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The protective effect of taurine on cyclophosphamide-induced testicular toxicity in rats

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Abstract: This study aimed to evaluate the protective effect of taurine (TAU) with regard to antioxidant, anti inflammatory and antiapoptotic pathways on cyclophosphamide (CP)-induced testicular toxicity in rats. Forty Sprague-Dawley male rats were used in this experimental study. The CP group animals received a single dose of 200mg/kg CP on Day 8 intraperitoneally (i.p). The other groups were treated with TAU (75, 150 and 300mg/kg) orally for 14 days prior to and following a single i.p injection of CP. Morphometrical analysis and histological examination of testicular tissue were performed. Serum testosterone, LH and FSH levels were measured in serum using commercial ELISA kits. The testicular injury induced by CP was evaluated in terms of oxidative stress, inflammation and apoptosis with a significant inflammatory and apoptotic response and an insignificant oxidative stress. TAU treatment resulted in improvement in body weight gain, oxidative stress, inflammation and apoptosis, some of which were significant. The improvement was more pronounced for antiapoptotic effect of taurine in the testis of CP-treated animals. It was concluded that TAU may prevent and/or treat the testicular toxicity by ameloirating oxidative stress, inflammation and apoptosis.

Keywords: Taurine, cyclophosphamide, oxidative stress, inflammation, apoptosis.

INTRODUCTION

Cyclophosphamide (CP), a bifunctional alkylating drug, is widely used in cancer chemotherapy for a variety of solid and hematological malignancies, as well as an immunosuppressive agent in the treatment of autoimmune disorders (Ahlmann and Hempel, 2016). Despite its wide range of therapeutic uses, CP causes cytotoxicity in normal human and experimental animal cells (Ramirez *et al*., 2019). The gonadotoxicity of CP is the primary cause of its acute toxicity. (Potnuri *et al*., 2018). CP interferes with the production of sperm in the testicles, resulting in infertility in men (Abarikwu *et al*., 2012). Furthermore, experimental investigations in male rats and mice treated with CP have indicated that reduced testicular weight, transient oligospermia, sperm motility and fertilization capacity, as well as abnormal alterations in the testis and epididymis, occur when male rats and mice are treated with CP (Oyagbemi *et al*., 2016, Elangovan *et al.*, 2006).

Taurine (TAU), 2-aminoethanesulfonic acid, is a non-proteogenic and essential amino acid for mammals and enriched in several tissues such as brain, retina, heart and skeletal muscles (Oja and Saransaari, 2007, Niu *et al*., 2018). TAU is synthesized in the liver from cysteine and methionine in the presence of vitamin B6, or it can be acquired through food, particularly seafood and meat (Nikkhah *et al*., 2021). TAU has been tested as potential pharmacological agents in many pathological cases, also has lot of physiological functions, including entering in cell volume regulation (Guizouarn *et al.*, 2000) and in inhibitory neuromodulation or neurotransmission (Wu and Prentice, 2010). TAU has been identified one of the most common free amino acids in the male reproductive system (Yang *et al*., 2010). However, it may act as an antioxidant in preventing sperm lipid peroxidation as a sperm motility factor (Partyka *et al*., 2017). Several studies have shown that TAU protects rat testes against NaAsO (2)- and cisplatin-induced oxidative stress and apoptosis (Das *et al*., 2009, Azab *et al*., 2020).

The aim of this study was to investigate protective effects of taurine on CP-induced testicular toxicity and to study the possible mechanisms -antioxidant and/or antiapoptotic- mediating this effect.

MATERIALS AND METHODS

Experimental animals and housing

Forty adult male Sprague-Dawley rats (8-10 weeks old, weighing 200-300g) were used in the experiment. They were housed in a temperature (24±1°C) and relative humidity of 65%-70% controlled room with a 12:12-h light-dark cycle with food and water ad libitum. The study was performed in accordance with the guidelines for the care and use of laboratory animals approved by the local Ethics Committee (approval number 2018-464-1).

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Chemicals

Taurine (Merck KGaA, Darmstadt, Germany), Cyclophosphamide (Endoxan®, Baxter Oncology GmbH, Germany), IL-2 (Shanghai YI biotech Co. Ltd. Rat Interleukin 2 ELISA Kit, China), IL-6 (Shanghai YI biotech Co. Ltd. Rat Interleukin 6 ELISA Kit, China), TAS, TOS Rel Assay Diagnostic, Turkey) ELISA kits were used for this study.

Study design

The forty male Sprague-Dawley rats were randomly divided into five groups (n=8/per group).

- Control group: Saline (i.g) for 14 days, at the same time SF (i.p) was administered on the 8th day.
- CP group: Saline (i.g) for 14 days and single dose CP (200 mg/kg, i.p) was administered on the 8th day.
- TAU75 group: Taurine (75 mg/kg, i.g) for 14 days and single dose CP (200mg/kg, i.p) was administered on the 8th day.
- TAU 150 group: Taurine (150 mg/kg, i.g) for 14 days and single dose CP (200mg/kg, i.p) was administered on the 8th day.
- TAU 300 group: Taurine (300 mg/kg, i.g) for 14 days and single dose CP (200mg/kg, i.p) was administered on the 8th day.

At the end of the 14 day treatment period and 24 hours after the last drug dose all animals were euthanized by cervical dislocation under high dose general anesthesia, and testicular tissues were removed. One of the testes was placed in phosphate buffer solution (NaCl: 8 g, KCl: 0.2 g, KH_2PO4: 0.2g, Na_2HPO4: 1.14g in 1L distilled water) for histological examination. The other testes was weighed and recorded. 100mg of tissue was placed in V-bottom capped tubes containing 100mg/ml (1:10 wt/vol) phosphate buffer solution to prepare testicular homogenate and stored at -20°C. Samples taken from -20°C were thawed at room temperature and then homogenized in semi-liquid, semi-ice form with the help of homogenizer and centrifuged at +4°C. After centrifugation samples were stored at +4°C.

Biochemical analysis

Oxidative stress assessment

Oxidative Stress Index (OSI): When calculating OSI, which is expressed as a percentage of the ratio of TOS levels to TAS levels, the following formula was used and the results were expressed as "arbitrary unit" (AU)(Feng *et al*., 2012).

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

Evaluation of cytokine levels

IL-2 and IL-6 ELISA kits were used to evaluate cytokine levels in homogenates obtained from testicular tissue.

Histological evaluation

Testicles were kept in 10% neutral buffered formaldehyde for 24 hours. Then, the detection of the transversely sliced testes was continued for 24 hours more. Slices from the lower, middle and upper parts of the testis were washed with tap water and then they dehydrated via using 70%, 80%, 90% and 96% ethanol series. It was held twice in xylol to make it light-permeable. Paraffin blocks were obtained by passing through liquid paraffin series. Hematoxylin-eosin staining technique was used for general histopathological evaluation and caspase 3 and Bcl-2 immunohistochemistry were used to evaluate the apoptosis (Ijaz *et al*., 2023). Images representing the findings of the examination performed under a binocular microscope were taken with a digital camera.

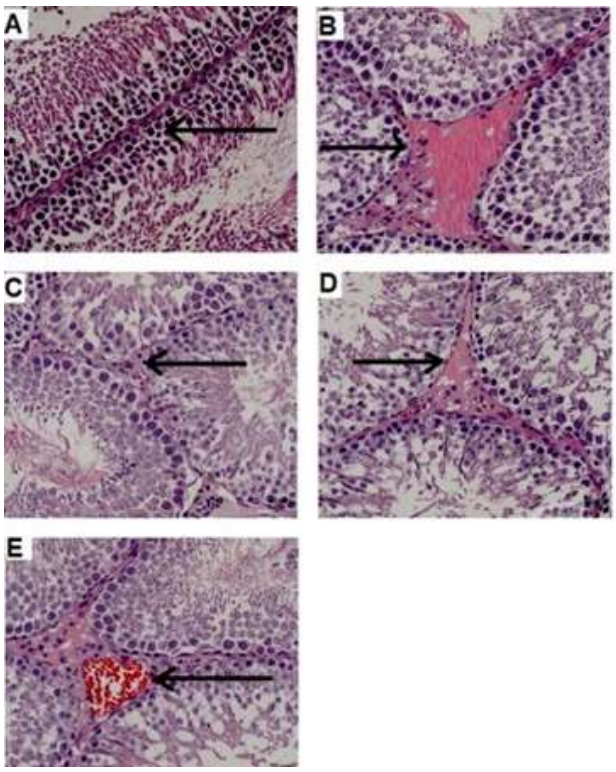

Fig. 1: Hematoxylin & Eosin staining in testis preparations (40x). (A) Control group showed seminiferous tubules with normal histological structure and interstitial area (arrow). (B) CP group showed intense hemorrhage and edema in the interstitial area and seminiferous tubules (arrow). (C) TAU75 group, the hemorrhage and edema were improved with decreased vacuolization and peritubular undulation compared to the CP group (arrow). (D) TAU150 group showed similarity with CP group in terms of vacuolization and hemorrhage and edema in the interstitial area (arrow). (E) TAU300 group showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area with decreased germ cell count peeled off into seminiferous tubule lumen (arrow).

STATISTICAL ANALYSIS

One-Way Analysis of Variance test was applied to the variables consisting of independent groups and showing

normal distribution. The variables that were not normally distributed were analyzed by Kruskal-Wallis OneWay Analysis of Variance on Ranks test. The probability values of $p<0.05$ were considered significant. All data analyzes were performed with SPSS 21.0 package programs.

RESULTS

Morphometric measurements

The body weight determined at the beginning (BW0) and at the end (BW1) of the study is shown in table 1. BW1 was significantly different in CP group compared to the controls ($p<0.001$) and in TAU150 group compared to CP group ($p<0.05$). The percentage change in body weight (BW%) was hıgher in control group compared to the CP group ($p<0.001$). BW% was significantly higher in TAU 150 group compared to CP group ($p<0.05$). In terms of the ratio of testicular weight to body weight, it was significantly higher in CP group compared to the control group ($p<0.001$) and significantly lower in TAU150 group compared to the CP group ($p<0.05$).

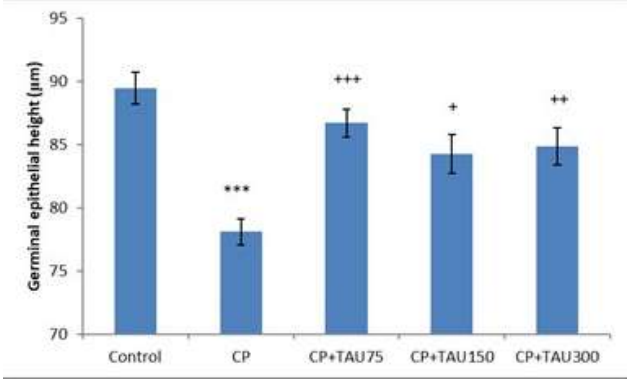

Fig. 2: Germinal epithelial height; results are given as mean ± SEM. ***, $p<0.001$ compared to control; +, $p<0.05$ ++, $p<0.01$ and +++$p<0.001$ compared to CP respectively. ANOVA analysis applied to germinal epithelial height

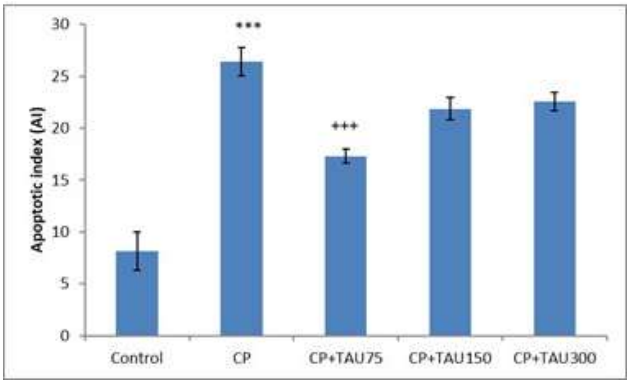

Fig. 3: Apoptotic index (AI); results are given as mean ± SEM***, $p<0.001$ compared to control; +++$p<0.001$ compared to CP ANOVA analysis applied to (AI)

Biochemical findings

IL-2 level was found to be increased significantly in CP-treated animals compared to the control animals ($p<0.01$). IL-6 level was slightly higher in CP group compared to the control group but slightly lower in TAU300 group compared to the CP group. OSI was slightly higher in CP-treated animals compared to the control animals. It was slightly lower in all three Taurine-treated groups compared to the CP group but there were no statistically significant difference ($p>0.05$) (table 2).

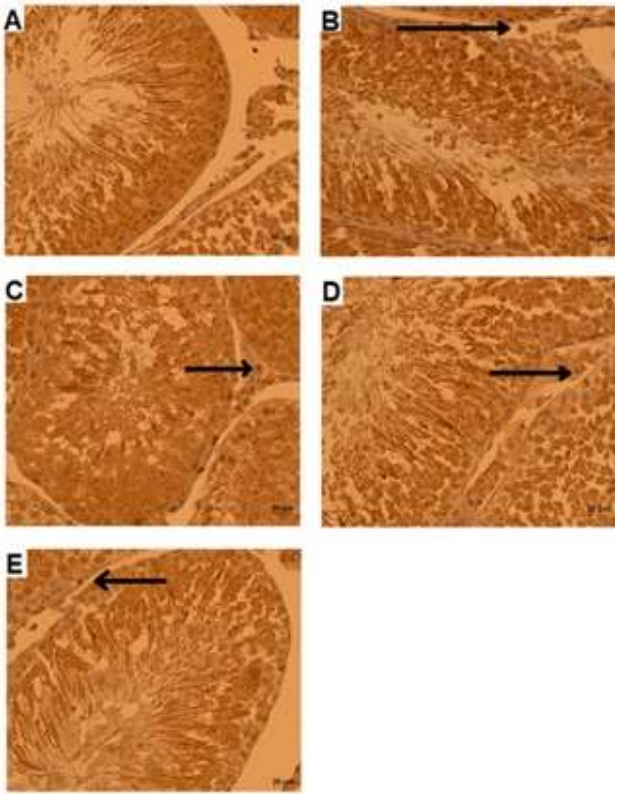

Fig. 4: Caspase-3 immune reaction of rat testis sections (40x) (A) Control group; (B) CP-treated group showing strong immunoreactivity for caspase-3 (arrows); (C) TAU75 showing weak immunoreactivity for caspase-3 (arrows); (D) TAU150 group showing weak immunoreactivity for caspase-3 (arrows); (E) TAU300 group showing weak immunoreactivity for caspase-3 (arrows)

Histological results

General histopathological assessment

CP group showed intense hemorrhage and edema in the interstitial area and a decrease in the thickness of the germinal epithelium. In the seminiferous tubules, changes such as peeling of germinal cells into the lumen, decrease in the thickness of the germinal epithelium and occasional vacuolization were observed. In testicular tissue of animals from TAU75 group, the hemorrhage and edema found in the interstitial area of CP group was improved with decreased vacuolization and peritubular undulation compared to the CP group.

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

	Control	CP	CP+TAU75	CP+TAU150	CP+TAU300
Body weight at the beginning (g)	217.50±13.46	205.00±17.61	232.50±9.58	231.88±13.99	158.75±14.21
Body weight at the end (g)	331.25±25.06	207.14±17.74[a***]	249.71±17.03	294.88±20.67[b*]	192.50±13.50
Left testis weight (mg)	1643.13±57.13	1483.00± 75.57	1565.29±81.73	1579.13±51.57	1489.00±73.77
Testis weight/ body weight	5.09 ± 0.26	7.31±0.31 [a***]	6.35±0.29	5.47±0.30 [b*]	7.91±0.53

Table 2: Oxidative stress status and cytokine levels in the groups ([a]compared to control **, p<0.01).

	Control	CP	CP+TAU75	CP+TAU150	CP+TAU300
OSI (AU)	2.07 ± 0.21	2.70 ± 0.13	2.01 ± 0.10	1.96 ± 0.27	2.41 ± 0.21
IL-2 (ng/L)	22.38 ± 4.71	100.14 ± 12.33 [a**]	81.00± 7.93	85.25 ± 7.01	95.13 ± 3.64
IL-6 (ng/L)	0.65 ± 0.34	0.89 ± 0.28	1.07 ± 0.27	1.10 ± 0.30	0.62 ± 0.27

In testicular tissue of TAU75 group animals, the edema and hemorrhage in the interstitial area of CP group was improved while peritubular fluctuation and vacuolization were found to be decreased compared to the CP group. TAU150 and TAU300 groups showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area with decreased germ cell count peeled off into seminiferous tubule lumen (fig. 1).

Germinal epithelial height being 89.43±1.27μm in control group was significantly decreased to 78.08±1.01μm in CP group (p<0.001) and significantly increased to 86.69±1.07, 84.28±1.53 and 84.86±1.49μm in Tau75, TAU150 and TAU300 groups respectively compared to CP group (p<0.001, p<0.05 and p<0.01) (fig. 2)

Apoptosis assessment

In the testis preparations immunochemically stained for Caspase-3, the apoptotic index being 8.11±1.84 in the control group was significantly increased to 26.40±1.33 in CP group (p<0.001) and significantly decreased to 17.31±0.70 in TAU75 group compared to CP group (p<0.001) (fig. 3 and 4.)

In testicular preparations immunohistochemically stained for Bcl-2, the immunoreactivity was significantly decreased in CP group compared to control group (p<0.001). On the other hand, TAU75 and TAU150 groups showed significantly increased immunoreactivity for bcl-2 compared to the CP group (p<0.05 for both). (fig. 5).

DISCUSSION

This study was conducted to investigate the potential protective effects of taurine on CP-induced gonadotoxicity in male rats. Rats were administered CP (200mg/kg, i.p) on experimental day 8 and TAU (75 mg/kg, 150mg/kg and 300mg/kg, p.o) was administered after a single dose of CP administration. Oxidative stress, inflammation, histological changes, and apoptosis were evaluated. Although it was observed the insignificant changes in oxidative stress and cytokine levels, TAU treatment improved testicular degeneration and reduced CP-induced apoptosis.

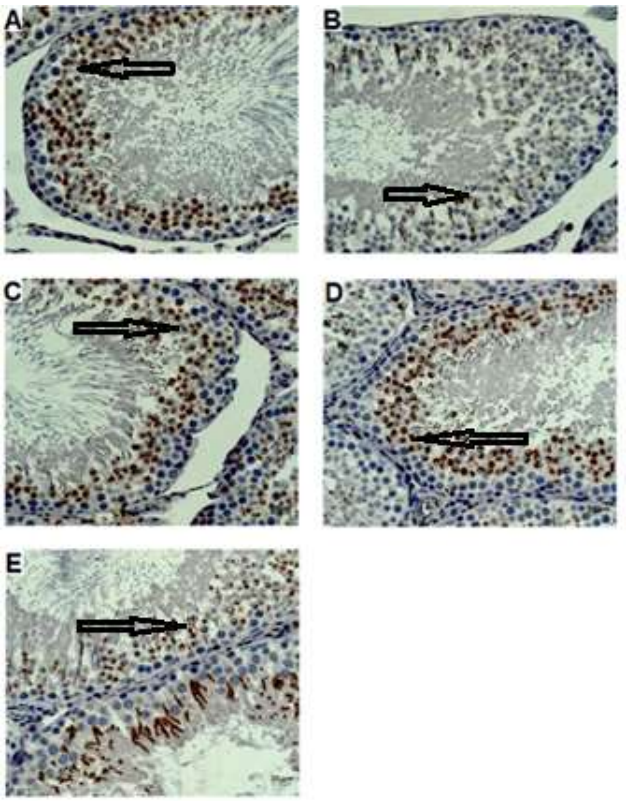

Fig. 5: Immunohistochemically stained testicular preparations for Bcl-2 (40x) A: Control group; B: CP group; C: TAU75 group, D: TAU150 group, E: TAU300); arrows indicate immunoreactivity for Bcl-2.

In the present study, the use of a single dose of CP (200 mg/kg) may have resulted in inconsistent findings reported in previous studies using lower dose (Kim *et al*., 2013) or longer duration CP treatment protocols (Rezvanfar *et al*., 2008).

CP-induced gonadotoxicity may be attributed to its active metabolite acrolein. This metabolite can lead to apoptosis or necrosis due to impaired redox balance, incomplete antioxidant system and increased oxidative stress (Hamzeh *et al*., 2018). The increased level of free radicals in the cell can induce peroxidation of lipids in cell membranes, which can compromise cellular integrity. Although this study found an insignificant increase in the oxidative stress index (OSI), there are several studies showing increased oxidative stress and decreased antioxidant capacity in rats treated with different treatment regimens and various doses of CP (Selvakumar *et al*., 2004, Can *et al*., 2022).

The antioxidant defense system, consisting of enzymatic and non-enzymatic antioxidants, provides protection with their direct participation in the elimination of free radicals in biological tissues, including testes (Adedara and Farombi, 2010). Although TAU has been reported to have antioxidant activity in vitro and in vivo (Azab *et al*., 2020, Feng *et al.,* 2012), we found no significant decrease in OSI in the TAU treatment groups and it may be related to inadequate treatment of TAU.

In our study, testicular sections of the CP group showed intense hemorrhage and edema in the interstitial area, and seminiferous tubules with wavy, occasional vacuolization in the peritubular tissue, with germinal cells peeling into the lumen. In the testicular tissue of the animals in the TAU75 group, hemorrhage and edema in the interstitial region of the CP group improved with reduced vacuolization and peritubular fluctuation compared to the CP group. CP is known to cause many histological changes in the testis and damage is increased at higher CP doses (Yuan *et al*., 2014). The histological findings in this study are similar to those found in previous studies using CP and several antioxidant and/or anti-inflammatory agents (Elangovan *et al*., 2006, Tripathi and Jena, 2008).

Oxidative stress is associated with mitochondrial dysfunction and the most beneficial effects of TAU are related to its antioxidant capacity (Marcinkiewicz and Kontny, 2014). Healthy cells contain anti-apoptotic genes such as *B-cell lymphoma 2 (Bcl2)* in their mitochondria. *Bcl2-associated X protein (Bax),* a pro-apoptotic protein, inhibits the anti-apoptotic *Bcl-2* gene and causes testicular damage (Abd El Tawab *et al*., 2014). Accordingly, the apoptotic index was significantly increased in the testicular preparations of our study in CP group supporting the idea that CP induces apoptosis in spermatogonia (Gu *et al*., 2017). On the other hand, the apoptotic index was significantly decreased in TAU75 group compared to previous studies showing TAU inhibits apoptosis in a dose-dependent manner in various cells (Verzola *et al*., 2002, Takatani *et al*., 2004). Reactive oxygen species (ROS), are constantly produced in all cells, however excessive ROS can induce oxidative damage in tissues. On the other hand, antioxidant agents protect against the hazardous effects of ROS. However, as long as not being excessive, some amount of ROS are necessary in tissues for protection from many damages including apoptosis. Thus, higher doses of taurine could interfere the protective function of ROS, while lower doses can enhance the antiapoptotic effects (Salganik, 2001).

CONCLUSION

In conclusion, this study demonstrates that CP treatment induces significant oxidative stress, which is severely impaired function and structure of testis and TAU has protective effects on CP-induced toxicity. These results might help in recovery from reproductive dysfunction in cancer patients that have been received CP.

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