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Effects of Panax ginseng on cisplatin-induced testicular damage of rats

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Panax ginseng'in sıçanlarda cisplatin ile oluşturulan testis hasarı üzerine etkileri

Abstract: Infertility has become an increasingly important health problem due to genetic, familial, hormonal, and congenital abnormalities, environmental and chemical reasons. This study aimed to investigate the effects of Panax ginseng (Pnx) root extract on cisplatin (CP) induced testicular damage of rats. Four animal groups were applied with different protocols as control, Pnx (200 mg / kg), CP (7 mg / kg), and CP + Pnx (200 mg / kg). At the end of the experiment, the body and testicular weights of the rats were measured. While free/total testosterone, total antioxidant capacity (TAC), and total oxidative species (TOS) levels were analyzed in blood samples, apoptotic cells were marked by TUNEL staining in testicular samples of rats. According to the results, free/total testosterone and TAC levels were decreased while TOS levels increased in injured rats' plasma. On the other hand, seminiferous tubule diameters widened, and the number of apoptotic cells increased in rats' testis. These variables were significantly improved with the consumption of Pnx. As a result, Pnx has a significant protective effect on testicular tissue; however, further studies are needed to elucidate its action mechanism.

Keywords: Apoptosis, Cisplatin, Panax ginseng, TAC, TOS

Özet: İnfertilite genetik, ailevi, hormonal nedenler, doğumsal anormallikler, çevresel ve kimyasal nedenlerle günümüzde artmakta olan önemli bir sağlık sorunu haline gelmiştir. Bu çalışma ile sıçanlarda sisplatin (CP) ile indüklenen testis hasarı üzerine Panax ginseng (Pnx) kök ekstraktının etkilerinin incelenmesi amaçlanmıştır. Sıçanlar kontrol, Pnx (200 mg/kg), CP (7 mg/kg) ve CP+Pnx (200 mg/kg) olmak üzere 4 gruba ayrılmıştır. Deney sonunda sıçanların vücut ve testis ağırlıkları ölçülmüştür. Sıçanların kan örneklerinde serbest/total testosteron, toplam antioksidan kapasite (TAK) ve toplam oksidan durum (TOD) seviyeleri analiz edilirken, testis örneklerinde TUNEL boyaması ile apoptotik hücreler işaretlenmiştir. Sonuçlara göre hasar oluşturulmuş sıçanların plazma örneklerinde serbest/total testosteron ve TAK düzeyleri azalırken, TOD miktarı artmıştır. Testis dokularında seminifer tübül çapları genişlemiş ve apoptotik hücre sayısı artmıştır. Pnx uygulaması ile bu değişkenlerin ciddi oranda düzeldiği saptanmıştır. Sonuç olarak, Pnx'in testiküler dokuda belirgin koruyucu etkinliği bulunmaktadır; fakat etki mekanizmasının aydınlatılabilmesi için ileri çalışmalara gereksinim vardır.

Anahtar Kelimeler: Apoptoz, Cisplatin, Panax ginseng, TAK, TOD

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1. Introduction

Cisplatin (CP) is an alkylating chemotherapeutic agent used alone or in combination with antineoplastic agents in mouth, head, neck, endometrium, lung, ovarian, and testicular cancers (Dasari et al., 2014). The therapeutic effect of CP is dose-dependent and cumulative (Hanigan et al., 2003). The most important known side effect of CP, which is frequently used in the clinic, is nephrotoxicity. Therefore, kidney functions are commonly followed in cancer chemotherapy, depending on CP (Barabas et al., 2008). The copper transporter Ctr1 mediates CP absorption and transport in mammals (Dasari et al., 2014). CP is known to induce DNA damage by binding to the N7 reagent center on purine residues and rising apoptotic cell death by blocking cell division in cancer cells. (Aly et al., 2020). Cancer cells cause more oxidation than normal cells with increased metabolic activity, oncogenic activation, and corruption of mitochondrial functions. One of the most critical contraptions is oxidative stress that plays a role in CP toxicity (Wang et al., 2020). Recent studies showed that CP induced genotoxicity and reproductive toxicity accompanied by severe oxidative stress (Aksu et al., 2016; Sadeghi et al., 2018). Similarly, CP reduced catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) expressions; however, it increased malondialdehyde (MDA) levels in the testicular tissues of mice (Zhang et al., 2020). On the other hand, CP caused lower testosterone levels and induced apoptosis via enhanced Bcl-2-associated X protein (Bax) and decreased B-cell lymphoma 2 (Bcl-2) levels in the testis of rats (Azab et al., 2020). Generally, CP-induced animals also exhibited lower sperm count and motility as well as higher abnormal sperm; however, testicular injury adversely affects spermatogenesis, sperm quality, and oxidative

stress parameters in the related studies (Ceylan et al., 2020; Kohsaka et al., 2020; Saad et al., 2020; Wang et al., 2020). These findings showed the CP-induced genotoxicity and reproductive toxicity, which might be closely related to oxidative stress. Until now, there is no effective treatment agent for CP-induced genotoxicity and reproductive toxicity, and finding new protective and therapeutic strategies against CP-induced adverse changes is mandatory.

Panax ginseng (Pnx) is an important medicinal herb and root extract, and has antioxidant effects with predominant metabolic, neurological, and urological activities. The use of Pnx in traditional Chinese medicine dates back to about 5000 years ago. Researches on Pnx have been increasing in recent years due to the biotransformation of ginsenosides in the roots or extracts of Pnx in the intestine and its high pharmacological activity and pharmacokinetics (Mancuso et al., 2017). Pnx consumption might reduce prenatal stress in rats (Kim et al., 2015). In another study, Korean Ginseng increased C-21 steroid metabolism in the testis' interstitial Leydig cells (Kim et al., 2011). However, many studies suggested that Pnx decreases oxidative stress and increases antioxidant capacity in various tissues (De Freitas et al., 2019; Kopalli et al., 2016; Kumar et al., 2003).

The purpose of this study is to examine the effects of Pnx, which has been used as a chemotherapeutic drug for centuries, on possible damages on the testis, and to develop an alternative treatment method in male infertility, which is a significant health problem today.

2. Materials and method

2.1. Reagents

Pnx pure 99% (2018040200019) was obtained from Aksu Vital Doğal Ürünler Gıda Sanayi ve Tic. A.Ş. CP (LRAB7778) and other reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Animals and treatments

The standard rodent chow diet and living conditions have been applied to the ten-week-old and approximately 200–300 g of 28 male Wistar rats. The rats were acclimated one week for the optimization, and then four groups were formed for different protocols: Control (n=7), Pnx (n=7), CP (n=7), and CP plus Pnx (n=7). Pnx (200 mg/kg) was given in 2 mL of saline by gastric gavage once a day for four weeks to the Pnx group; however, after this, single dose of CP (7 mg/kg) was administered intraperitoneally (i.p.) to CP and CP plus Pnx groups. Three days after, rats were anesthetized with ketamine (100 mg/kg) and xylazine 10 mg/kg) at the end of the experiment, blood and testis samples were collected rapidly. The Ethical Animal Research Committee of Afyon Kocatepe University (AKUHADYEK 268-17) approved this study's animal procedures.

2.3. Determination of the TAC, TOS, and total/free testosterone levels in the plasma

Non-fasted rats' cardiac blood was centrifuged immediately at +4°C and 1,000*g* for 30 min. The resulting supernatant is utilized and stored at −85 °C until the total antioxidant capacity (TAC) (Erel, 2004) and total oxidant status (TOS) (Erel, 2005) and testosterone levels are measured. TAC and TOS Assay Kits (Rel Assay Diagnostics, TR) were used for the measurements. The TAC results were presented as mmol Trolox Eq/L; however, TOS results were given as μmol H_2O_2 Eq/L for plasma. The oxidative stress index (OSI) was calculated according to the formula; OSI = [(TOS, μmol H_2O_2 Eq/L) / (TAC, (mmol Trolox Eq / L) x 100]. Free and total testosterone levels were determined by an ELISA (eBioscience, USA) kit according to the manufacturer's instructions,

2.4. Histopathological evaluations

Tissue samples taken from sacrificed rats were fixed in Bouin's fixative and then histologically processed and embedded in paraffin blocks. Five μm sections were taken on polylyzed slides which were stained with Hematoxylin & Eosin (HE) for the general histomorphological appearance. Besides, 25 different tubules were selected from each section, and Johnsen scoring was applied. According to this scoring; 10: Tubules perfect and complete spermatogenesis, 9: Numerous spermatozoa, irregular spermatogenesis, 8: There are only a few sperm, 7: There are no spermatozoa, but many spermatids, 6: Just a few spermatids, 5: No spermatozoa or spermatids, but many spermatocytes, 4: Only a few spermatocytes are present, 3: Only spermatogonia are present, 2: No germ cells, 1: Neither germ cells nor Sertoli cells are present. Then, the data were compared statistically. Testicular injury and spermatogenesis in the sections were evaluated histopathologically using Johnsen's mean testicular biopsy score (MTBS) criteria (Johnsen, 1970).

2.5. Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay (TUNEL)

Testicular tissues were detected by TUNEL assay according to the instructions of ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA, USA). 5 μm sections were taken from paraffin blocks and stained according to the manufacturer's protocol. Dark brown stained cells were considered positive. TUNEL-positive cells were counted in 6 randomly chosen areas (400X).

2.6. Statistical analysis

All data is represented as mean ± standard error of the mean (SEM) throughout the study and compared for differences using the Prism 6.01 GraphPad software. Student's t-test for unpaired data or one-way ANOVA followed by the *Bonferroni* post hoc analysis were used to compare different groups. Johnsen's scoring was made using the Chi-Square test. All statistical tests were performed at a p-value less than 0.05.

3. Results

3.1. The effects of Pnx and CP on body and testis weight

As shown in Table 1, both Pnx and CP did not affect the bodyweights of rats. Similarly, there is no change in the ratio of right and left testis weight to body weight in the Pnx-treated healthy rats. However, CP significantly decreased the ratio of right testis weight to body weight, and there was a tendency toward reduction in left testis weights, but it was not significant. This reduction remained at the levels of Control rats with Pnx-treatment given before CP administration. In this respect, Pnx-treatment significantly prevented the decrease in CP-dependent testicular weights (Table 1).

Table 1. The initial and terminal body weights, right and left testicular weights, and their ratio to the bodyweight of Control, Pnx, CP, and CP plus Pnx groups.

Groups	Control	Pnx	CP	CP+Pnx
Initial body weight (g)	254.9±11.5	227,1±14.3	291.6±14.6	267.9±14.2
Terminal body weight (g)	297±19.3	299.6±15	355±14.2	307.7±15.4
Right testis absolute weight (g)	3.44±0.21	3.41±0.05	3.57±0.18	3.79±0.18
Left testis absolute weight (g)	3.24±0.23	3.72±0.27	3.76±0.14	4.04±0.23
Right testis weight to body weight (g/100g BW)	1.16±0.07	1.14±0.01	1.01±0.05*	1.23±0.06#
Left testis weight to body weight (g/100g BW)	1.19±0.07	1.24±0.09	1.06±0.04	1.31±0.08#

Values are expresses as mean ± SEM, n = 7; * $p < 0.05$, significantly different from control; #$p < 0.05$, significantly different from CP.

3.2. The effects of Pnx and CP on endocrine parameters, TAC, TOS, and OSI levels in the plasma of rats

The levels of total and free testosterone in the plasma samples were established by using ELISA kits. Accordingly, there was no change between Pnx and control groups on the total and free testosterone levels (Fig. 1a,b). Giving CP to the healthy rats significantly reduced total and free testosterone levels compared to controls. On the other hand, as shown in Figures 1a and b, there were preventive effects with Pnx against CP-administration, achieving a significance level. Plasma TAC and OSI levels did not change, but TOS levels decreased with Pnx-feeding (Fig. 1c,d,e). Furthermore, reduced TAC and increased TOS and OSI levels were found after CP-injection in healthy rats' plasma compared to controls. As shown in Figures 1c, d, and e, there was specific prevention with Pnx-treatment to impaired TAC, TOS, and OSI levels of CP injected rats.

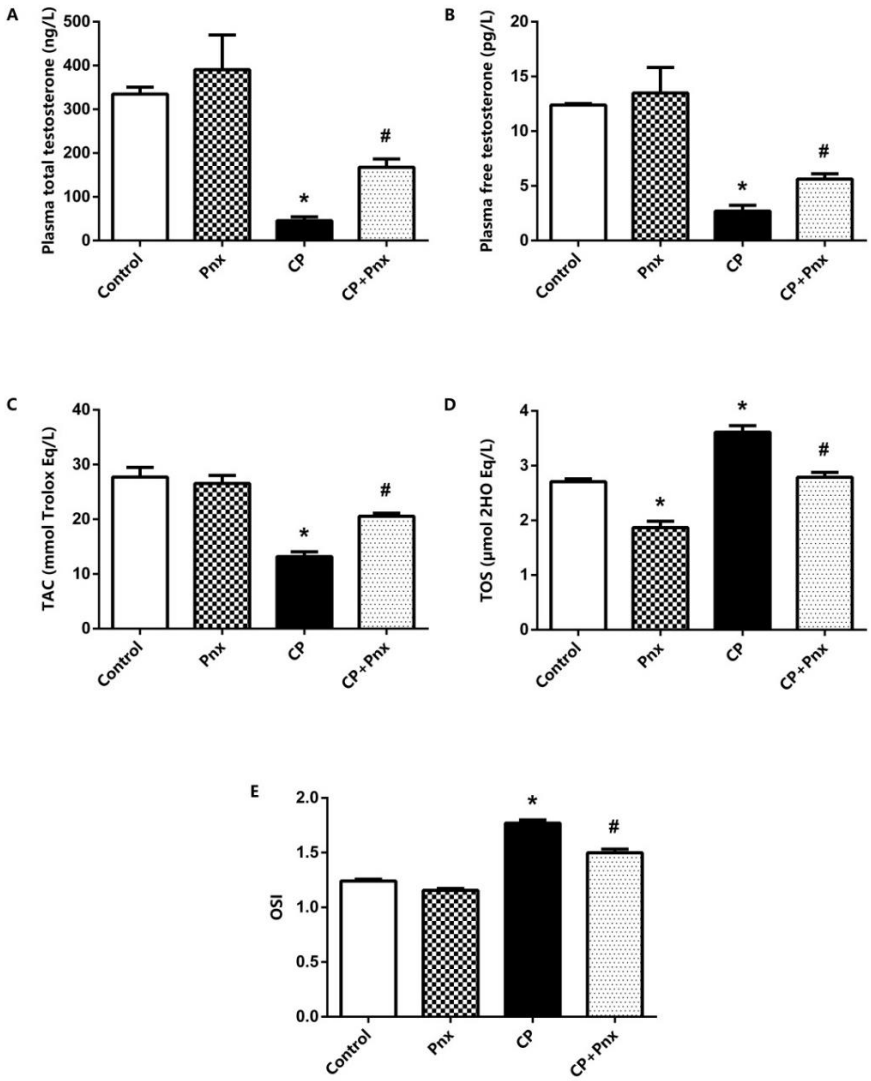

Figure 1. Changes in total testosterone (A), free testosterone (B), TAC (C), TOS (D), and OSI (E) levels in the plasma of the rats. Values are expressed as mean ± SEM, and each bar represents the means from at least six rats. *P<0.05, significantly different from the Control; #P<0.05, significantly different from the CP-treated rats.

3.3. The effects of Pnx and CP on testis histology; results of TUNELassay and Johnsen's score

Dietary Pnx did not change TUNEL (+) spermatogenic and Leydig cells and Johnsen's scores in healthy rats' testicular tissues compared to controls (Table 2; Fig. 2a,b). There was a significant increment in TUNEL (+) spermatogenic and Leydig cells; however a reduction in Johnsen's scores with CP-administration in the testis of healthy rats. On the other hand, Pnx-feeding prevented these changes in the CP groups. Seminiferous tubules showed normal histological appearance in control and Pnx groups. Testes of cisplatin-treated rats showed loss of sperm production and lots of degenerated cells. Pnx improved the histopathological morphology of testes in animals. Testes showed typical histological structure in the seminiferous tubules with complete spermatogenesis.

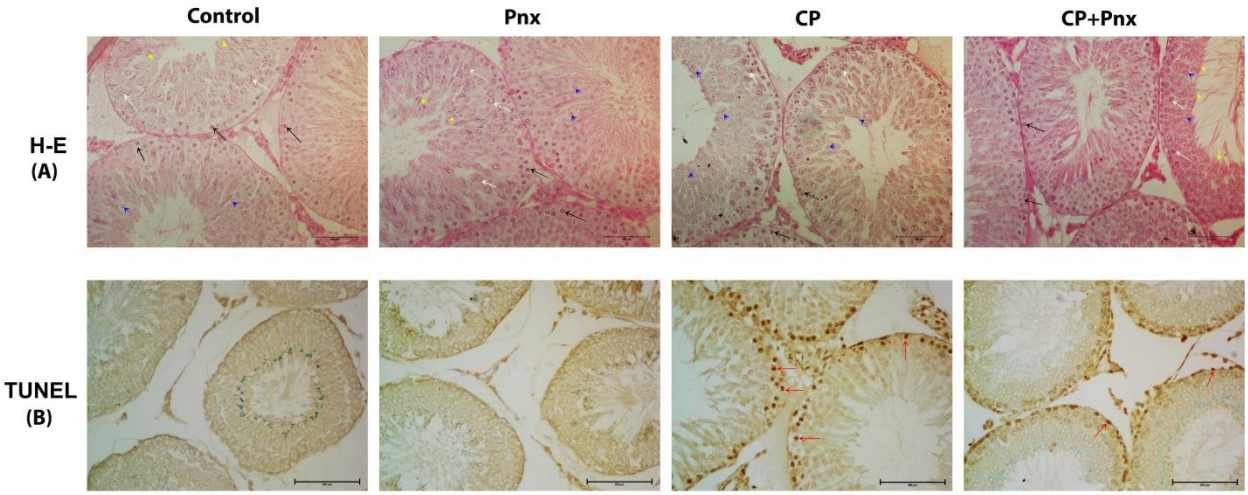

Figure 2. (A) Histological features of testis sections from Control, Pnx, CP, and CP+Pnx groups. In all groups, spermatogonia are indicated by the black arrow. The white arrow indicates primary spermatocytes, the blue arrow indicates spermatids, and many spermatozoa are indicated by the yellow arrow (200X magnifications, H-E). (B) Spermatogenic and Leydig cells apoptosis detected via TUNEL assay (200X magnifications). TUNEL (+) cells have dark Brown nuclei and are indicated by the red arrow.

Table 2. Johnsen's score of tubules and number of TUNEL (+) spermatogenic and Leydig cells of Control, Pnx, CP, and CP plus Pnx groups.

Groups	Control	Pnx	CP	CP+Pnx
TUNEL (+) spermatogenic cells	8.7±1	8.4±0.4	463±22*	159±8#
TUNEL (+) Leydig cells	3.4±0.2	3.3±0.2	32.6±1.4*	12.9±1#
Johnsen's score	10±0.1	10±0.1	7±0.3*	9±0.3#

Values are expressed as mean ± SEM, n = 7; *p < 0.05, significantly different from control; #P < 0.05, significantly different from CP.

4. Discussions

Herein, we demonstrated dietary Pnx prevented oxidative stress and dysmorphology in the CP-induced degeneration of testis via rising plasma TAC, total & free testosterone, and testicular Johnsen's scores, and reducing plasma TOS levels, testicular TUNEL (+) spermatogenic cells, and TUNEL (+) Leydig cells.

CP has been considered the most frequently used chemotherapeutic agent against various solid tumors because of its significant therapeutic effects (Barabas et al., 2008). However, it also damages many tissues such as the kidney, brain, liver, and testis due to its intense cytotoxic activity (Dasari et al., 2014; Hanigan et al., 2003). Many studies examining its effects on the testis have been repeatedly shown that CP causes mitochondria-mediated oxidative stress, apoptosis, inflammation, and gonadotoxicity (Aly et al., 2020; Ceylan et al., 2020; De Freitas et al., 2019). Recently, research investigating Ginger juice's effects on CP-induced testicular damage showed that rats' body weights did not change at the end of the fifth day after CP administration. However, testicular weights significantly decreased (Famurewa et al., 2020). A similar study found a decrease in testicular tissue weight at the end of the fifth day after CP injection in the hamsters (Wang et al., 2020). In this study, the ratio of right testis weight to body weight decreased at the end of the third day after CP application, but body weight did not change. Moreover, left testis weights were reduced, but they could not reach the significance level because, unlike other studies in our study, animals were decapitated at the end of the third day after CP application. Several studies also showed that the reason for CP-mediated lower testis weight is related to decreased sperm count and epididymal weight (Wang et al., 2020; Yucel et al., 2019). On the other hand, Pnx-feeding increased testicular weights to body weights in the CP-administered rats. This finding is the indicator of the protective efficacy of Pnx against CP-mediated testicular damage. Recent studies showing the antioxidant, anti-inflammatory, and cytoprotective effects of Pnx also support our results. (Kim et al., 2017; Kopalli et al., 2016; Zhu et al., 2020). It is known that CP used for therapeutic purposes causes the increment of reactive oxygen species and the suppression of antioxidant structures in the blood or tissues (Rashid et al., 2013). Besides, the studies showed that CP-administration causes an increase in testicular MDA, 4-hydroxynonenal, and caspase-3 levels while reducing superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels of rats (Saad et al., 2020; Majd et al., 2021). In another study, CP caused a

reduction in TAC and an increment in TOS in rats' plasma samples (Geyikoglu et al., 2017). We determined lower TAC and higher TOS levels in CP-treated rats' plasma in the present study. Herein, it was shown that CP increases reactiCP-treated species and reduces the antioxidants' impact (Saad et al., 2004). Moreover, the unconfronted reactive oxygen species enhance DNA and mitochondrial membrane damage via lipid peroxidation (Sadi and Sadi, 2010). Therefore, these changes due to CP have already been expected. However, Pnx-feeding significantly increased plasma TAC and reduced TOS levels in the CP-induced testicular damage. De Freitas et al. showed that Pnx metabolite (GIM-1) prevented oxidative stress and apoptosis in human Sertoli cells via increased SOD, CAT, GPx, and caspase-3 levels (De Freitas et al., 2019). In another study, in GC-2 sperm cells, Pnx prevented hydrogen peroxide-induced oxidative stress via increased GPx expression (Kopalli et al., 2016). These results are consistent with our current study and point to the cytoprotective efficacy of Pnx.

One of the leading causes of reproductive dysfunction is oxidative stress (Lonare et al., 2016). Reactive oxygen species may lead to testosterone synthesis disorder via impaired cyclic adenosine monophosphate (cAMP) in Leydig cells. Kong et al. expressed that testosterone synthesis is a complex process that involved many served enzymes. (Kong et al., 2017). When gonadotropin luteinizing hormone is released, it results in cAMP release and protein kinase A activation, which initiates a series of enzyme cascade reactions (Liu et al., 2015). In various studies examining the effects of CP on testicular and reproductive function, it has been shown that plasma or testicular total and free testosterone levels are also reduced; these results have often been associated with oxidative stress (Azab et al., 2020; Ceylan et al., 2020; Famurewa et al., 2020; Jourabi et al., 2020; Kohsaka et al., 2020; Majd et al., 2021; Saad et al., 2020; Wang et al., 2020). In the present study, we also demonstrated that CP-induced oxidative stress significantly decreased total and free testosterone levels in rats' plasma. However, the antioxidant effects of Pnx increased both total and free testosterone productions. This result indicated that Pnx might promote the testosterone secretion of Leydig cells via suppressing oxidative stress which was induced by CP in rat testes. Kohsaka et al. reported that CP causes an increase in TUNEL (+) spermatogenic and Leydig cells apoptotic index; however, it reduces Johnsen's score in rats' testes (Kohsaka et al., 2020). Research on the protective effect of caffeic acid phenethyl ester on testicular damage caused by CP showed that CP decreased Johnsen's score and damaged spermatogenic cells (Ceylan et al., 2020). Sperm count, viability, and motility were reduced by CP-administration, while sperm dysmorphology increased in rats' testes. CP-administration decreased sperm count, viability, and motility, while sperm dysmorphology increased in rats' testes (Jourabi et al., 2020). Our results suggest augmented TUNEL (+) spermatogenic and Leydig cells in CP-induced damaged rats. A significant pathological effect that included a reduced Johnsen's score was revealed in rats treated with CP. However, treatment for four weeks with Pnx prevented TUNEL (+) spermatogenic and Leydig cells and Johnsen's score. These results indicated that the rat model of testicular lesion induced by CP was successfully established, and Pnx had protective effects on CP-induced basic toxicity.

In conclusion, an animal model of CP-induced testicular damage and Pnx intervention was established in the current study. Results indicated that CP might lead to testicular toxicity via oxidative stress and inhibiting testosterone synthesis in rats. Moreover, Pnx has preventive effects on CP-induced modulations. Further research is required to clarify its preventive effects and mechanism.

Conflict of interest

The authors report no conflicts of interest related to this study.

Authors' Contributions

EA, KK, HG, HHD, SÇ, and MBP performed the research. EA, KK, and MBP helped during the experimental work, statistical analysis and in writing the manuscript. MBP drafted the manuscript. EA, HG, and MBP conceived and designed the study and critically revised the manuscript.

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