



# Impact of saponin on cyclophosphamide -induced testicular damage

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## ABSTRACT

**Introduction:** Cyclophosphamide (CP) is the most common chemotherapy drug that has toxic effects on the male reproductive system. This experimental study aimed to assess the protective impact of saponin (SP) on the testicular damage induced via CP.

**Methods:** Twenty-four male mice were separated randomly into 4 groups (N=6): 1. Control group, 2. CP, 3. SP and 4. CP + SP. CP (15mg/kg/weekly) and SP (2.5 mg/kg/daily) were intraperitoneally injected for 35 consecutive days. At the end of the study, the testicles were removed for the histological and biochemical (MDA, SOD, and GPX) evaluation.

**Results:** CP induced degenerative changes in testicular tissues and SP treatment reduced these alterations. The results indicated that CP increased MDA levels and reduced SOD, GPX, and testosterone levels ( $P<0.05$ ) in contrast to the control group. In the CP+SP group, a significant reduction ( $P<0.01$ ) in MDA levels and the elevation of SOD, GPX, and testosterone levels were observed.

**Conclusion:** It seems saponin may reduce CP-induced reproductive toxicity in male mice.

### Keywords:

Cyclophosphamide

Oxidative stress

Saponin

Testis

## Introduction

Many factors can affect reproductive potential function and cause infertility. In recent years, infertility rates have increased, with many cases attributed to male factors such as sperm disorders in shape, count, and motility, as well as hormonal irregularities like low testosterone secretion. Among the myriad factors affecting the male reproductive system, oxidative stress emerges as a significant contributor. Exposure to toxins, environ-

mental pollutants, and chemotherapy drugs like cyclophosphamide (CP) increases the production of reactive oxygen species (ROS), upsetting the balance between oxygen radicals and antioxidant defense mechanisms, thereby causing oxidative tissue damage (Salimnejad et al., 2018). CP, a chemotherapy agent, is known to induce liver, renal, and testicular damage in animals. Free radicals generated by CP treatment can bind to sperm membranes to generate peroxy radical, which in turn,

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induce lipid peroxidation, modify sperm motility and quality. Additionally, free radicals induce necrotic spermatocytes and collapse in seminiferous tubules (Pavin et al., 2018; Timar and Banaei 2022).

Saponins, the herbal medicines, contain the bioactive components with various pharmacological properties, such as lipid lowering, anti-oxidative, anticancer, anti-inflammatory and, immune-regulatory effects. Saponins derived from *Panax japonicus* (SPJ) are believed to exert hepatoprotective and neuroprotective activities (Golmohammadi and Banaei 2021; Yuan et al., 2014). Ginseng saponins, for instance, inhibit catecholamine (CA) production, exerting hypotensive action. Furthermore, saponins, when administrated at low doses, reduce plasma renin activity through a dose-dependent hypotensive response. Some studies suggest that saponins induce endothelial nitric oxide synthase (eNOS) in animal models as a consequence of nitric oxide (NO) and subsequent nitric oxide (NO) production in animal models, potentially contributing to their hypotensive activity (Jang et al., 2011; Rezagholizadeh et al., 2022).

Based on the pharmacological effects of saponins and the mechanisms underlying cyclophosphamide therapy, we hypothesized that saponin may have a beneficial effect on cyclophosphamide-induced oxidative stress. Hence, this study aimed to investigate the potential antioxidative effects of saponins in the context of cyclophosphamide treatment.

## Materials and Methods

In this study, twenty-four male NMRI mice, each 2 months of age, were selected for investigation, with no female mice included. The mice were housed under normal conditions, with a 12-hour light-dark cycle and a temperature of  $22 \pm 2$  °C, in an animal facility. They were provided with ad libitum access to standard animal feed and water. This experiment was approved by the code of ethics (IR.ARUMS.REC.1398.554) in the ethics committee of Ardabil University of Medical Sciences, and all procedures followed the committee's protocols. The mice were randomly assigned to one of four groups (N=6 per group): 1. Control, 2. Cyclophosphamide (CP), 3. Saponin (SP), and 4. Cyclophosphamide + Saponin (CP + SP). CP (sigma-USA) was injected intraperitoneally (IP) once a week (15 mg/kg) for five weeks and SP (sigma-USA) was injected daily (2.5 mg/kg, IP) for 35 days.

### *Sampling*

At the conclusion of the study, the animals were anesthetized using a combination of xylazine (10 mg/kg) and ketamine (50 mg/kg), following which blood samples were collected via cardiac puncture. Additionally, the testicles of the mice were excised to measure oxidative stress markers such as malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD), along with assessing tissue damage.

### *Tissue MDA measurement*

The tissue malondialdehyde (MDA) assay was based on a reaction with thiobarbituric acid (TBA) (Ahmadiasl et al., 2014).

### *Tissue SOD and GPx assessment*

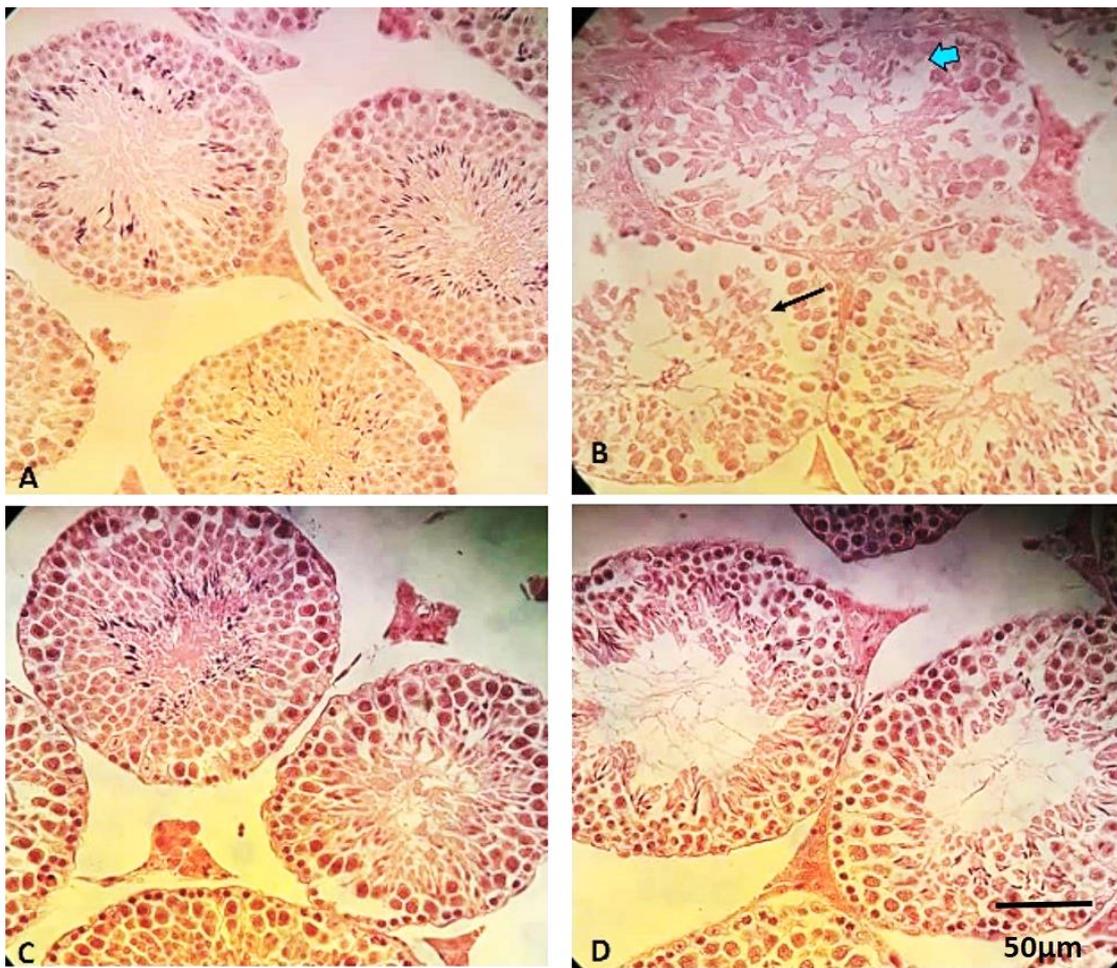
To estimate GPx and SOD levels, testicular tissue was homogenized in 1.15% KCl solution. The analysis of GPx and SOD levels was conducted using the ZellBio kit (Germany), following the relevant protocol provided by the manufacturer (Banaei et al., 2016).

### *Histopathological study*

To evaluate tissue damage, the testicles were fixed in 10% formalin and embedded in paraffin blocks after tissue processing. Subsequently, sections of 5 µm thickness were prepared using a microtome and mounted onto microscopic slides. For histopathological examination, the testicular sections were stained with hematoxylin and eosin (H&E), and images were captured for further analysis. Morphometric parameters, including the height of the germinal epithelium and the outer diameter of the seminiferous tubules, were measured using Image J software. Fifty seminiferous tubules were randomly selected from each slide for analysis (Golmohammadi et al., 2020a; Kalwar et al., 2019). Additionally, the spermatogenesis index was determined for each seminiferous tubule using the Johnsen Score method, which assigns a score from 1 to 10 based on the level of spermatogenesis. Finally, the mean Johnsen score for each sample was calculated (Mazani et al., 2020).

### *Assessment of serum levels of testosterone*

Blood samples were centrifuged at 2000 x g for 15 minutes at +4°C, and the resulting sera were stored at -70°C. Testosterone concentrations were determined using an Enzyme-Linked Immunosorbent Assay (ELISA)



**FIGURE 1.** Photomicrograph of testes in difference group (H&E staining). Control (A) group testes show normal histology, in CP group (B) degenerative change (blue arrow) and cell detachment (black arrow) were noted, SP (C) and CP + SP (D) groups have normal histology (40 × H&E).

kit (Testosterone ELISA, DIA.METRA S.R.L DKO 002), and absorbance readings were measured at 450 nm using an Epoch Microplate Spectrophotometer (United States) (Golmohammadi et al., 2020b).

#### Statistical analysis

The results are reported as Mean ± SEM and were statistically analyzed using one-way ANOVA followed by Tukey tests. A P-value of less than 0.05 ( $P < 0.05$ ) was considered statistically significant.

## Results

The one-way ANOVA analysis indicated levels of MDA [ $F(3, 16) = 6.689$ ,  $P = 0.004$ ], GPx [ $F(3, 16) = 1.466$ ,  $P = 0.261$ ], SOD [ $F(3, 16) = 0.908$ ,  $P = 0.459$ ], testosterone [ $F(3, 16) = 4.020$ ,  $P = 0.026$ ], the outer diameter [ $F(3, 16) = 2.608$ ,  $P = 0.087$ ], thickness of

germinal epithelium [ $F(3, 16) = 1.593$ ,  $P = 0.230$ ], and mean Johnson score [ $F(3, 16) = 5.745$ ,  $P = 0.007$ ] in adult male mice.

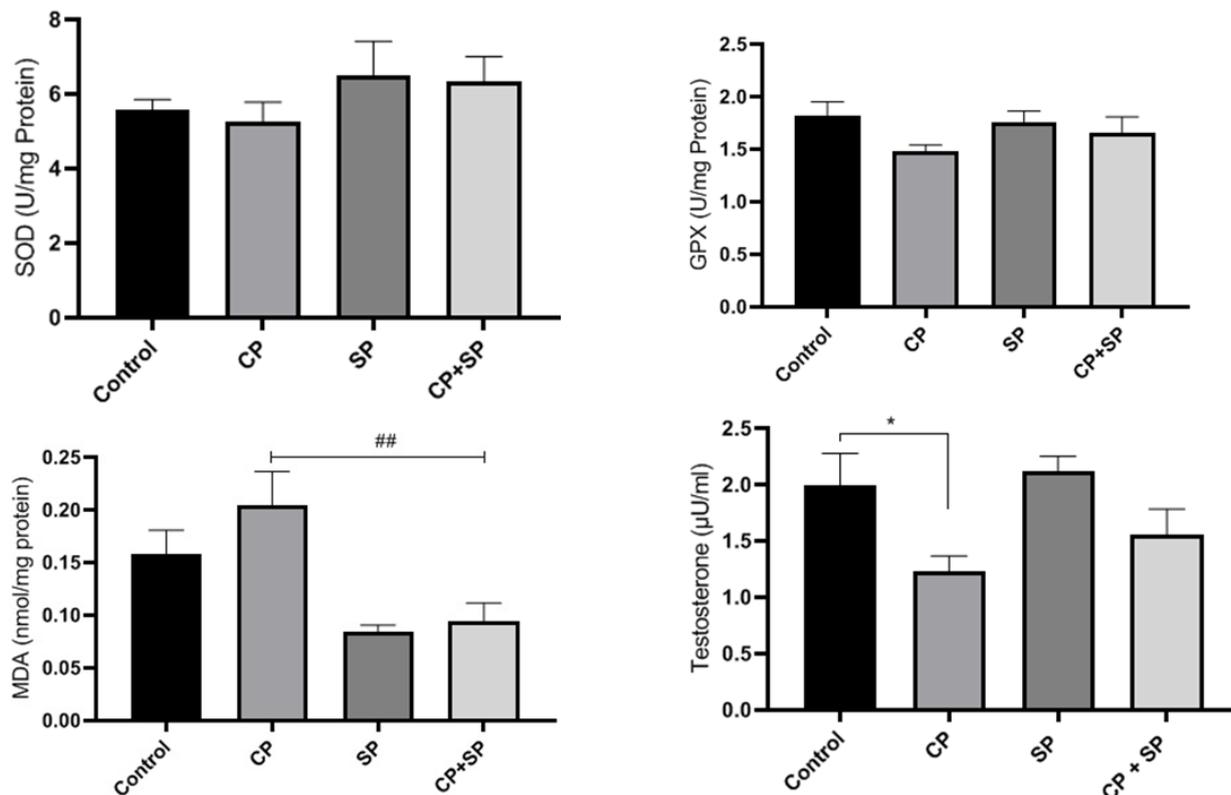
#### *The effect of saponin and CP on histopathological changes*

The histological assessment revealed a normal structure in the seminiferous tubules and interstitial tissue in the testes of the control group (Figure 1A). In contrast, the CP group exhibited degenerate seminiferous tubules, tissue injury, and cell detachment of the germinal epithelium (Figure 1B). However, in the treated groups, the extent of testicular damage was reduced compared to the CP group (Figure 1C, 1D). Morphometrical studies indicated changes in the outer diameter and height of the germinal epithelium across the groups, although these changes were not statistically significant. Further-

**TABLE 1:** The effect of saponin (SP) on histopathological changes induced by cyclophosphamide (CP)

Parameters	Control	CP	SP	CP+SP
Outer diameter (µm)	144.5±5.71	148.8±1.89	148.9±3.18	134.6±4.51
Thickness of germinal epithelium (µm)	79.72±5.48	78.18±1.38	82.10±1.55	83.16±4.53
Mean Johnsen score (MJS)	7.55±0.26	5.55±0.48**	6.16±0.36	5.77±0.26

\*\* Significantly decreased when compared with control group,  $P < 0.01$ .



**FIGURE 2.** The MDA, SOD, GPx and the serum levels of testosterone in different groups. \* $P < 0.05$  versus control group and \*\* $P < 0.01$  compared with CP group.

more, the mean Johnsen score (MJS) was significantly reduced ( $P < 0.01$ ) in the CP group compared to the control group (Table 1).

*The effect of SP and CP on the MDA level*

Lipid peroxidation evaluation revealed that CP significantly increased MDA levels, whereas in the groups treated with saponin, this level was significantly decreased compared to the CP group ( $P < 0.01$ , Figure 2).

*The effect of saponin and CP on the GPx and SOD levels*

In the CP group, the levels of GPx and SOD in the testis insignificantly declined compared to the control

group. Although the SP-treated groups showed an elevation in GPx and SOD activity compared to the CP group, the difference was not statistically significant (Figure 2).

*The effect of saponin and CP on the serum testosterone levels*

The serum levels of testosterone were significantly reduced in the CP-treated group ( $P < 0.05$ , Figure 2), whereas in the saponin-treated groups, this level was increased compared to the CP group.

**Discussion**

This study demonstrated that CP caused degenerative changes in testicular tissue and treatment with SP

reduced these alterations. Our results showed that CP increased MDA levels and reduced the antioxidant enzymes (SOD and GPX) in testicular tissue.

There is a balance between free radical generation and the antioxidant system in the body. However, exposure to chemotherapy drugs like CP results in oxidative stress and an imbalance between the antioxidant defense system and free radical production. Oxidative stress leads to lipid peroxidation in cell membranes and causes reproductive toxicity. Researchers suggest that sperm is more sensitive to free radicals than other cells due to low antioxidant levels and high levels of unsaturated fatty acids in cell membranes (Gu et al., 2021). Additionally, sperm morphology prevents antioxidant enzymes from effectively protecting sperm membranes from acrosome to tail (Melek et al., 2015). Thus, normal reproductive function and sperm quality depend on the availability of antioxidants.

We found that CP-induced oxidative stress in testicular tissues was confirmed by decreased antioxidant enzymes and high lipid peroxidation characterized by SOD and GPX levels. MDA, an important indicator of the degree of lipid peroxidation, is implicated in CP toxicity in testis tissues. These findings highlight a close association between CP toxicity and the induction of oxidative stress and lipid peroxidation (Salimnejad et al., 2018; Salimnejad and Soleimani Rad 2018). According to our results, the decreased MDA and increased SOD and GPX levels observed with SP treatment emphasize the antioxidant and protective properties of saponin.

ROS, MDA overproduction and reduced antioxidant enzymes play a major role in the pathogenesis of CP therapy. CP induces ROS production, inhibiting the antioxidant defense system and resulting in redox imbalance in tissues. The peroxidation of mitochondrial membrane can lead to oxidative phosphorylation, mitochondrial dysfunction, the accumulation of reactive radicals. Saponins, with antioxidant, anti-inflammatory, and angiogenesis activities, may have therapeutic potential effects on CP-induced damage in animal models (Gür et al., 2021; Liu et al., 2019).

Testicular oxidative stress leads to hypo-spermatogenesis and infertility. Saponins possess bioactive components that play an important role in preventing free radical generation and improving antioxidant enzymes (SOD and GPX) (Koczurkiewicz et al., 2019; Wan et al., 2021). Histopathological evaluation revealed marked

cell detachment and degeneration in the CP treated group. However, SP administration improved the histopathological changes created by CP in testis tissues. Also, we found that MJS and thickness of the germinal epithelium were reduced in the cyclophosphamide group compared to the control group. However, SP therapy improved MJS, germinal epithelium thickness, and seminiferous tubules against CP damage. This protective effect of saponin may be due to its potent antioxidant activities, as confirmed by the reduction of pathological modifications in the testicular structure.

Testosterone, the male sex hormone, plays a vital role in spermatogenesis and serves as a marker of testicular activity and spermatogenesis in men. This hormone, secreted by Leydig cells, diffuses into the seminiferous tubules in the interstitial spaces, exerting a tropic effect on spermatogenesis. Low levels of testosterone can lead to decreased seminiferous tubules, germinal epithelium thickness, and hypo-spermatogenesis, ultimately resulting in reproductive disorders and infertility (Masala et al., 1997). In the present study, it is demonstrated that saponin administration enhanced the reduction in testosterone levels caused by CP therapy and resulted in an elevation of hormone concentration. Previous findings have shown that CP induces reproductive toxicity, such as oligospermia, seminiferous tubule atrophy, and low testosterone secretion. The results indicate that CP can disrupt the redox balance in organs, ultimately leading to pathophysiological tissue alterations, including testicular toxicity and infertility (Masala et al., 1997; Yoo and Tanaka 2020).

## Conclusion

Indeed, the findings suggest that CP increases lipid peroxidation of cell membranes, reduces antioxidant enzymes in testicular tissue, and triggers oxidative stress. However, saponins, owing to their potent antioxidant activities, were effective in preventing CP-induced damage and mitigating its adverse effects on testicular tissue. Therefore, it is recommended that saponin could serve as a protective agent for the testis against oxidative stress.

## Acknowledgments

This study was approved by Ardabil University of Medical Sciences (Ethics code: IR.ARUMS.REC.1398.554).

## Conflict of interest

The authors report no conflicts of interest.

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