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Research Article / Araştırma Makalesi

The Effect of Berberine on the Prevention and/or Treatment on Cyclophosphamide-Induced Testicular Damage in Rats

Sıçanlarda Berberinin Siklofosfamide Bağlı Testiküler Hasar Üzerindeki Önleme ve / veya Tedavi Edici Etkisi

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Abstract

The protective and/or therapeutic effect of berberine on cyclophosphamide-induced testicular injury was investigated in rats. A total of 40 male Sprague-Dawley rats were divided into 5 groups (n=8 per group). The control group received saline and CP, BER75, BER150 and BER300 groups received single dose of intraperitoneal 200 mg/kg CP on day 8. Berberine (75 mg/kg, 150 mg/kg and 300 mg/kg) was administered orally for 7 days before and after the CP injection. At the end of the protocol, testes were removed for histological examination, immunohistochemical staining, and biochemical assessments. In our study, CP did not cause a significant change in body weight and testis weight, whereas berberine treatment significantly decreased body weight. Although no significant change was observed in terms of oxidative stress markers and cytokine levels, degenerative changes and apoptotic findings were found in the CP group; which were improved significantly with increasing doses of berberine treatment. In conclusion, we found that treatment with berberine may have anti-degenerative and anti-apoptotic potential rather than altering oxidative stress markers and/or inflammatory pathways in CP-induced testicular damage.
Keywords: Berberine, cyclophosphamide, Rat, Testicular injury

Özet

Bu çalışmada Berberinin sıçanlarda siklofosfamide bağlı testis yaralanması üzerindeki koruyucu ve/veya terapötik etkisi araştırıldı. Toplam 40 erkek Sprague-Dawley sıçan 5 gruba ayrıldı (n = 8). Sekizinci günde tek doz olarak kontrol grubuna intraperitoneal salin, CP, BER75, BER150 ve BER300 gruplarına ise intraperitoneal 200 mg/kg CP uygulandı. Serum fizyolojik, 75 mg/kg, 150 mg/kg ve 300 mg/kg berberin, enjeksiyondan 7 gün önce ve 7 gün sonra oral olarak uygulandı. Protokolün sonunda testisler histolojik inceleme, immünhistokimyasal boyama ve biyokimyasal değerlendirme için çıkarıldı. Çalışmamızda CP vücut ağırlığı ve testis ağırlığında önemli bir değişikliğe neden olmazken, berberin tedavisi vücut ağırlığını anlamlı ölçüde düşürdü. Oksidatif stres belirteçleri ve sitokin düzeyleri açısından anlamlı bir değişiklik görülmemekle birlikte CP grubunda dejeneratif değişiklikler ve apoptotik bulgular saptanmıştır, bu bulgular artan berberin tedavisi dozları ile önemli ölçüde iyileşmiştir. Sonuç olarak berberin ile tedavinin, CP'ye bağlı testis hasarında oksidatif stres belirteçlerini ve/veya inflamatuvar yolları değiştirmekten çok anti-dejeneratif ve anti-apoptotik potansiyele sahip olabileceğini bulduk.
Anahtar Kelimeler: Berberin, siklofosfamid, Sıçan, Testis hasarı

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1. Introduction

Cyclophosphamide (CP) is an immunosuppressive agent used in the treatment of various epithelial tumors such as ovarian, breast and small cell lung carcinomas, as well as some hematological malignancies (1) and also in the treatment of some chronic autoimmune diseases such as preventing graft rejection (2). CP, an inactive prodrug that requires enzymatic bioactivation, is transformed into phosphoramide mustard and acrolein as a result of enzymatic reactions.

Phosphoramide mustard, which is the active alkylation agent of cyclophosphamide, causes the addition of alkyl groups to the nitrogen and oxygen atoms of guanine in DNA. The alkylated guanine loses its affinity for cytosine and leads to DNA crosslinking. Cross-linking of DNA strands causes cell growth, differentiation of mitotic activity and impairment of cell function (3, 4). This leads to disruption in DNA replication and transcription.

On the other hand, there are some studies in which acrolein, the other active metabolite of CP, has a negative effect on human fertility along with hemorrhagic cystitis and apoptotic changes in the testis (5, 6) There are some other studies reporting decreased testicular weight due to CP treatment (2). CP can also induce aplasia of the germinal epithelium of the testis, ultimately oligospermia and even azoospermia(7). There are studies showing a significant decrease in sperm concentration and motility after CP exposure (2). The mechanisms for negative effects on reproductive system may include induction of DNA damage, peroxidation of critical thiol groups in proteins, membrane lipid peroxidation, oxidative stress in mitochondria, and reduction of enzymes involved in the tricarboxylic acid cycle (8). It is also known that exposure to reactive oxygen species (ROS) reduces ATP concentration and sperm motility (9).

Berberine is a herbal quaternary benzylisoquinoline alkaloid and has a history of at least 3000 years of use in Ayurveda and Chinese medicine due to its potent antimicrobial, antiprotozoal and antidiarrheal effect (10). Its active ingredients are berberine, berbamine and palmatine (11). Berberine is now also produced by chemical synthesis and the chloride or sulfate salt of berberine is often used for clinical purposes. Clinical studies have shown that berberine has a wide spectrum of pharmacological effects including antihypertensive, antiarrhythmic, antihyperglycemic, anticancer, antidepressant, anxiolytic, neuroprotective, antioxidant, anti-inflammatory, analgesic and hypolipidemic activity (12-14).

In non-obese diabetic (NOD) mice, berberine reduced the production of pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), interleukin-6 (IL-6), interferon-γ (IFNγ) and IL-17 and increased antiinflammatory cytokines such as IL-10 (15-18).

All these studies suggest that berberine may be clinically effective in preventing and/or treating testicular damage. We could not find any study examining the effect of berberine on testicular damage induced by CP and the aim of our study is to investigate the effects of berberine in the prevention and/or treatment of testicular damage induced by CP in adult male rats.

2. Materials and Methods

A total of 40 adult male Sprague-Dawley rats weighing approximately 200-300 g were used in the experiments. Animals were housed in well-ventilated rooms at 24 ± 1 ° C, on a 12-hour dark, 12-hour light cycle and fed with standard animal food and tap water. All experiments were carried out after obtaining approval from Local Ethics Committee (Date and number 2018-658).

The animals were divided into 5 groups, eight in each group: control (C), cyclophosphamide-treated (CP) and 75, 150 and 300 mg/kg berberine-treated groups (BER75, BER150 and BER300). Control and CP groups were given saline (0.9%) daily by gavage for 14 days, while BER75, BER150 and BER300 groups received 75 mg/kg, 150 mg/kg and 300 mg/kg berberine (Fluoro Chem, UK) for 14 days by gavage,

respectively. Berberine was dissolved in saline. All groups except the control groups received a single intraperitoneal injection of 200 mg/kg cyclophosphamide (Endoxan®, Baxter Oncology GmbH, Germany) on day 8, while control group received a single intraperitoneal injection of 0.2 ml of saline (0.9%) on the same day.

At the end of the treatment protocol on the day 15, general anesthesia was induced with intraperitoneal injection of 50 mg/kg ketamine (Ketalar®, Pfizer, NY, USA) and 5 mg/kg xylasine (Rompun®, Bayer, Turkey) and sacrification was performed by cervical dislocation. Animals were weighed at the beginning (BW1) and end (BW2) of the experiment. Body weight change (BW%) was calculated as percentages (BW% = (BW2–BW1)/100).

Both testicles of each rat were removed immediately. One of them was placed in containers containing phosphate buffer solution (NaCl: 8 g, KCl: 0.2 g, KH2PO4: 0.2 g, Na2HPO4: 1.14 g in 1L distilled water) and stored for subsequent histological examination. The other testis was cleaned from blood and other contaminants, weighed and recorded. Approximately 100 mg of tissue sample taken from the latter testis was stored in PBS (1:10 w/v) at -20 ° C. The frozen testicular samples were thawed at room temperature and homogenized in a semi-liquid form, which was then centrifuged at + 4 ° C at 15000rpm for 15 minutes and supernatants were used for determination of cytokine levels and oxidative status

Total Antioxidant Status Level (TAS) (Rel Assay Diagnostic, Turkey) and Total Oxidant Status Level (TOS) (Rel Assay Diagnostic, Turkey) in testicular homogenates were measured by using commercially available kits. Oxidative Stress Index (OSI) was calculated by the following formula and the results were expressed as "arbitrary unit" (AU) (19).

$$OSI = \frac{\text{TOS, µmol H2O2 equiv./lt}}{\text{TAS, mmol Trolox equiv./lt x 10}}$$

Commercially available IL-2 (Shanghai Yl biotech Co. Ltd. CHINA) and IL-6 (Shanghai Yl biotech Co. Ltd. CHINA) rat ELISA kits were used to evaluate cytokine levels in testicular homogenates.

The testicles stored for histological examination were kept in 10% neutral buffered formaldehyde for 24 hours. The testes were then divided into transverse slices and the fixation was continued for an additional 24 hours. Slices of samples taken from the upper, middle and lower parts of the testis were washed with tap water and then dehydrated by passing through 70%, 80%, 90% and 96% ethanol series, respectively. It was held in xylol twice to make it transparent. Paraffin blocks were obtained after passing the liquid paraffin series. Sections with a thickness of 4-5 µm were taken with a microtome, and hematoxylin-eosin (H&E) staining technique was used for general histopathological evaluation. Caspase 3 and Bcl-2 immunohistochemistry was used to evaluate the apoptosis.

2.1.Statistical analysis

Data are expressed as mean±standard error. OneWay Analysis of Variance test was applied to variables that consist of independent groups and showed normal distribution, and variables that did not show normal distribution were analyzed with Kruskal-Wallis OneWay Analysis of Variance on Ranks test. Significance level was set at $p< 0.05$. All statistical analyzes were made with SPSS 21.0 package program.

3. Results

3.1.Morphometric Findings

Body and testis weight measured in animals at the beginning and end of the experimental procedure are shown in Table 1.

There was no significant difference between the groups in terms BW_0 and BW_1 ($p> 0.05$). The TW/BW was not significantly different between control and CP groups ($p> 0.05$). Treatment with 300 mg / kg berberine also did not cause a significant change in TW/BW, while it was significantly higher in the Ber75

and Ber150 groups compared to both the control and the CP groups (p< 0.001 and p< 0.05 compared to control group; p< 0.001 and p< 0.05 compared to CP group) (Table 1).

Table 1. Body and testis weight of animals.
([a] compared to control and [b] compared to CP; *p<0.05 and ***p<0.001)

	Control	CP	CP+Ber75	CP+Ber150	CP+Ber300
BW1 (g)	208.75 ± 22.24	180.63 ± 18.77	156.88 ± 12.25	200.63 ± 16.51	208.75 ± 12.46
BW2 (g)	314.13 ± 29.72	275.88 ± 25.51	192.25 ± 10.7	248.71 ± 18.35	265.50 ± 18.34
Testis weight (TW) (mg)	1538.75 ± 66.33	1520.75 ± 66.15	1351.63 ± 27.23	1592.43 ± 63.04	1594.88±67.49
TW / BW	5.24 ± 0.30	5.26 ± 0.20	7.14 ± 0.29[a,b***]	6.53 ± 0.33[a,b*]	6.10 ± 0.23

BW% was slightly decreased in CP group compared to the control group (p> 0.05). However, berberine treatment at doses of 75 mg / kg, 150 mg / kg and 300 mg / kg resulted in a significant decrease in BW% compared to both control and CP groups (for all groups p< 0.001 compared to control group; for Ber 75 and Ber150 groups p< 0.001 and for Ber300 group p< 0.01 compared to CP group) (Fig 1).

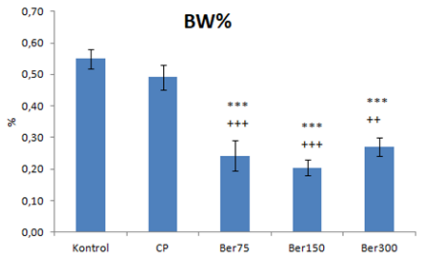

Figure 1. The percentage of body weight change (BW%) on the day 15. (***p<0.001 as compared to control group; ++p<0.01 and +++p<0.001 as compared to CP group)

3.2.Biochemical findings

IL-2 levels in the testicular homogenates did not show statistically significant difference between the groups (p> 0.05) (Fig 2)

IL-6 levels in testicular homogenates were found to be significantly higher in the CP group compared to the control group (p< 0.05) (Fig 3).

Although being slightly higher in CP group compared to the controls and slightly lower in berberine-treated groups compared to CP group, OSI showed no significant difference between the groups (p> 0.05) (Fig 4).

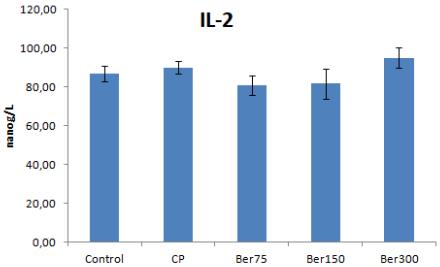

Figure 2. IL-2 levels measured in testicular homogenates (ng/L)

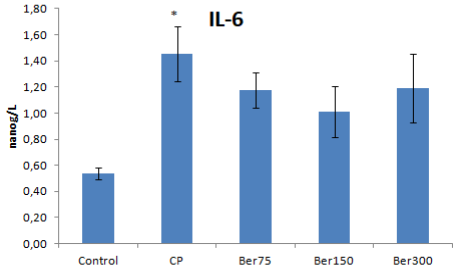

Figure 3. IL-6 levels measured in testicular homogenates (ng/L) (*p<0.05 as compared to control group)

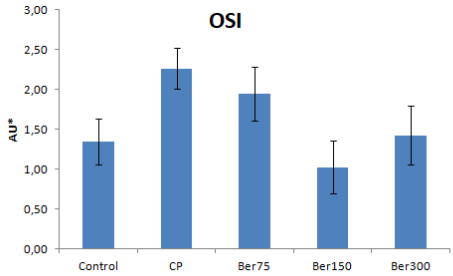

Figure 4. Oxidative stress index (OSI) (AU*: arbitrary unit)

3.3. Histopathological findings

Hematoxylin-eosin-stained images of testicular sections are shown in Fig 5 and 6. In the CP-treated group, seminiferous tubules exhibited an irregular shape and disorganized spermatogenetic cells were observed in the tubule wall. While spermatogenetic cells were seen in the lumen of some tubules, the diameter of the tubule decreased and the tubule lumen disappeared due to cell loss in some other tubules. Berberine treatment was found to reduce or improve the damaging effects of CP on testicle in a dose-dependent manner with almost a normal histological appearance found in Ber300 group (Fig 5 and 6)

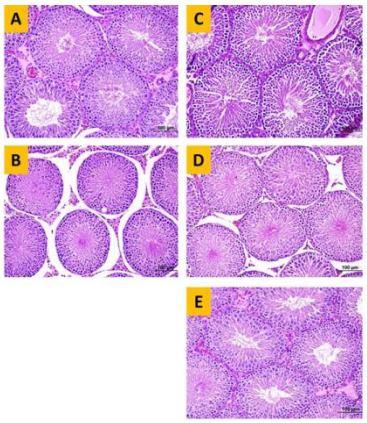

Figure 5. H&E staining of testicular sections (x20 magnificance).
A: control group; B: CP group; C: Ber75 group; D: Ber150 group and E: Ber300 group

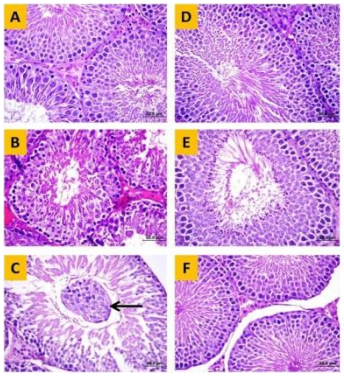

Figure 6. H&E staining of testicular sections (x40 magnificance)
A: control group; B: CP group; C: CP group with seminiferous tubules exhibiting an irregular shape and disorganized spermatogenetic cells observed in the tubule wall (arrow) D:Ber75 group; E: Ber150 group and F: Ber300 group

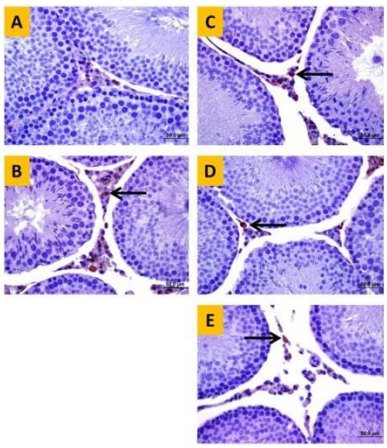

Figure 7. Caspase 3 immunoreaction in testicular sections. Arrows indicate positively-stained Leydig cells (x40 magnificance)
A: control group; B: CP group; C: Ber75 group; D: Ber150 group and E: Ber300 group

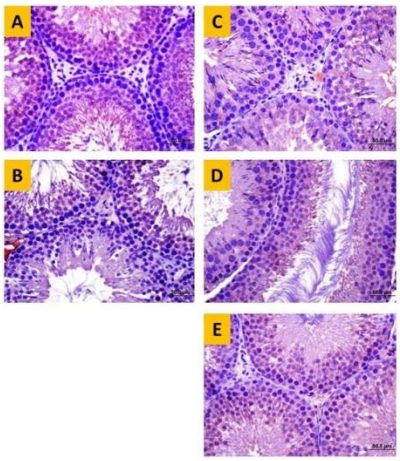

Figure 8. Bcl-2 immunoreaction in testicular sections (x40 magnificance)
A: control group; B: CP group; C: Ber75 group; D: Ber150 group and E: Ber300

Immunohistochemical staining for caspase 3 revealed that Leydig cells located in the interstitial area showed a different level of caspase 3 reaction between groups, while spermatogenetic cells did not show any significant apoptotic finding in any group. While Leydig cells did not show a positive reaction in the control group, the positive reaction was observed with the highest numbers and density in the CP group. On the

other hand, the number and density of positively stained Leydig cells decreased as the dose of berberine increased (Fig 7).

In testis preparations stained immunohistochemically for Bcl-2, spermatogenetic cells showed a significant positive staining in the control group with no or weak staining in the CP group. On the other hand, the positivity of the staining in spermatogenetic cells increased as the dose of berberine increased (Fig 8).

4. Discussion

In this study, 75, 150 and 300 mg/kg doses of berberine improved the degenerative histological changes induced by a single intraperitoneal injection of 200 mg / kg CP in the testicular tissue of adult male rats in a dose-dependent manner. Although oxidative stress markers and cytokine levels measured in testicular homogenates did not show a significant finding, the immunohistochemical staining revealed that CP induces apoptosis in rat testicular tissue which was improved with berberine treatment in a dose-dependent manner.

Although there was no difference betweeen the groups in terms of body weights at the beginning and end of the treatment, Increase % of body weight was significantly decreased in all three berberine-treated groups compared to both CP and control groups ($p<0.001$ in all berberine-treated groups compared to controls; $p<0.001$ in BER75 and BER150 groups and $p<0.01$ in BER300 group compared to the CP group). How do you explain the effects of berberine reduced body weight gain?) Although previous studies reported significantly decreased body weight gain with the CP-treatment (20-24), we administered a single intraperitoneal injection of CP at a dose of 200 mg/kg which was much lesser and short term compared to others. Therefore, this inconsistency might result from less and short exposure to CP in our study (Table 1). In fact, body weight loss is a common side effect of CP treatment possibly because of a direct effect on energy metabolism or its antiproliferative effects on adipocyte precursors (25). In addition, Xie et al. found that there was a decrease in general mobility and appetite in animals, especially in the first week after CP administration (24).

On the other hand, significantly decreased body weight gain in berberine-treated groups may be attributed the well-known antidiabetic effect of berberine. Previous studies reported a significant decrease in body weight with berberine treatment which was suggested to be due to changes in the expression of some genes that control energy consumption (26) or to a decrease in fat accumulation (27). In addition the diuretic effect of berberine may have also caused a decrease in body weight. In the study of Bashir and Gilani, the diuretic effect of berberine was shown in a dose-dependent manner (28).

In order to evaluate the testicular toxicity of CP, relative testicular weight was estimated as TW/BW. Accordingly, CP did not cause a significant difference in TW/BW compared to the controls ($p> 0.05$) and only BER75 and BER150 groups showed significantly increased TW/BW compared to the CP group ($p<0.001$ and $p<0.05$, respectively) (Table 1). In many of the previous studies, CP treatment at different doses and/or durations was reported to cause a significant decrease in absolute and/or relative testicular weight. It has been suggested that this decrease may be due to decreased sperm production and histological changes such as apoptosis, degeneration and parenchymal atrophy (20, 22, 23).

In our study, histological examination revealed irregularity in seminiferous tubules and disorganization in spermatogenetic cells in CP-treated group. In addition, spermatogenetic cells poured into the tubule lumen and a decrease in tubule diameter was observed. On the other hand, the histological appearance of the testes improved with increasing doses of berberine. In addition to these degenerative and parenchymal changes, caspase-3 and Bcl-2 immunohistochemical staining revealed apoptotic changes in the testicular tissue with CP treatment, while berberine showed a dose-dependent antiapoptotic effect (Figures 5-8). As mentioned above, these histological changes

can also be expected to result in a decrease in testicular weight. However, the lack of a significant change in either absolute or relative testicular weight after CP treatment in our study may be due to short-term use of CP. As a matter of fact, in most studies with a decrease in testicular weight, CP exposure was prolonged up to 5-10 weeks.

In many previous studies, increased oxidative stress has been shown in rats treated with CP with a decrease in GSH-Px and G6PD (21) and an increase in lipid peroxidation (20). Oxidative status is in equilibrium with ROS production and elimination of ROS in the cell. Disruption of this balance causes damage to the cell (29). In our study, although not being statistically significant, the OSI, which shows the status of oxidative and antioxidative systems was slightly higher in the CP group compared to the control group ($p > 0.05$).

On the other hand, a decrease in oxidative stress markers (TBARS) was observed with a rich content of berberine (100 mg / kg) in the testicular oxidative stress models induced by various agents. It has been shown that berberine extract can protect the cell against ROS damage by decreasing TBARS and NO levels and increasing the amount of decreased GSH (30). The researchers observed that these results are consistent with previous studies showing that the berberine fraction, they have used, has a strong antioxidant capacity (30-32). In our study, berberine treatment resulted a slight but insignificant decrease in OSI ($p > 0.05$) which may be resulted from the fact that unlike other berberine extracts, we used a chemical pure form of berberine including no additional alkaloids such as berberubine, columbamine, bermamine, palmatine, jatrorrhizin (33).

It is well known that oxidative stress also initiates the apoptotic cascade (34). ROS activates proapoptotic proteins and result in sitochrome-c release via voltage-gated anion channels (35). In a healthy cell, mitochondria contain antiapoptotic genes such as Bcl-2. On the other hand, Bax, one of the proapoptotic proteins that can migrate to mitochondria, can cause testicular damage by inhibiting the antiapoptotic Bcl-2 gene (20, 36). Pro-apoptotic proteins also cause cytochrome-c to migrate out of mitochondria and bind to apoptotic protease activating factor-1 (Apaf-1), which forms a complex called apoptosome (37). Apoptosome then binds with procaspase-9 and activates the caspase-9 form. This active caspase triggers the activation of caspase 3, 7 and 12 (38). Activation of caspase-3 mediated pathways also causes reproductive (germ) cell apoptosis (39-41).

Berberine has been shown to prevent apoptosis by increasing the antiapoptotic protein Bcl-2 and reducing apoptotic proteins such as cytochrome-c, Bax, and caspases(42). There is also a study showing that berberine can prevent Alzheimer's disease by reversing GSK-3β activation that targets Bcl-2 protein (43). Based on all these studies, it can be assumed that berberine may have a protective effect in CP-induced testicular apoptosis by both its antioxidant and antiapoptotic effects.

It is known that cyclophosphamide triggers an inflammatory reaction in animals with different mechanisms. Cytokines (42, 44-46) and PPARγ pathway (47) are thought to play a role in these proinflammatory events.

Berberine can achieve its anti-inflammatory effect by inhibition of the MAPK pathway, inhibition of the NF-KB pathway, by RhoGTPase inhibition and PPARγ activation (48).

There are studies showing that berberine treatment reduces TNF-α and IL-6 in cultures of various cells (49-52). The anti-inflammatory activity of berberine has been also observed in splenocytes, kidneys, and liver of NOD mice (17, 18). We also measured cytokine levels but only found a slight but statistically insignificant decrease only in IL-6 levels ($p > 0.05$). In fact, all above-mentioned studies were conducted on diabetic animal models, which might be confusing due to the inflammatory nature of diabetes itself.

As a result, we found degenerative changes in testicular tissue with CP, which were improved dose-dependently with berberine treatment. The role of oxidative stress, inflammation and apoptosis in this histological damage was evaluated and although no significant change was found in

terms of oxidative stress markers and cytokine levels in testicular homogenates, the apoptotic changes induced in testis by CP administration was significantly prevented and/or treated by berberine in a dose-dependent manner. Our results suggest that berberine may have beneficial effects in preventing and/or treating testicular damage due to CP. Because these effects in animals are likely to be able to seen in humans, more animal and clinical studies should be conducted to confirm all these results.

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Ethics

Ethics Committee Approval: The study was approved by Eskişehir Osmangazi University HADYEK Ethical Committee (Number: 658, Date:14. 03.2018).

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