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# Protective effect of baicalin on cyclophosphamide-induced oxidative stress and morphologic damage in testis tissue

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**ABSTRACT**: The purpose of this study is to examine the effect of baicalin on cyclophosphamide (CP)-induced testicular damage in rats. Twenty-eight Wistar albino rats were assigned to the control, baicalin, CP, and CP+Baicalin groups. A single dose of CP (200 mg/kg) was induced intraperitoneally (i.p.) in the CP and CP+Baicalin groups. Baicalin (100 mg/kg/day, i.p.) was administered in the baicalin and CP+ Baicalin groups for 6 days. After sacrification, the testes, epididymis and blood samples were taken. Testis tissues were investigated for general morphology, proliferating, and apoptotic cells. Oxidative stress parameters in testis tissue and hormone levels in serum were examined by biochemical analysis. Sperm analysis was performed on smear samples obtained from the epididymis. In CP + Baicalin group, decreases in both the number of abnormal spermatozoa and degeneration of seminiferous tubules were observed compared to the CP group. Also, decreased apoptotic cells and increased proliferative cells were detected in these seminiferous tubules. Furthermore, the treatment with Baicalin reversed the changes observed in the markers of oxidative damage including malondialdehyde, total oxidant status, and oxidative stress index, and markers of the antioxidant defense system including glutathione, total antioxidant capacity, superoxide dismutase, and catalase except for glutathione s-transferase (GST) in testicular tissue. Decreased serum levels of follicle-stimulating hormone, luteinizing hormone, and testosterone related to CP significantly improved in CP + Baicalin group. **Conclusion:** Baicalin attenuates CP-induced testicular damage by inhibiting oxidative stress possibly due to its antioxidant properties. Considering on the proposed beneficial effects of Baicalin against testicular damage induced by CP, it might have the potential to be a promising agent for male infertility related to chemotherapy.

**KEYWORDS**: Cyclophosphamide; Baicalin; Testis; Histology; Oxidative stress.

## 1. INTRODUCTION

Cyclophosphamide (CP), an alkylating anticancer agent, is broadly utilized for treating a variety of neoplastic and autoimmune diseases, but it has serious side effects, including testicular damage. The development of oxidative stress, lipid peroxidation, DNA damage, and reduced glutathione levels are the primary causes of CP-induced reproductive damage. Infertility has remained the leading comorbidity in patients exposed to CP therapy. Some antioxidant agents such as melatonin, α-tocopherol, and squalene have been proposed to tackle CP-induced damage and prevent adverse effects [1].

*Scutellaria baicalensis Georgi,* a traditional Chinese herb, contains a variety of flavonoids, polyphenols, and essential oils. Among flavonoids, baicalin (7-glucuronic acid, 5,6-dihydroxyflavone) is a significant phytochemical marker and crucial active component. It exhibits a wide variety of biological effects, including antioxidant, antidiabetic, antimicrobial, anti-inflammatory and anticancer activities [2]. Tan et al. reported that baicalin can reduce adriamycin-induced nephrotic syndrome by suppressing fibrosis-related genes and inflammatory responses [3]. Baicalin was also demonstrated to have a strong inhibitory effect on lipid peroxidation induced by cisplatin on human erythrocytes [4]. El-Ela et al. observed that possible cardioprotection against doxorubicin-induced cardiotoxicity was induced by inhibiting the inflammatory TLR4/NF-kB pathway while activating the protective Wnt/b-catenin pathway [5]. In a study about its potential anticancer effects, baicalin repressed the growth of osteosarcoma cells and triggered cell cycle arrest and apoptosis. It has been proposed that the antitumor impact of baicalin in these cells may be

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attributed to the suppression of the AKT pathway and a reduction in the protein levels of cyclin D1, CDK4, and the Bcl-2/Bax ratio [6]. Fouad et al. [7] discovered that baicalin, in a dose-dependent manner, reduced the expression of Fas ligand and decreased the histopathological damage in rat testes subjected to torsion/detorsion. Furthermore, baicalin, whether used alone or in a low-dose combination with apigalin, could protect against chloroquine-induced male infertility. The proposed protection was achieved through their antioxidant and anti-apoptotic effects, as well as their ability to restore the normal hormonal balance in the hypothalamic–pituitary–gonadal axis, with baicalin being particularly effective [8].

A limited number of studies have questioned the role of baicalin in various types of testicular injury [7,9-12]. Particularly, the effect of baicalin against CP-induced testicular damage has not been documented yet. Our objective of this study was to test the potential role of baicalin in CP-induced testicular damage and related male infertility in rats.

## 2. RESULTS

### 2.1 Body and Testicular Weight Assessment

The rat change of body weight (%) decreased significantly in the CP group in comparison with the control and baicalin group ($p<0.0001$, $p<0.001$, respectively) (Figure 1A). Compared with the CP group, rat change of body weight (%) in the CP+Baicalin group significantly increased ($p<0.05$). There was no statistical difference in testicular weight among all groups (Figure 1B).

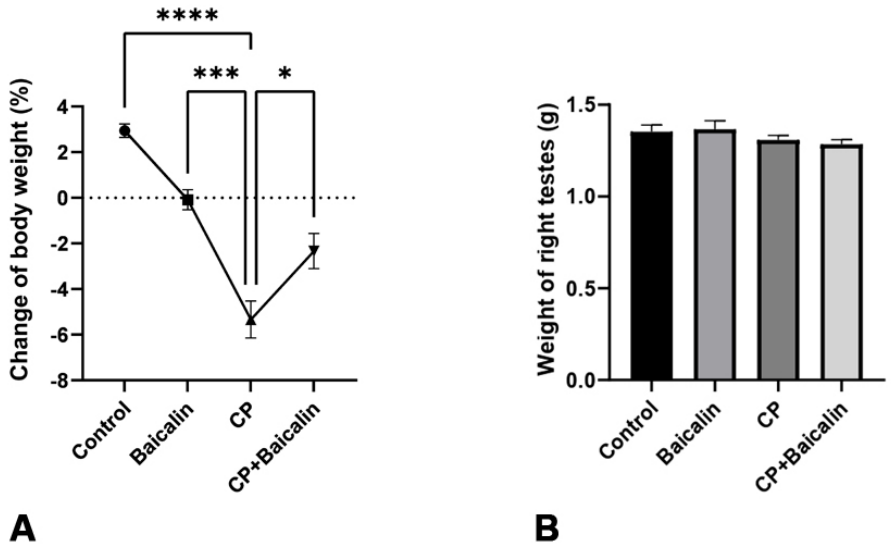


**Figure 1.** Changes in the rat body weight (A) and weight of right testes (B) in the experimental groups. *: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$.

### 2.2 Sperm Account and Morphological Results

The number of epididymal spermatozoa was 111±3.8 million/mL in the control group, 101 ± 7.1 million/mL in the baicalin group, 52 ± 7 million/mL in the CP group, and 89.7 ± 8.9 million/mL in the CP+Baicalin group. The sperm count in the CP group was significantly lower than in the control and baicalin groups ($p<0.0001$). The CP+Baicalin group had considerably higher sperm counts than the CP group ($p<0.0001$) (Figure 2.D).

A small number of spermatozoa with head, neck, and tail defects were observed in the control and baicalin groups in addition to those with normal morphology. The rate of normal sperm morphology in the CP group was considerably lower than in the control and baicalin groups ($p<0.0001$). Baicalin treatment of CP-induced rats caused a substantial rise in the rate of normal sperm morphology ($p<0.01$). Administration of CP caused a significant increase in the rates of the head and tail anomalies compared to the control ($p<0.0001$, for both) and baicalin ($p<0.0001$, for both) groups. Moreover, the rates of the head and tail

anomalies in the CP+Baicalin group were significantly lower than the CP group ($p<0.0001$ and $p<0.01$, respectively) (Figure 2.$D_1$).

**2.3 Histopathological Results**

Light microscopic examination revealed that control and baicalin groups had normal testicular morphology with regular seminiferous tubules. Sperms were present in the lumen of the seminiferous tubules, which were lined by germinal epithelium made up of spermatogenic and Sertoli cells (Figure 2.B, $B_1$, C, and $C_1$). In the CP group, degeneration of seminiferous tubules with the irregular basement membrane, reduction in the spermatogenic germ cell line, disorganized germinal epithelium, dilatation and vacuole formation between the germinal epithelial cells, and exfoliation of germ cells in the lumen were prominent in many areas (Figure 2.$B_2$ and $C_2$). Mild degeneration was still present in some tubules besides quite regular morphology of the seminiferous tubules in the CP+Baicalin group (Figure 2.$B_3$ and $C_3$). Additionally, Johnsen's score was considerably lower in the CP group than in the control and baicalin groups ($p<0.0001$). Also, a significant increase in Johnsen score was detected in the CP + Baicalin group compared to the CP group ($p<0.0001$) (Figure 2.$D_2$). The results of the measurement of epithelial thickness, area, and diameter of the seminiferous tubule are presented in Figure 3. Examination of these morphometric parameters revealed a substantial reduction in the CP group compared to the control and baicalin groups ($p<0.0001$). Compared to the CP group, a significant increase in the epithelial thickness, area, and diameter of the seminiferous tubules was detected in the CP + Baicalin group ($p<0.001$, $p<0.0001$ and $p<0.0001$, respectively).

**2.4. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Results**

TUNEL-positive cells were detected in all experimental groups (Figure 4.A-$A_3$). In comparison to the control and baicalin groups, the apoptotic index was noticeably higher in the CP group ($p<0.0001$). However, baicalin treatment in CP-administered rats led to a substantial decrease in the apoptotic index in comparison to the CP group ($p<0.0001$) (Figure 4.C).

**2.5. Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry Results**

The seminiferous tubules of all experimental groups contained PCNA-positive cells. (Figure 4.B-$B_3$). The proliferation index in the CP group substantially decreased in comparison to the control and baicalin group ($p<0.0001$, Figure 4.$C_1$). Treatment with baicalin increased the proliferation index significantly in the CP+Baicalin group compared with the CP group ($p <0.001$, Figure 4.$C_1$).

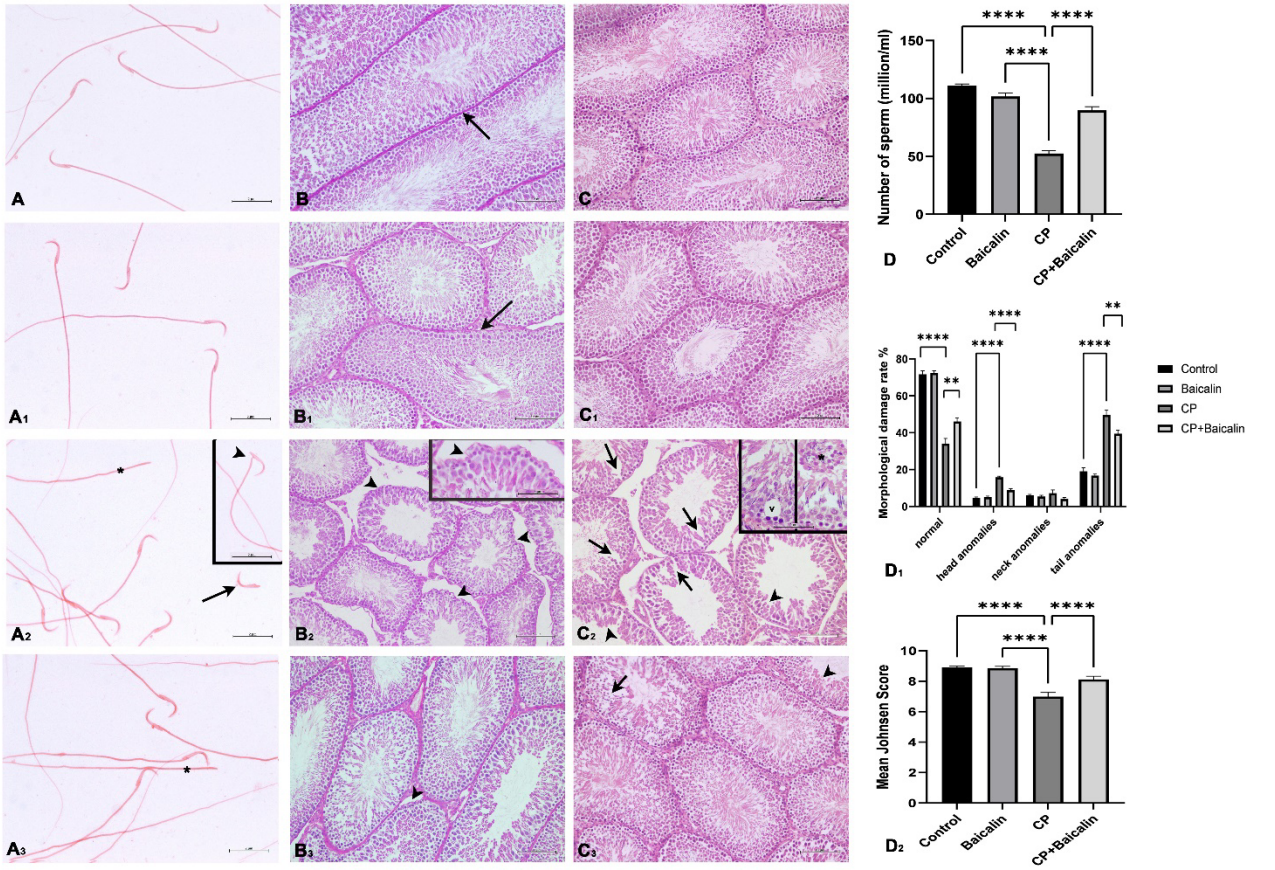


**Figure 2**. Representative light micrographs of spermatozoa (A-$A_3$) and testis tissue (B-$B_3$ and C-$C_3$) and the graph of the number of sperm (D), sperm morphological damage ratio ($D_1$) and Johnsen's histopathological scores ($D_2$) in control (A, B, C), baicalin ($A_1$, $B_1$, $C_1$), cyclophosphamide (CP, $A_2$, $B_2$, $C_2$) and CP+Baicalin ($A_3$, $B_3$, $C_3$) groups. Typical spermatozoa and spermatozoa with head (*), neck (arrowhead) and tail (arrow) anomalies were observed in control, baicalin, CP group, and CP+Baicalin groups (A-$A_3$). In the control and baicalin groups, normal morphology of seminiferous tubules with regular germinal epithelium (C and $C_1$) and basement membrane (arrows, B and $B_1$) were observed. In the CP group, degenerated seminiferous tubules with irregular basement membranes (arrowheads, $B_2$), large dilatation (arrows, $C_2$), and vacuoles (v, $C_2$) between the germinal epithelial cells, reduced thickness of the germinal epithelium (arrowheads, $C_2$) and exfoliation of germ cells in lumen (*, $C_2$) were detected in this group. In the CP+Baicalin group, besides quite regular morphology of seminiferous tubules, irregular basement membrane (arrowheads, $B_3$), dilatation between the germinal epithelial cells (arrow, $C_3$) and reduced thickness of the germinal epithelium (arrowhead, $C_3$) were observed in some tubules. Eosin Y staining: A-$A_3$, PAS staining: B-$B_3$ and Hematoxylin and Eosin (H&E) staining: C-$C_3$. Original magnification: x1000 (A-$A_3$ and inset $A_2$), x200 (B-$B_3$, C-$C_3$) and x400 (inset $C_1$ and $C_2$). **: p<0.01, ****: p<0.0001.

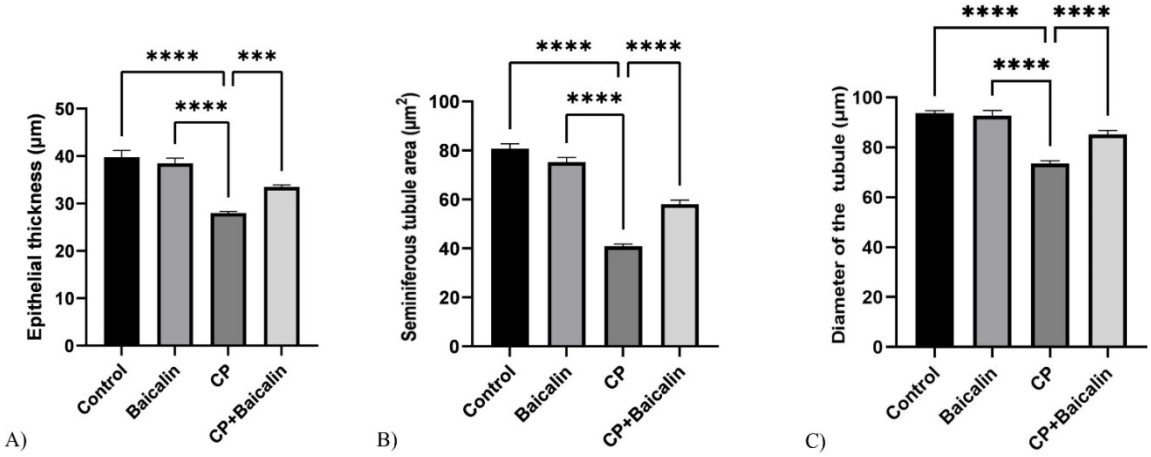


**Figure 3.** Epithelial thickness (A), seminiferous tubule area (B), and diameter of the tubule (C) in experimental groups. Cyclophosphamide (CP). ***: p<0.001, ****: p<0.0001.

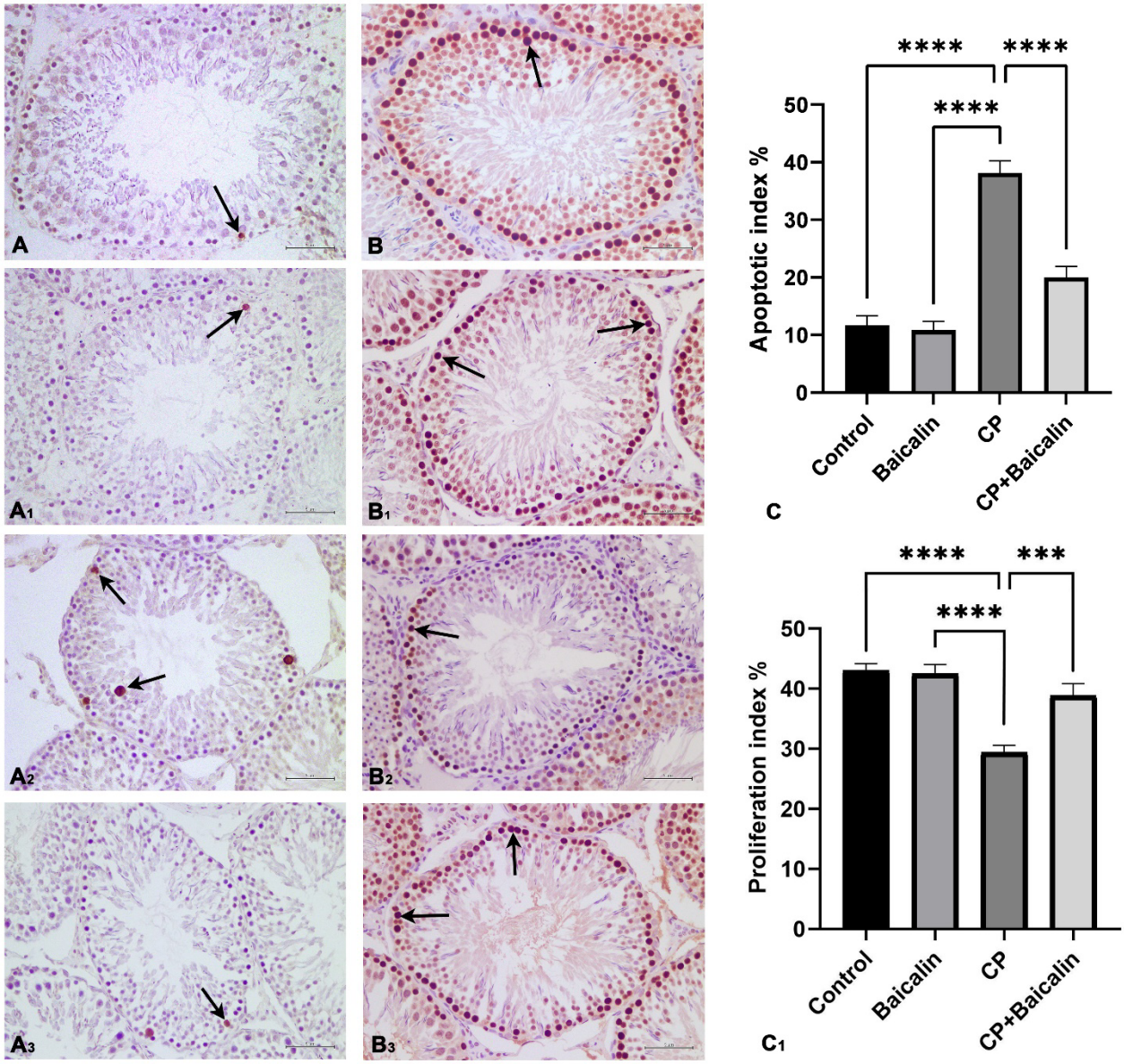


**Figure 4**. Representative light micrographs of TUNEL (A-$A_3$) and PCNA (B-$B_3$) stained testis tissue and graph of apoptotic index (C) and proliferation index ($C_1$) in experimental groups. The arrows showed TUNEL-positive (A-$A_3$) and PCNA-positive (B-$B_3$) cells. Cyclophosphamide (CP), Original magnification: x400 (A-$A_3$ and B-$B_3$), ****: $p<0.0001$, ***: $p<0.001$.

### 2.6 Testicular Antioxidant and Oxidant Parameters Results

Superoxide dismutase (SOD), catalase (CAT) and glutathione s-transferase (GST) activities and the glutathione s-transferase (GSH) and malondialdehyde (MDA) levels in testis tissue were given in Figure 5. In the CP group, SOD, CAT, and GST activities decreased in a significant manner compared with the control group ($p<0.05$, $p<0.001$, and $p<0.05$, respectively). Similarly, SOD, CAT, and GST activities were significantly lower in the baicalin group than in the CP group ($p<0.05$, $p<0.01$ and $p<0.01$, respectively). Baicalin treatment increased the levels of SOD and CAT in the CP+Baicalin group in comparison to the CP group, while there were no significant differences in GST activities between these groups ($p<0.001$ and $p<0.05$, respectively). The level of GSH was significantly lower in the CP group compared with the control and baicalin group ($p<0.05$). In addition, the level of MDA increased in the CP group when compared with the control and baicalin groups ($p<0.01$ and $p<0.0001$, respectively). However, treatment with baicalin in CP-induced rats reversed the levels of GSH and MDA, and these changes were statistically significant ($p<0.05$ and $p<0.01$, respectively).

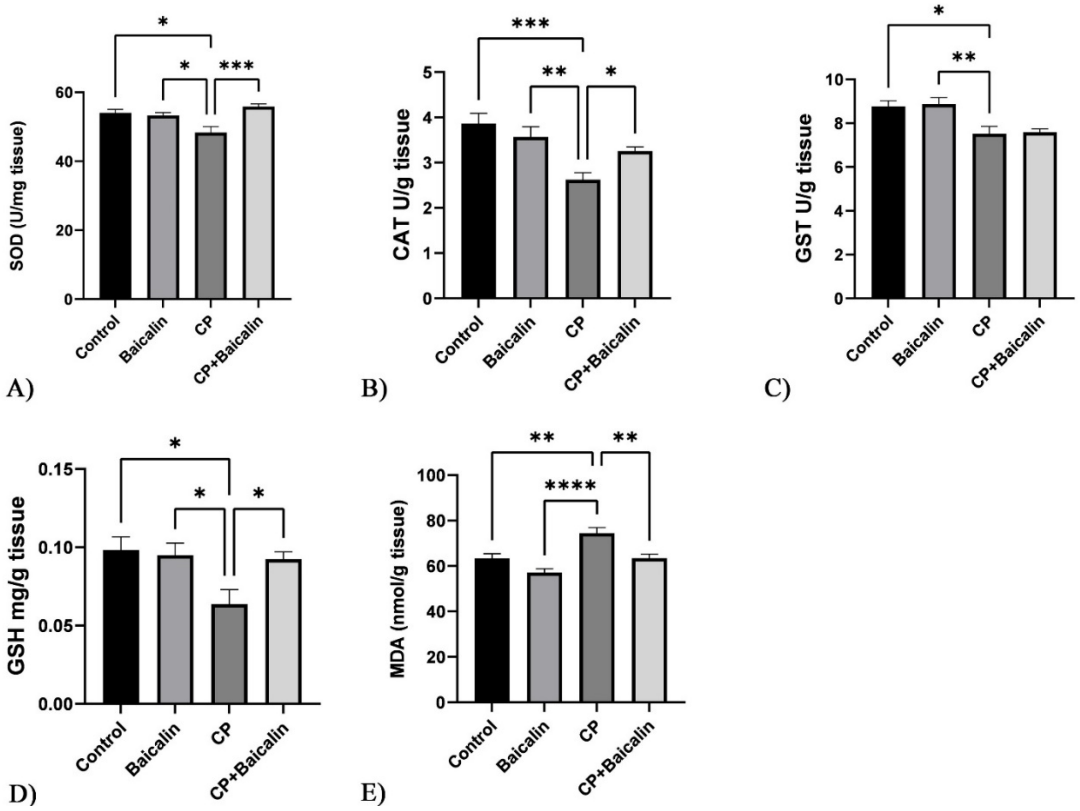


**Figure 5**. SOD (A), CAT (B), GST (C) activities and GSH (D), MDA (E) levels in testicular tissue of the experimental groups. SOD: superoxide dismutase; CAT: catalase; GST:glutathione-S-transferase; GSH: glutathione; MDA: malondialdehyde. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

The levels of total oxidant status (TOS), total antioxidant capacity (TAC) and oxidative stress index (OSI) levels in the testis tissue were shown in Figure 6. A statistically significant decreased TAC level and increased TOS and OSI levels were found in the CP group when compared with the control (p<0.0001, p<0.01 and p<0.0001, respectively) and baicalin (p<0.0001, p<0.05 and p<0.001, respectively) groups. Treatment with baicalin significantly reversed TAC, TOS, and OSI levels in the CP+Baicalin group in comparison to the CP group (p<0.05, p<0.05 and p<0.001, respectively).

### 2.7 Serum Testosterone, LH and FSH Results

The levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) are given in Figure 7. The control group had substantially elevated testosterone, LH, and FSH levels than the CP group (p<0.0001, p<0.0001 and p<0.001, respectively). Similarly, the baicalin group had high testosterone, LH, FSH levels compared with the CP group (p<0.0001, p<0.0001 and p<0.01, respectively). In comparison to the CP group, baicalin treatment significantly increased testosterone, FSH, and LH levels in the CP+Baicalin group (p<0.0001, p<0.0001 and p<0.01, respectively).

## 3. DISCUSSION

In our study, we detected adverse outcomes associated with CP, which included a decrease in sperm count, an increase in abnormal sperm count, morphological degeneration and increased oxidative stress in testicular tissue. Moreover, serum sex hormones including testosterone, LH, and FSH were decreased in accordance with the biochemical and histological changes. As a natural antioxidant, baicalin remarkably addressed all aspects of CP-induced testicular damage by improving these changes.

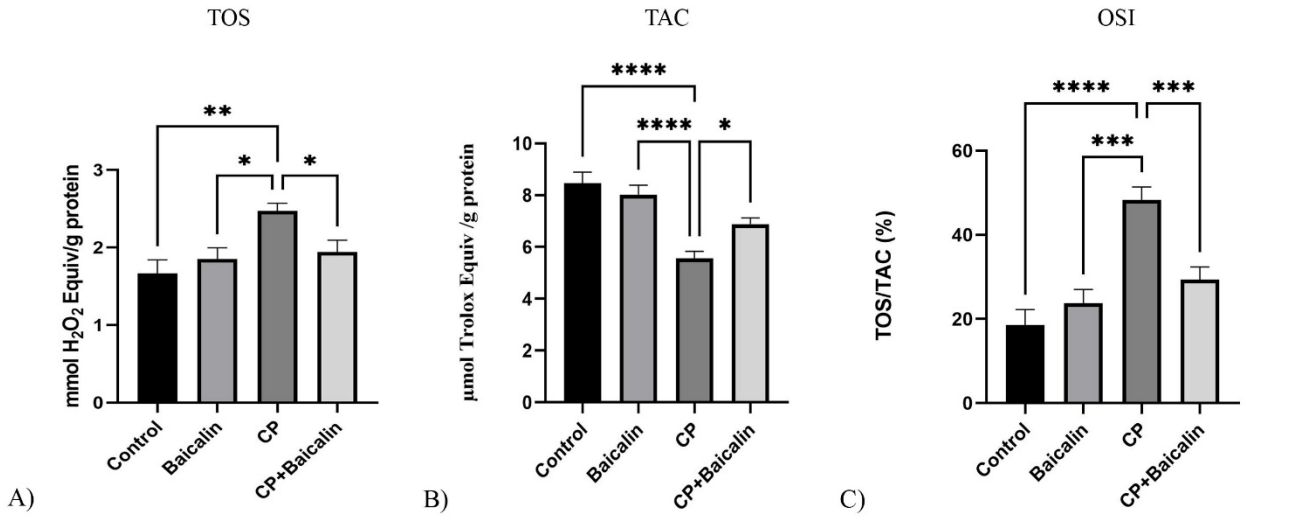


**Figure 6.** TOS (A), TAC (B) and OSI (C) levels in testicular tissue of the experimental groups. TOS: total oxidant status, TAC: total antioxidants capacity, OSI: oxidative stress index. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

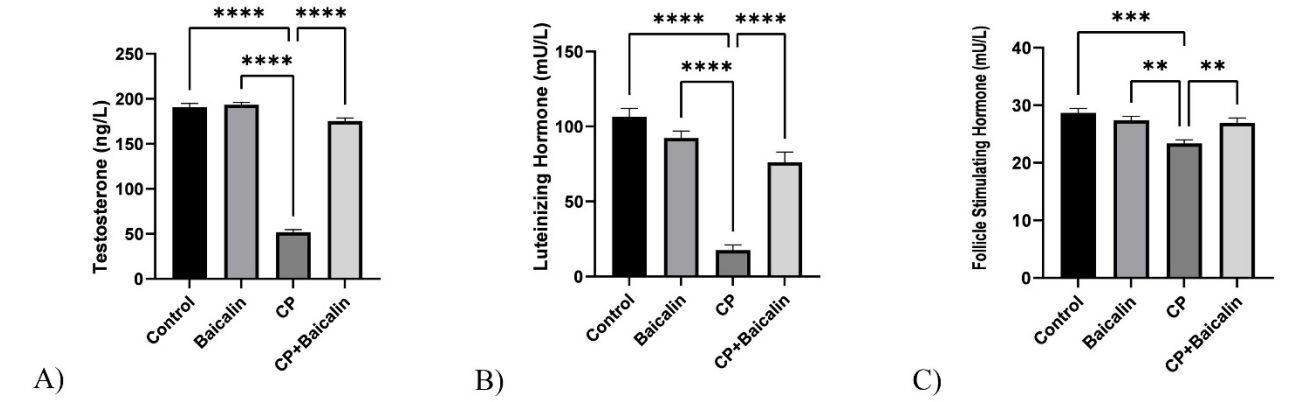


**Figure 7.** The levels of testosterone (A), luteinizing hormone (B) and follicle-stimulating hormone (C) in experimental groups. **: p<0.01, ***: p<0.001, ****: p<0.0001

Cyclophosphamide is extremely toxic to germ cells in the spermatogonial stage due to the rapid division potential of these cells. Studies have reported that CP significantly enhances the abnormality in sperm head and tail morphology and fosters a decrease in sperm count [1,14]. In our study, CP caused sperm head and tail morphologic damage and decreased sperm count. Fan et al. [12] showed that baicalin treatment increased rat pregnancy rates and sperm motility significantly. Our findings about sperm parameters revealed baicalin significantly increased sperm count and lessened the damage to the head and tail morphology. Kanno et al. [15] showed that CP (100, 150, 200 and 250 mg/kg, once a week, i.p., for six weeks) decreased both mice body and testicular weight and the reduction in body weight could be attributed to the systemic toxicity of CP and the lack of nutrition as a result of CP-induced stress. In our study, though CP reduced body weight, it was slightly improved in the CP+Baicalin group. Contrary to the findings of Kanno et al. [15] showed that the testis weight was not significantly different among groups in the present study. This situation might be due to the dose of CP, duration of treatment and animal sensitivity, which was supported by the previous studies in the literature [16,17].

CP has negative effects on the antioxidant defense and increases the production of reactive oxygen species (ROS) and lipid peroxidation [18]. The reduction of antioxidant enzyme activities in testes was found to be related to uncontrollable $H_2O_2$ production [19]. Baicalin could eliminate ROS, prevent lipid peroxidation, and preserve antioxidant defenses in the face of oxidative stress [20,21]. Tekeli et al. [11] reported that baicalin administration to rats exposed to the pesticide emamectin benzoate provided adequate

protection against the adverse effects by ameliorating oxidative stress parameters including testicular nitric oxide, MDA, and GSH levels, and glutathione peroxidase (GPX), glutathione reductase (GR), GST, SOD, and CAT activities and, therefore, reducing histological damage. At the cellular level, the protective effect of baicalin against methotrexate-induced testicular toxicity was demonstrated in mitochondria by improving the activities of antioxidant (CAT, SOD, GSH, and GPX) and tricarboxylic acid cycle enzymes and the levels of ROS and thiobarbituric acid reactive substances. In the hepatotoxicity model induced by fluoxetine, Ganguly et al. [20] demonstrated that the administration of 100 mg of baicalin led to a decrease in MDA and advanced oxidation protein products levels and an increase in GSH levels, as well as enhanced activities of SOD, CAT, and GST in the liver. Meanwhile, treatment with 50 mg of baicalin improved oxidative stress biomarkers, with the exception of CAT. Similarly, Du et al. [22] showed that baicalin increased SOD activity in cancer cells without affecting catalase and GST. Our findings showed that baicalin significantly reduced MDA and TOS levels and increased antioxidants (SOD-CAT activities, and GSH-TAC levels), with the exception of GST activity in accordance with the literature. In our opinion, the lack of alteration in GST activity in the CP+Baicalin group might be linked to the use of a 100 mg/kg dose in the present study. Using higher doses of baicalin would potentiate the impact of baicalin on antioxidant enzyme activities. Moreover, with regard to oxidants/antioxidants, the altered response to baicalin therapy among different organs might be another reason.

Morphologic damage in testicular tissue of CP-induced rats was shown as the reduced thickness and vacuolization of the germinal epithelium, incomplete spermatogenic lineage, exfoliation of germ cells, perivascular fibrosis, edema, hemorrhage and congestion [23,24]. In a study by Hosseini et al. [25], CP caused the destruction of the Sertoli and Leydig cell populations, as well as abnormalities in sperm morphology. As reported by Alkhalaf et al. [23], we think that decreased testosterone synthesis and morphologic damage after CP administration could be interrelated with each other. In line with the literature, our study revealed a significant decrease in testosterone level and an increase in degenerated seminiferous tubules in the CP group, which were significantly reversed by baicalin.

CP generates oxidative stress by increasing ROS levels and targets steroidogenic enzyme activities, resulting in decreased testosterone, LH, and FSH production [26]. It is also hypothesized that CP suppresses FSH secretion, primarily through inhibin hormone release by Sertoli cells, and the sensitivity of Sertoli cells to FSH changes with CP administration [27]. Therefore, both the findings of the present study and those in the literature suggest that CP might alter hormone secretion via Leydig and Sertoli cell dysfunction and the pituitary-gonadal axis disruption. In an experimental study, baicalin reduced heat stress-induced cell apoptosis in bovine Sertoli cells by modulating cell survival rate through activation of the Fas/FasL pathway and upregulation of a highly stress-inducible 72-kDa protein expression [9]. Baicalin administration significantly reversed the levels of testosterone, FSH, and LH levels in the CP+Baicalin group compared to CP in the present study. In our opinion, this beneficial hormonal effect of baicalin could be attributed to its protective action on testicular tissue.

Cyclophosphamide inhibits cell growth and differentiation in the testis, primarily by cross-linking DNA strands, damaging germinal epithelial integrity, depleting PCNA immunoreactivity [14,28] and activating apoptosis [1]. In our study, we examined the potential role of baicalin treatment in the prevention of testicular tissue apoptosis caused by CP. The morphological evaluation revealed that 200 mg/kg baicalin improved the pathological condition of renal tissue and reduced the occurrence of apoptosis in the mouse sepsis model [29]. In a heat stress model by Sui et al. [10], baicalin alleviates the testicular damage by increasing anti-oxidative enzyme activities and possibly by inhibiting the Fas/FasL apoptosis pathway. It was found that baicalin inhibits bleomycin-induced apoptotic protein expression and exerts a suppressive effect on bleomycin-induced pulmonary fibrosis and fibroblast proliferation in rat lung tissue [30]. Moreover, baicalin was shown to increase hepatic PCNA and cyclinD1 expression in acetaminophen induced acute liver injury in a mouse model [31]. Our results showed that baicalin reversed the decreased expression of PCNA and the increased TUNEL positivity in the CP+Baicalin group in line with the literature.

## 4. CONCLUSION

Cyclophosphamide impaired spermatogenesis, and caused testicular damage by increasing lipid peroxidation, apoptotic cell numbers, and ROS levels, and decreasing proliferative cell numbers. Additionally, baicalin treatment reversed all of these harmful effects of CP on testicular tissue. Baicalin could alter the biochemical milieu of the testicular tissue by changing the balance between antioxidant enzymes and oxidative products in favor of antioxidants. The beneficial effect of baicalin on damaged testicular tissue might be due to its antioxidant and anti-apoptotic function. Taking the proposed curative effects of baicalin

on CP-induced testicular damage into consideration, it might be a promising agent against male chemotherapy-related infertility and it could have potential implications in clinical practice.

## 5. MATERIALS AND METHODS

### 5.1 Chemicals

Cyclophosphamide was obtained from Baxter Oncology GmbH, Germany. Baicalin (CAS number: 21967-41-9, purity ≥95%) was purchased from Cayman Chemical Company, USA.

### 5.2 Animals

The Marmara University Animal Care and Use Committee approved the experimental procedure (approval code: 23.2021.mar). Two-month-old male Wistar Albino rats (200-250g) were kept in a standard laboratory condition containing 22 ± 2° C temperature with a 12-hr day/night cycle and they were fed with commercial rat food and tap water *ad libitum*.

### 5.3 Experimental design

Rats were enrolled into the control (n=6), Baicalin (n=6), CP (n=8), and CP+Baicalin (n=8) groups: A single dose of CP (200mg/kg) was administered intraperitoneally (i.p.) to rats in the CP and CP+Baicalin groups on the first day of the experiment. Baicalin was dissolved in 0.1% dimethylsulfoxide (DMSO, diluted in saline) solution. Rats received baicalin (100mg/kg/day, i.p.) for six days in the Baicalin and CP+Baicalin groups. The baicalin dosage was determined based on the literature [7]. Control and CP groups were given an equivalent volume of 0.1 % DMSO (i.p.) solution without baicalin for six days. After the sacrification of rats on day 7 of the experiment, blood samples and right testes were collected for biochemical analysis. Moreover, the left testes with epididymis were taken out for sperm and histopathological analyses.

All rats were weighed at both the beginning and end of the research. The percentage of weight loss or gain (weight change/initial weight x100) was computed for each animal's body weight gain or loss. The weight of the right testes was also measured at the end of the research.

### 5.4 Assessment of the sperm count and morphology

The caudal part of the epididymis was separated from the left testicle and sliced into small pieces in 10 ml of phosphate-buffered saline (PBS) solution in a laboratory dish on a heater set at 37 °C. For sperm counting, samples were prepared and transferred to the Thoma slide as previously described by Wang [32]. To evaluate the sperm morphology, the smears were prepared and stained with 1% Eosin-Y. Two hundred spermatozoa were examined for head, neck, and tail morphology at 100x objective with oil immersion under a photomicroscope [33,34].

### 5.5 Light microscopic preparation and morphological evaluation

The left testes were fixed in 10% neutral buffered formalin solution, dehydrated in graded alcohol solutions, cleared in xylene and embedded in paraffin. Four micrometer-thick paraffin sections were stained with H&E for general morphological examinations and Periodic Acid Schiff (PAS) to detect alteration in the basement membrane. All stained slides were examined under a light microscope by two histologists (Olympus DP72, Tokyo, Japan) and photographed with an attached camera (Olympus DP22, Tokyo, Japan).

A hundred cross-sectioned seminiferous tubules were included in the calculation of the epithelial thickness, diameter and area of the tubule using the Image J program in H&E stained slides taken from each sample. The epithelial thickness and diameter of the tubule were determined by measuring the four different sides [35]. The seminiferous tubule damage in these one hundred tubules was also assessed using a modified Johnsen's scoring system that has a scale from 1 (absence of germinal epithelium of the seminiferous tubule) to 10 (complete spermatogenesis) [36].

### 5.6 TUNEL analysis

TUNEL staining was performed in accordance with the instructions in the commercial kit's user manual (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, S7101, Millipore, USA). Twenty seminiferous tubules in each section were evaluated for counting of the TUNEL-positive cells. The ratio of seminiferous tubules with three or more TUNEL-positive cells to all seminiferous tubules was utilized to calculate the apoptotic index [34].

### 5.7 Immunohistochemistry

Three-µm-thick sections were incubated overnight in the oven at 37°C and deparaffinized in xylene. Following dehydration with ethanol series, the sections were kept in 3% hydrogen peroxide in methanol to block the endogenous enzyme activity. Sections were treated with citrate buffer solution (10 mM, pH 6.0) for 20 minutes in a microwave for antigen retrieval. After washing with PBS, a protein-blocking solution (SHP125, Scytek, USA) was applied to slides for the non-specific binding of antibodies. In a humidified environment, sections were exposed to anti-proliferating cell nuclear antigen (anti-PCNA) primary monoclonal antibody (Bsm-33035M, Bioss, USA, dilution 1:400) overnight at 4°C temperature. Sections were treated with an anti-polyvalent secondary antibody (SHP125, Scytek, USA) after a PBS wash. Following a second PBS wash, sections were incubated with streptavidin peroxidase (SHP125, Scytek, USA). After washing with PBS, DAB chromogen (ACK125, Scytek, USA) was applied to slides to visualize the labelling. Sections were coverslipped with entellan after counterstaining with Mayer's hematoxylin. A total of 20 seminiferous tubules were examined in each section for PCNA immunohistochemistry. The ratio of the number of PCNA-positive cells to the total number of cells in each seminiferous tubule was utilized to calculate the proliferation index [34].

### 5.8 Biochemical Analyses

For biochemical analyses, testis tissue homogenates were done using saline solution and were stored at -20°C. SOD, CAT, and GST activities and GSH and MDA levels were determined using the methods described previously by Oktay et al. [37] in a study. Moreover, TOS, TAC and OSI were determined according to previously described methods by Kaypaklı et al. [13].

#### *5.8.1 Hormone Measurements*

Testosterone (E0259Ra, BT Lab, Korea), LH (E0179Ra, BT Lab, Korea), and FSH (E-EL-R0391, Elabscience, USA) levels in rat serum were ascertained by enzyme-linked immunosorbent assays (ELISA) in line with the owner's recommendations.

### 5.9 Statistical Analysis

All statistical calculations were performed using GraphPad 9.0 Software (GraphPad Software, San Diego, CA, USA). After confirming the normal distribution of data by the Shapiro-Wilk test, one-way ANOVA and Tukey's multiple comparison tests were done. The results were given as the mean ± standard error of the mean (SEM). $P< 0.05$ values were regarded as significant.

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