analysis

One-way analysis of variance followed by the Tukey *post hoc* test was used to evaluate differences between the experimental groups. Data are given as mean±standard deviation.

Results

1. Sperm parameter analysis

Our findings demonstrated that in rats treated with CP, the sperm count and the percentage of motile and viable sperm were significantly lower than those in the control group (p<0.001). Conversely, the percentage of immotile sperm was significantly higher (p<0.001). In rats receiving WS treatment, both sperm quantity and quality were dramatically increased. Sperm parameters also significantly improved in the WS pre-treatment group compared to the WS post-treatment group (Table 2).

Table 1. Primer sequences of the genes analyzed in this study

Gene		Primer sequences (5'-3')	Product length (bp)
Bax	Sense	GCTACAGGGTTTCATCCAG	
	Antisense	TCCACATCAGCAATCATCC	174
Bcl2	Sense	AGCGTCAACAGGGAGATG	
	Antisense	CCACAAAGGCATCCCAG	118
CycloA	Sense	GGCAAATGCTGGACCAAACAC	
	Antisense	TTAGAGTTGTCCACAGTCGGAGATG	196

2. Testis histomorphometry evaluation

Our findings indicate that animals treated with CP exhibited significantly fewer spermatogonia (p<0.001) (Figure 2) and a reduced diameter of the seminiferous tubules compared to the control group (p<0.001) (Figure 3). In rats treated with WS, both pre-treatment and post-treatment groups showed significant preservation of spermatogonia relative to the CP group (p<0.001). Notably, the quantity of spermatogonia was higher in the WS pre-treatment group than in the WS post-treatment group (p<0.001) (Figure 2). Additionally, pre-treatment with WS significantly increased the diameter of the seminiferous tubules compared to the CP group (p<0.0001) (Figures 3, 4).

3. Expression of the *Bax* and *Bcl2* genes

To investigate the anti-apoptotic effects of WS, we analyzed gene expression in testicular tissue, focusing on an apoptotic gene (*Bax*) and an anti-apoptotic gene (*Bcl2*), using qRT-PCR. As anticipated, CP significantly upregulated *Bax* expression (p<0.001) (Figure 5) and downregulated *Bcl2* expression (p<0.001) (Figure 6) 1 month post-injection. The mRNA levels of *Bax* were notably lower in the groups treated with WS compared to the CP group (Figure 5). Conversely, *Bcl2* gene expression was significantly higher in both the WS-treated and WS pre-treated groups (p<0.001) (Figure 6).

Discussion

In this study, we evaluated the effects of WS on the side effects of CP in rat testicles. We found that WS administration to rats with CP-induced damage reduced apoptosis and improved testicular histology and sperm parameters. CP is a chemotherapy drug that tar-

Table 2. Sperm parameter analysis in different experimental groups

Group	Count ($\times 10^6$)	Viability (%)	Progressive (%)	Immobility (%)
Control	43.75±9.6	81.58±5.5	61.0±7.4	7±2.7
CP	12.25±2.5[a)]	31.76±12.6[a)]	16.25±6.3[a)]	93.33±11.5[a)]
WS	41.17±1.4[b)]	97.63±1.7[b)]	67.50±2.9[b)]	10.±5.5[b)]
CP+WS pre-treatment	33.4±5.8[b)]	87.92±5.0[b)]	62±18.2[b)]	8.33±2.6[b)]
CP+WS post-treatment	31.65±7.3[b)]	66.45±9.7	23.7±5	16.25±4.8[b)]

Values are presented as mean±standard deviation.
CP, cyclophosphamide; WS, *Withania somnifera*.
[a)]Significant vs. control group; [b)]Significant vs. CP group.

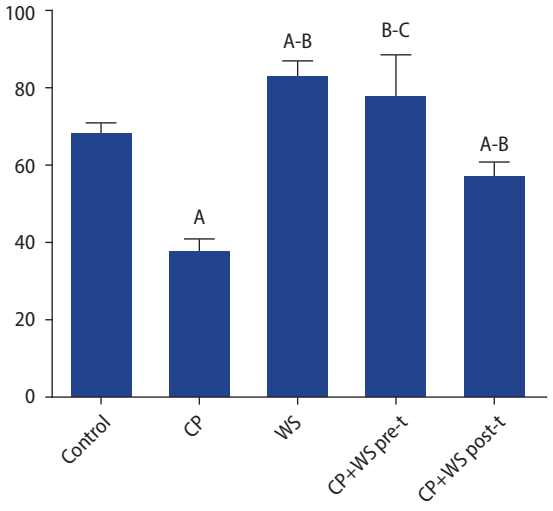

Figure 2. Comparison of the number of spermatogonia in different experimental groups. Following *Withania somnifera* (WS) therapy, there were noticeably more spermatogonia. This increase in the pre-treatment (pre-t) group occurred more rapidly than in the post-treatment (post-t) group. CP, cyclophosphamide; A, significant vs. control; B, significant vs. CP; C, significant vs. post-t group.

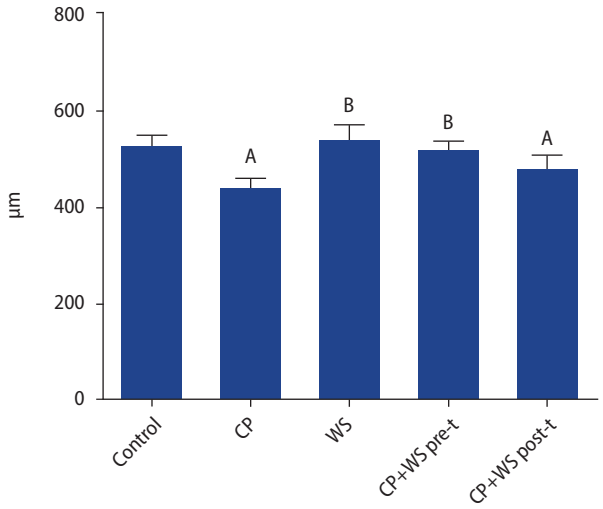

Figure 3. Comparison of the seminiferous diameter 1 month after *Withania somnifera* (WS) therapy. The WS and WS pre-treatment (pre-t) groups showed a significantly higher increase in seminiferous diameter than the cyclophosphamide (CP) group. post-t, post-treatment; A, significant vs. control; B. significant vs. CP.

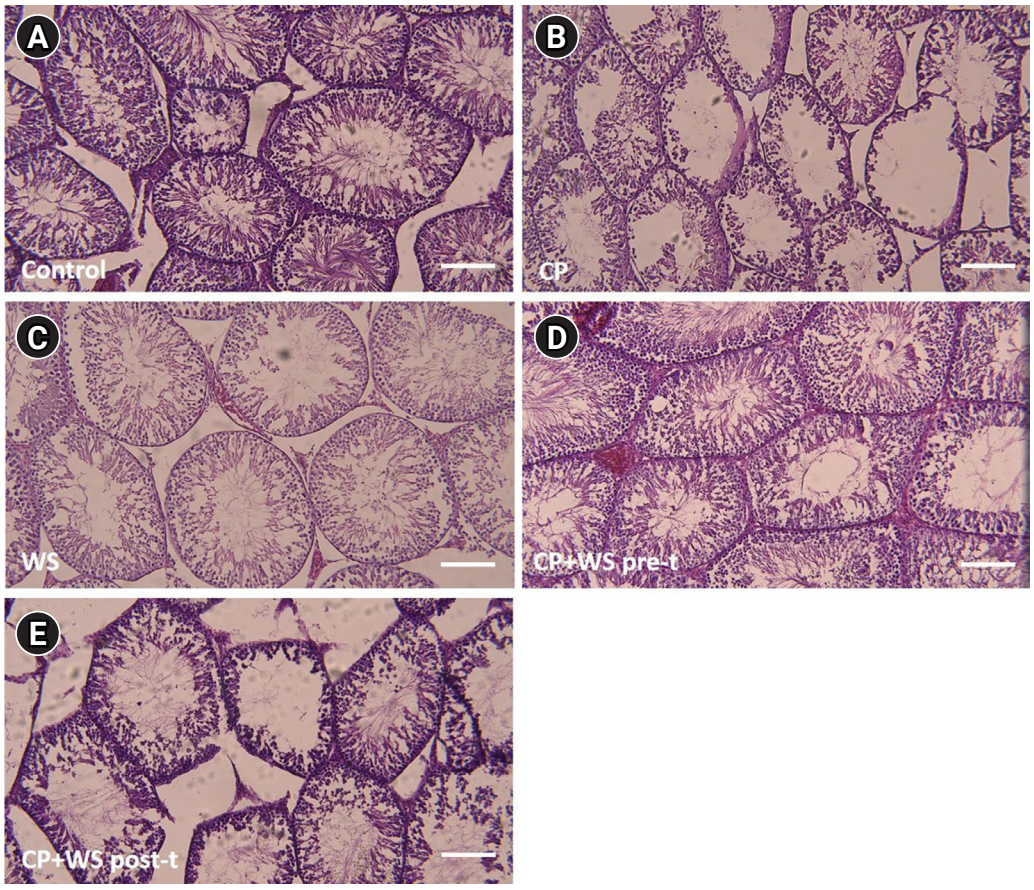

Figure 4. Light microscopy of testes tissue stained with H&E in different experimental groups. (A) Control, (B) cyclophosphamide (CP), (C) *Withania somnifera* (WS), (D) CP+WS pre-treatment (pre-t), and (E) CP+WS post-treatment (post-t) group. The bar represents 50 μm.

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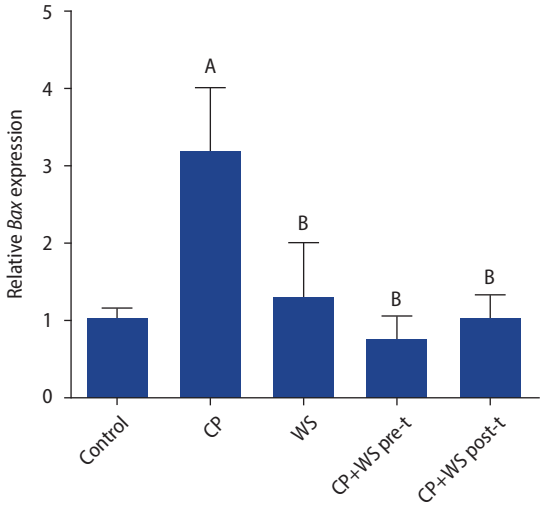

Figure 5. Real-time polymerase chain reaction analysis of Bax mRNA expression in different experimental groups. Following treatment with *Withania somnifera* (WS), the expression of *Bax* was drastically reduced in all treatment groups compared to the cyclophosphamide (CP) group. pre-t, pre-treatment; post-t, post-treatment; A, significant vs. control; B, significant vs. CP.

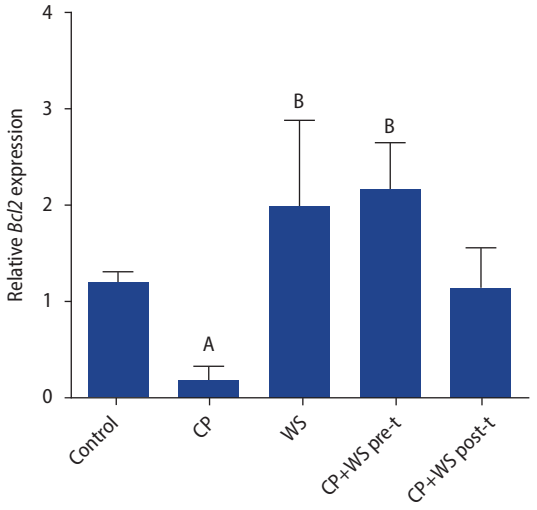

Figure 6. The mRNA expression of *Bcl2* 1 month after *Withania somnifera* (WS) therapy. The WS and WS pre-treatment (pre-t) groups showed a significantly higher increase in the expression of Bcl_2 than the cyclophosphamide (CP) group. The WS post-treatment (post-t) also increased the expression of *Bcl2*, but it was not significant. A, significant vs. control; B, significant vs. CP.

gets proliferating cells in the testis, such as spermatogonia, and has adverse effects on reproductive health [2]. Our data showed that 1 month after a single dose of CP, there was a decline in sperm quality, a reduction in the number of spermatogonia, and a decrease in the diameter of the seminiferous tubules, while the expression of genes associated with apoptosis was elevated. CP is thought to exert its damaging effects on DNA and spermatogenesis by specifically targeting protamines [7] and to promote apoptosis by upregulating the expression of several genes, including Bax and some caspase family members [25]. Furthermore, CP reduces the levels of key antioxidant molecules in the testis—glutathione, superoxide dismutase, and catalase—thereby increasing the vulnerability of the testis to oxidative damage [25]. Several studies have suggested the use of substances, primarily composed of antioxidants from chemical or natural sources, as prophylactic treatments to protect against CP-induced reproductive toxicity [7].

In this study, we used an aqueous-alcoholic extract of WS root to mitigate the detrimental effects of CP on the testis. It is important to note that WS influences male fertility [26]; however, no research has yet explored its effects on testicular damage induced by CP. WS is a natural compound known for its anti-inflammatory, anti-apoptotic, and antioxidative properties, and withanolides are its primary bioactive constituents [18]. For our experiments, the animals received an oral dose of 500 mg/kg of the WS aqueous extract via gavage. This dosage was selected based on prior studies that have confirmed its effectiveness. Notably, oral administration of WS root extract at doses of 500 and 1,000 mg/kg has shown beneficial effects in the treatment of systemic lupus erythematosus [27]. Furthermore, a daily oral dose of 500 mg/kg of WS extract in male Wistar rats has been found to inhibit oxidative stress in the hippocampus [28], while also reducing tissue inflammation in Wistar rats with methotrexate-induced arthritis [29]. Pawar et al. [30] demonstrated that treatment with WS extract led to a significant increase in antioxidant enzymes. At a high concentration (500 µg/mL), the extract inhibited nitric oxide (NO), hydrogen peroxide (H_2O_2), and lipid peroxidation [30].

Our results indicated that pre-treatment with WS mitigated testicular damage and improved all sperm parameters in rats with CP-induced damage. Additionally, it appeared to reduce apoptosis in testicular tissue by decreasing Bax expression and increasing Bcl2 expression. Ambiye et al. [31] demonstrated that oligospermia patients who received 675 mg of WS three times daily for 90 days experienced improvements in sperm count, motility, semen volume, and serum testosterone levels. In another study by Nasimi Doost Azgomi et al. [32], men with idiopathic infertility who took six capsules containing 5 g of WS daily for 90 days showed recovery in sperm parameters, including count, motility, and morphology.

Gupta et al. [33] showed that the metabolic profiles of seminal plasma in infertile patients, which include alanine, lactate, citrate, glutamate, glycerophosphocholine, histidine, and phenylalanine, significantly improved after 3 months of oral administration of WS. These improvements correlated with changes in enzymatic, hormonal, and clinical variables [33]. It is important to note that the

mechanisms by which WS affects the testis are complex and multifaceted. WS appears to protect the reproductive system via two distinct mechanisms [18]. The first is an oxidative mechanism that involves the regulation of antioxidant enzymes and the modulation of antioxidant activity. The second is a non-oxidative mechanism that affects the hypothalamic-pituitary axis in the pituitary gland, along with its anti-stress activities mediated through this axis [34]. In the latter case, the hypothalamic-pituitary-adrenal axis seems to be the primary pathway, as indicated by decreased cortisol levels and increased hormone levels in men, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which reduce stress levels and enhance fertility. Additional research indicates that the active compounds in WS stimulate the thyroid gland to secrete more triiodothyronine (T3) and thyroxine (T4) hormones, leading to a decrease in thyroid-stimulating hormone levels via the hypothalamic-pituitary-thyroid axis [35].

It is believed that the antioxidant activity of WS plays a significant role in preventing inflammation [35]. WS also exhibits anti-inflammatory properties by inhibiting cyclooxygenase activity and suppressing the activation of inflammatory markers mediated by nuclear factor κB (NF-κB) and interleukins [36]. Furthermore, WS modulates various signaling pathways, including NF-κB, Janus kinase (JAK)-signal transducer and activator of transcription (STAT), and activating protein-1 (AP-1), to reduce inflammation [37]. Its derivatives have been found to affect the testis, male reproductive cells, and the homeostasis of endocrine glands, potentially enhancing male fertility [18]. Studies have shown that WS treatment increases the levels of zinc, copper, gold, and iron ions in seminal plasma. These ions improve semen quality by acting as essential cofactors for enzymes in the seminal fluid [38]. These cofactors are crucial for the proper functioning of testicular antioxidant enzymes, which are all reduced following CP injection [25].

WS extract can also imitate the action of gamma-aminobutyric acid (GABA) [13]. By acting on GABA receptors, WS extract promotes the secretion of gonadotropin-releasing hormone and causes the release of FSH, LH, and testosterone [31]. These hormones are essential for normal spermatogenesis and semen quality [31], which are all disturbed by CP [7]. It has been shown that withaferine, the primary constituent of WS, exerts its neuroprotective effects by deactivating Bax and activating Bcl2 [39]. In an experimental model of ischemia and reperfusion, Paul et al. [27] demonstrated that pre-treating rats with 50 mg/kg of WS for one month can reduce apoptosis in the myocardium, preserve myocytes, and protect them from cell death. Our findings suggest that pre-treating animals with WS for a month may help maintain the quality of semen and protect testicular tissue against apoptosis. Therefore, it is conceivable that pre-treatment with WS could mitigate the detrimental effects of CP on testicular tissue.

In conclusion, WS pre-treatment significantly protected testicular tissue from injury caused by CP. The expression of genes related to apoptosis and histopathological changes were markedly improved by the herbal extract of WS. These protective effects of WS therapy may be attributed to its potent anti-apoptotic and antioxidant properties. It has the potential to be used as an adjunct therapy to mitigate the adverse effects of CP in patients.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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