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Protective Effect of Taurine on Thiopurine-Induced Testicular Atrophy in Male Albino Rats

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Abstract

Background: Though the use of Azapress (Azathioprine) in cancers and autoimmune diseases has proved therapeutic effectiveness in numerous prospective clinical trials, some cases of testicular toxicity has been reported, that were referred to the evolved oxidative stress and inflammatory milieu. Taurine (TAU) is an amino acid found abundantly in brain, heart, and reproductive organ cells and with reported antioxidant and anti-inflammatory benefits.

Objective: The aim of the work was to investigate the protective effects of Taurine against Azapress-induced testicular dysfunction in male albino rats and unravel the contributing mechanisms.

Material and methods: Forty adult male albino rats were allocated into four equal groups; (i) normal control rats (Control group), (ii) Azapress group (AZP, 1mg/day for four weeks); (iii) Taurine group(Tau; 100 mg/kg bw/day for 6 weeks), (iv) Taurine and AZP group).

Results: AZP caused alterations in sperm parameters, increased DNA damage, and sex hormones disturbance. Moreover, significant decreased levels of superoxide dismutase (SOD) and catalase (CAT) activities, and upregulated levels of the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), as well as apoptotic markers; Bcl2 and caspase-9 expression it were evident in the testicular tissues. In contrast, taurine pretreatment significantly alleviated these toxic effects that were further evidenced histologically.

Conclusion: Our data suggest that oxidative stress and inflammation are involved in AZP-induced destruction in the male reproductive system and that co-administration of taurine exerts a protective effect against AZP-induced male reproductive testicular atrophy. This could open new horizon to its usage as an add-on complementary approach to chemotherapy supportive care.

Keywords: Taurine; Testicular atrophy; Azapress; Bcl2, Caspase-9; DNA fragmentation; IL-1β

Introduction

Thiopurines were first described in the 1950s by Gertrude Elion and George Hitchings and comprised three chemical structures: 6-thioguanine (6-TG), mercaptopurine (MP) and azathioprine [1]. The latter is an immunomodulatory drug, available as Azapress® and Imuran® in the market and often used to treat inflammatory bowel disease, autoimmune disorders, prevent rejection of transplanted organs and acts as an anticancer drug [2]. It acts via multifaceted pathways; it inhibits purine metabolism leading to DNA damage [3], its anti-inflammatory effect is mainly mediated via inhibition of the small GTPase Rac1, leading to apoptosis of activated T-lymphocytes, whereas high chemotherapeutic dosages in oncological treatment are associated with inhibition of DNA synthesis [4]. Upon its administration, it is rapidly converted into several toxic and non-toxic metabolic compounds, including the active 6-mercaptopurine (6-MP) which is formed through a conjugation reaction with glutathione (GSH) and leads to the depletion of GSH) [5]. This conversion can occur spontaneously or by enzymatic conversion through glutathione S-transferase, leading to surge of reactive oxygen species (ROS). 6-MP is metabolized by xanthine oxidase (XO) to thiouric acid, a reaction that is also known to create ROS [6]. The major reported adverse effect of thiopurines is immune suppression, with consequent lowering of infection-fighting white blood cells, hence increasing the vulnerability to infection [7].

The aforementioned antimetabolic drugs interfere with the availability of precursors of purine nucleotides by competing with them in the synthesis of DNA or RNA. Despite their effectiveness, these drugs may cause drug-induced toxicity with increased risk of death, even when used in standard doses [8]. One of these major drug–related disorders is developing testicular atrophy and infertility with a possible contributing mechanism of the genetic polymorphisms of thiopurine methyltransferase (TPMT) enzyme, which is responsible for thiopurine metabolism. Population studies have shown that patients with low enzymatic activity have a high risk for severe potentially fatal toxicities [9]. The common side effect of azathioprine treatment in both animal and human is bone marrow depression and lymphocyte depletion, which are anticipated findings for immunosuppressive drugs. However, its active metabolite, 6-mercaptopurine (6- MP) inflicts damage on rapidly dividing cells, such as bone marrow, intestinal epithelium and the reproductive organs in adults [10,11].

Amino acids have been recognized as important signaling mediators in different cellular functions. Taurine is a sulfur-containing

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amino acid, which does not contribute to protein synthesis, and is traditionally considered as an inert molecule without any reactive groups. Besides the well-known conjugation with bile acids, taurine has a number of other physiological functions such as intracellular osmolyte for volume regulation and some antioxidant properties [12,13]. It can be obtained either exogenously through dietary source as poultry, beef, pork, seafood, and processed meats or endogenously through biosynthesis from methionine and cysteine precursors. Both sources are important to maintain the physiologic levels of taurine, and either can help to compensate the other in cases of deficiency. Moreover, it has been found in variable amounts in the liver, muscle, kidney, pancreas, spleen, small intestine, lungs and in the male and female reproductive organs. Its supplementation has been proposed to have beneficial effects in the treatment of epilepsy, heart failure and cystic fibrosis [14]. It has also been reported that taurine can be biosynthesized by male reproductive organs [15]. It has been localized to Leydig cells of the testes, the cellular source of testosterone in males, as well as the cremaster muscle, efferent ducts, and peritubular myoid cells surrounding seminiferous tubules. Taurine has been detected in the testes of humans and has been identified as the major free amino acid of sperm cells and seminal fluid [16].

It has become increasingly apparent that oxidative stress plays a major role in a broad range of human diseases and in many diseases including the destruction of male rat reproductive system. Taurine, by virtue of its antioxidant activity, has been shown to play a crucial role as a cytoprotectant and in the attenuation of apoptosis [17]. There is a growing consensus that oxidative stress is linked to mitochondrial dysfunction and that the beneficial effects of taurine are due to its antioxidant properties [18], added to its ability to improve mitochondrial function by stabilizing the electron transport chain and inhibiting the generation of reactive oxygen species [19]. Levels of taurine in spermatozoa are correlated with sperm quality, presumably by protecting against lipid peroxidation through taurine's antioxidant effects, as well as through the spermatozoa maturation by facilitating the capacitation, motility, and the acrosomal reaction of the sperm [20,21].

To the best of our knowledge, there was no previous focus on the effect of taurine supplementation on AZP induced testicular atrophy in a rat model. Accordingly, this study aimed to evaluate the effect of AZP on the testicular functions and unravel the possible protective effects of TAU combination with AZP to alleviate such adverse effects.

Material and Methods

Material

- Commercial chow diet (balanced diet), containing 67% carbohydrates, 10% fat, and 23% protein as the energy sources (overall calories: 3.6 kcal/g), was purchased from El Gomhorya company (Cairo, Egypt).
- Azapress® (AZP): manufactured by EXCELLA Gmbh and Co. Feucht, Germany.
- Taurine (TAU): 2-amino ethane sulfonic acid was supplied by GALL Pharma, Austria- Pharmaceuticals.

Methods

Experimental design: Forty adult male albino rats, 7-8-weeks old, weighing 130-150 g were purchased from the Nile Pharmaceuticals Company, Cairo, Egypt. They were housed in laboratory standard cages (25 × 30 × 30 cm) 5 rats/cage, under specific pathogen-free conditions in facilities maintained at 21-24°C with a 40-60% relative humidity and 12 hr light/dark cycle. All animals have free access to chow diet and water *ad libitum*. They were acclimated for one week prior to initiation of the experiment in the laboratory of Physiology, Faculty of Medicine AI-Azhar University. All Animal Care Committee procedures were approved. The principles of laboratory animal care were followed, as well as specific national laws were applicable.

The rats were divided into 4 equal groups (10 rats each)

- **Group I (Control)**: Rats were served as control group. They received normal balanced chow and saline orally by gastric gavage tube for 10 weeks.
- **Group II (TAU)**: Rats received TAU daily at a dose of 100 mg/kg b/w, orally. It was freshly prepared by dissolving it in 0.9% saline for 6 weeks according to Abd El-Twab et al. [22].
- **Group III (AZP)**: Rats received normal diet for six weeks, and then they were treated with AZP dissolved in distilled water and given orally by gastric gavage in a dose of 1mg/day, for four weeks [11].
- **Group IV (TAU/AZP)**: Rats received TAU daily at a dose of 100 mg/kg b/w, orally and then were given AZP by the same dose and duration as AZP group.

Serum and tissue collection: At the end of the experiment, after overnight fasting, blood samples were collected from retro-orbital venous plexus by capillary tubes under light phenobarbitone anesthesia. The blood was then centrifuged at 3000 rpm for 15 minute for serum collection. Serum was separated in aliquots in Eppendorf tubes and stored frozen at -80°C until analysis for detection of sex hormone activities. Finally the animals were decapitated and both testes were removed, the right one was used for preparation of the homogenate to be used for determination of DNA fragmentation, oxidative stress marker activities, pro-inflammatory cytokines, and gene expression for caspase 9 and Bcl2 in the testis. The left was rapidly immersed in Bouin's fixative for 24 hr for histology and immunohistochemistry.

Sperm count: The two fresh cauda epididymis were used to study sperm abnormalities including, sperm motility, epididymal count and vitality. They were hold in 4 ml of saline solution (0.9% NaCl); by squeezing process, the sperms became free in the saline solution. A haemocytometer slide was used for sperm counting; the sperms were counted in four squares at 40 magnifications. The motility assessment was expressed as percentage motile forms. The epididymal filtrate was then mixed in equal volume with eosin-nigrosin stain and a smear made of it was used for epididymal sperm vitality [23]. The caudal epididymal sperm reserve was determined using standard hemocytome tric method [24].

Tissue preparation of the homogenate: The right testis was washed in ice-cold saline and kept in 1 ml physiological saline (0.9% NaCl). The testes were sliced into small pieces, and then homogenized in 1 ml physiological saline. The homogenates were centrifuged at 20,000 xg for 30 min at 4°C. The supernatants were collected and stored at −20°C until DNA, SOD, CAT, TNF-α and IL-1β assay were conducted.

Biochemical Analysis

Sex hormone assay

Serum levels of testosterone, Luteinizing hormone (LH) and Follicle stimulating hormone (FSH), were estimated using enzyme-linked immunosorbent assay (ELISA) kits (Diagnostic System Laboratories Inc., USA), according to the manufacturer's instruction.

Superoxide dismutase (SOD) activity

SOD activity in testicular cells was estimated following the method described by Kono (1978) [25]. SOD was generated by the oxidation of hydroxylamine hydrochloride. The reduction of nitroblue tetrazolium to blue formAZPn mediated by superoxide anions was measured at 560 nm under aerobic conditions. Addition of SOD inhibited the reduction of nitroblue tetrazolium and the extent of inhibition was taken as a measure of enzyme activity.

Testicular catalase (CAT) activity

CAT activity was estimated following the method described by Aebi [26]. Briefly, 0.5 mL of the post-mitochondrial supernatant was mixed with 50 mM phosphate buffer (pH 7.0) and 20 mM H2O2. The estimation was done using spectrophotometer following the decrease in absorbance at 240 nm.

Testicular tumor necrosis factor alpha (TNF-α) and interleukin-1B (IL-1B) level

Proinflammatory cytokines including TNF-α and IL-1β levels in testicular cells were assessed and quantified (pg/mg protein) by using ELISA technique (R and D systems, USA).The DNA fragmentation was determined according to the chemical method of Collins et al. [27]. After tissue homogenization, centrifugation and precipitation of DNA was done by addition of 1 ml TTE solution and vortexing vigorously to allow the release of fragmented DNA. DNA was hydrolyzed by adding 160µl of 5% TCA to each pellet and heating 15 minutes at 90˚C in a heating block. Colorimetrical quantitation on staining with diphenylamine (DPA) was assessed at a wave length 600 nm against blank reagent and the values are given as % fragmented DNA.

Protein extraction and Western Blotting for Bcl2

Testis was homogenized in RIPA buffer supplemented with protease inhibitors using the liquid nitrogen grinding, followed by incubation on ice for 10 min. The samples were centrifuged thoroughly to obtain protein supernatants. The protein concentrations were determined using a BCA Protein Assay Kit (Pierce). Twenty mg protein for each sample was resolved on 12% Bis-Tris or 4-12% gradient Bis/Tris gels (Life Technologies) and then transferred to PVDF membranes (Millipore). After blocking in 10% skim milk, the immunoblotting membrane was probed with indicated antibodies and visualized by ECL kit (Pierce). Lastly, images of indicated protein bands were recorded on the BioMax film (Kodak), and quantification was conducted by using Image J software (Bio-Rad). Antibodies used in this study were diluted as anti-Bcl2 (1:500, Santa Cruz Biotechnology).

Determination of caspase-9 enzymatic activity

The activity of caspase-9 was measured by Caspase-Glo-9 assay kit according to the manufacturer's instructions (Promega).

Histopathological Analysis

Light microscopic examination

After removal of the left testis, it was weighed and rapidly immersed in Bouin's fixative for 24 hr. Then, washed in several changes of 70% ethanol, dehydrated, cleared and embedded in paraffin [28]. The tissue was sectioned at 5 µm thick, mounted and stained with Hematoxylin and Eosin (H and E) for studying the general structure, Masson's Trichrome stain for staining the collagen fibers [29], and Periodic Acid-Schiff reaction (PAS) for demonstration of mucopolysaccharides [30].

Immunohistochemical study

BCL2 the immunohistochemical technique was used to evaluate the protein expression of BCL2 in the testes of the different experimental groups. BCL2 is an oncoprotein that inhibits the programmed cell death (apoptosis) [31]. Positively charged paraffin sections were deparaffinized in xylene and rehydrated using ascending grades of alcohol. The process of antigen retrieval was performed in 10 mM sodium citrate buffer. Endogenous peroxidase activity was blocked using 0.03% hydrogen peroxide for 5 min. at room temperature. Tissue sections were washed gently with phosphate buffered saline (PBS) and then incubated with anti-apoptotic protein BCL2 (1:50) biotinylated primary antibodies for 15 min. Sections were gently washed with the buffer and kept in a buffer bath in a humid chamber. A sufficient amount of streptavidin biotin peroxidase was then added and incubated for 15 min. Diaminobenzidine-substrate chromagen (DAB) was added to the sections and incubated for 7 min., followed by washing and counterstaining with hematoxylin for 5 sec [32,33].

Histomorphometrical analysis

For the estimation of spermatogenesis in testicular tissue, different indices were used. In 20 randomly selected round and nearly round seminiferous tubules, the seminiferous tubule diameter (STD), the epithelial height (EH) the tubular differentiation index (TDI), the repopulation index (RI) and the spermiogenesis index (SPI) were measured for each testis in H and E stained sections. In addition, optical density of PAS and Masson's trichome stained sections respectively, were measured too. To determine TDI, the number of seminiferous tubules that have more than three layers of germinal cells derived from type A of spermatogonia was calculated. To find out RI, the ratio of active spermatogonia (having lightly stained nuclei) to inactive spermatogonia (with darkly stained nuclei) was calculated and to determine the SPI, the ratio of the number of seminiferous tubules with spermatozoids versus empty tubules was calculated according to Movahed et al. [34]. A lica Qwin 500 LTD image was used to count the apoptotic cells in 10 high power fields (HPF) and optical density of BCL2 in all groups.

Statistical analysis

All the data were expressed as mean ± standard error (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc multiple comparison test using the program Statistical Package for the Social Sciences (SPSS). The values of $P<0.05$ were considered significant.

Results

Effect of AZP and TAU on rat's body and testis weights, levels of serum testosterone, LH and FSH (Table 1)

Since there was no difference between control group receiving TAU and control group, only those for control are utilized in the current study for statistical comparison. As illustrated in Table 1, there was a significant decrease in the body and testis weights in AZP treated group (8.7%, 22.4%, respectively), when compared to control group. TAU treatment successfully normalized the body and testis weights when compared to AZP treated group, as illustrated in Table 1. Moreover, AZP had a negative effect on the steroidogenic hormones, reflected as significant decrease in the serum levels of testosterone (41.74%), LH (71.6%) and FSH (52.4%), when compared to control group. Upon TAU treatment, the aforementioned altered levels were mitigated to reach nearly the normal levels.

Groups	Group I	Group II	Group III	Group IV
Parameters	**(Control)**	**(TAU)**	**(AZP)**	**(TAU/AZP)**
body weight (g)	285 ± 2.5	289 ± 2.9	260^{a} ± 1.59	$275^{a,b}$ ± 4.3
Testicular weight (g)	1.25 ± 0.5	1.85 ± 0.4	0.97^{a} ± 0.3	1.19^{b} ± 0.7
Testosterone (ng/ml)	5.15 ± 0.35	4.8 ± 0.5	3.02^{a} ± 0.54	4.97^{b} ± 0.15
Luteinizing hormone (LH) (mIU/ml)	3.01 ± 0.33	3.2 ± 0.5	0.85^{a} ± 0.45	2.95^{b} ± 0.45
Follicle stimulating hormone (FSH) (mIU/ml)	4.1 ± 0.25	4.3 ± 0.4	1.95^{a} ± 0.51	3.98^{b} ± 0.33
Data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc multiple comparison test (n=10). Difference between groups was considered statistically significant when P ≤ 0.05. (a) Significant values versus group control, (b) Significant values versus AZP group.				

Table 1: Effects of TUA, AZP, and their combination on the body and testicular weights, serum testosterone (ng/ml), Luteinizing hormone (LH) (mIU/ml) and Follicle stimulating hormone (FSH) (mIU/ml).

Group	Group I	Group II	Group III	Group IV
Parameters	**(Control)**	**(TAU)**	**(AZP)**	**(TAU/AZP)**
TNF-α (pg/mg protein)	31.95 ± 2.2	33.8 ± 2.5	70.65^{a} ± 4.6	$40.05^{a,b}$ ± 2.1
IL-1β (pg/mg protein)	34.05 ± 1.5	35.2 ± 1.7	61.15^{a} ± 3.1	$39.52^{a,b}$ ± 1.3
SOD (U/mg protein)	1.8 ± 0.1	1.92 ± 0.2	0.9^{a} ± 1.35	1.5^{b} ± 2.95
CAT (U/mg protein)	6.1 ± 1.2	6.7 ± 1	3.5^{a} ± 2.55	5.9^{b} ± 4.45
Data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc multiple comparison test (n=10). Difference between groups was considered statistically significant when P ≤ 0.05. (a) Significant values versus group control, (b) Significant values versus AZP group.				

Table 2: Effects of TUA, AZP, and their combination on testicular levels of TNF-α (pg/mg protein), IL-1β (pg/mg protein), Superoxide dismutase (SOD) (U/mg protein), and Catalase (CAT) (U/mg protein) in experimental groups.

Groups	Group I	Group II	Group III	Group IV
Parameters	**(Control)**	**(TAU)**	**(AZP)**	**(TAU/AZP)**
Sperm Motility (%)	90.45 ± 5.01	88.5 ± 4.5	51.59^{a} ± 1.59	86.13^{b} ± 4.32
Caudal epididymal sperm count (10^{6}/ml)	95.01 ± 2.15	92.3 ± 2.2	30.75^{a} ± 2.66	89.99^{b} ± 3.65
Sperm vitality (Live: Death ratio)	14.15 ± 1.02	13.8 ± 1.3	5.95^{a} ± 0.65	13.01^{b} ± 1.25
Data were expressed as Mean ± SEM. and analyzed using one-way ANOVA followed by Bonferroni post hoc multiple comparison test (n=10). Difference between groups was considered statistically significant when P ≤ 0.05. (a) Significant values versus group control, (b) Significant values versus AZP group.				

Table 3: Effects of TUA, AZP, and their combination on percentage of sperm motility, epididymal count (10^{6}/ml) and sperm vitality (Live: Death ratio).

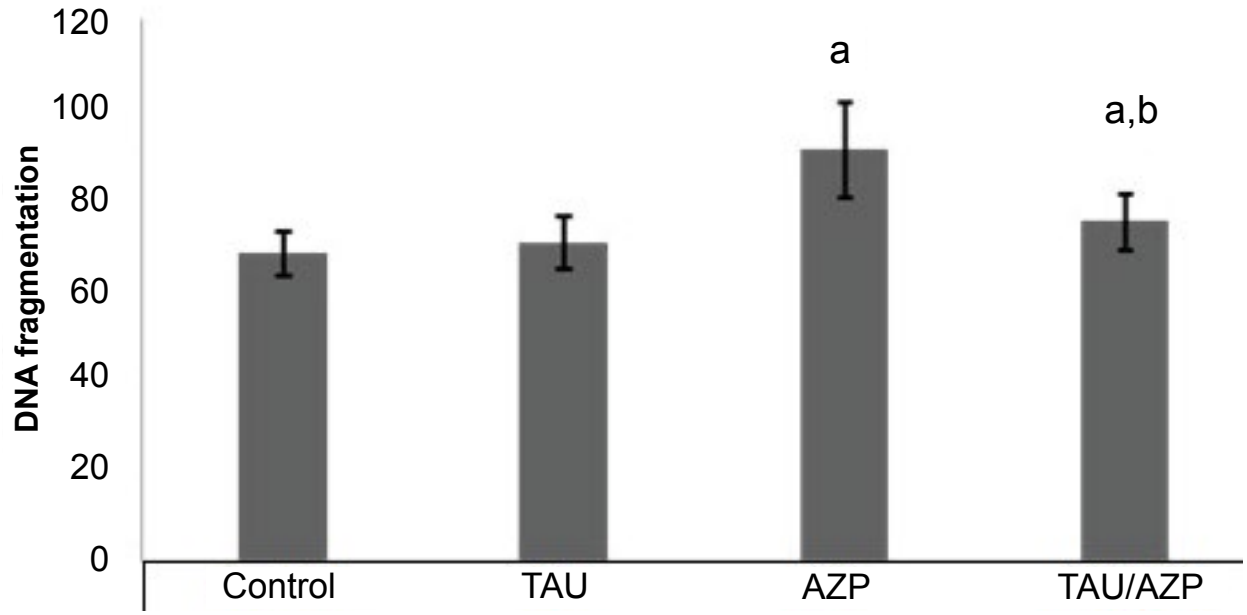

Figure 1: The percentage of DNA fragmentation in different groups. Data were presented as means ± SEM (n=10), (a) Significant difference from control; (b) Significant difference from AZA group; all at P<0.05.

Effect of AZP and TAU on rats' testicular pro-inflammatory cytokines TNF-α and IL-1β and antioxidant enzymes, SOD and CAT (Table 2)

The testicular levels of pro-inflammatory cytokines including TNF-α and IL-1β were significantly increased in AZP supplemented rats (2.2,1.8 folds, respectively), compared to the control animals. TAU supplementation for six weeks ameliorated successfully the elevated cytokine levels, when compared to AZP treated rats, data presented in (Table 2). AZP supplementation for four weeks induced a robust oxidative stress, reflected by significant reduction of testicular activities of CAT and SOD (42.6, 50%, respectively), when compared to normal rats. TAU supplementation for six weeks significantly increased their level when compared to AZP supplemented group.

Effect of AZP and TAU on rat's epididymal sperm count, motility and viability (Live:Death ratio) on AZP treated rats (Table 3)

Significant reduction of sperm motility (42.9%), caudal epididymal sperm count (67.6%) and vitality (57.8%) were observed in AZP treated

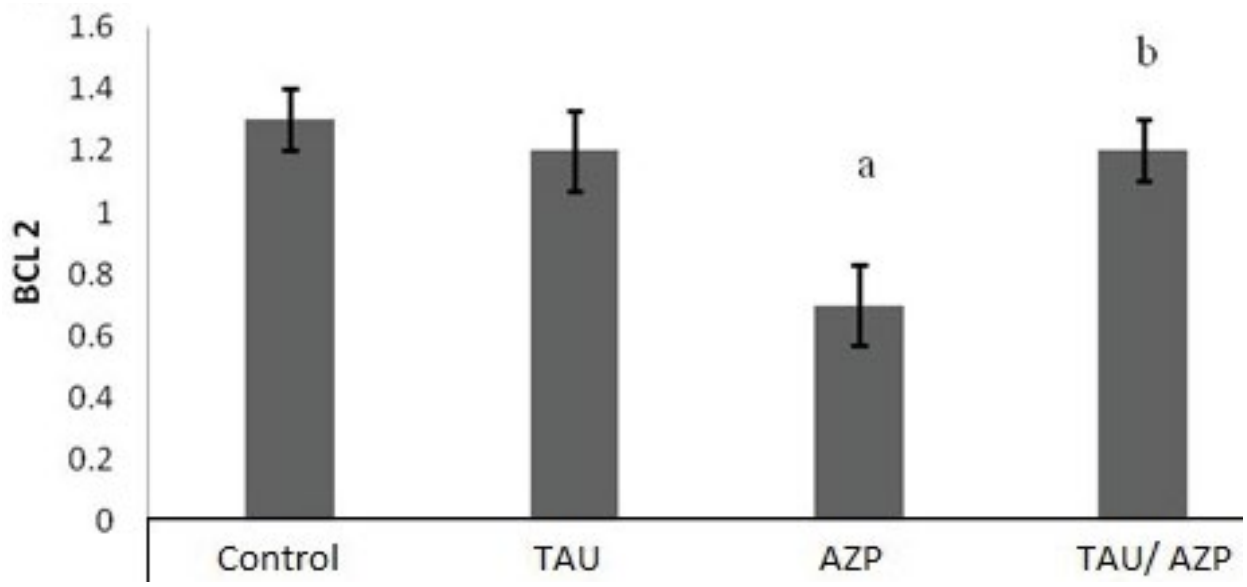

Figure 2: Transcriptional expression of BCL2 measured by real-time qPCR. Data were presented as means ± SEM (n=10), (a) Significant difference from control; (b) Significant difference from AZA group; all at P<0.05.

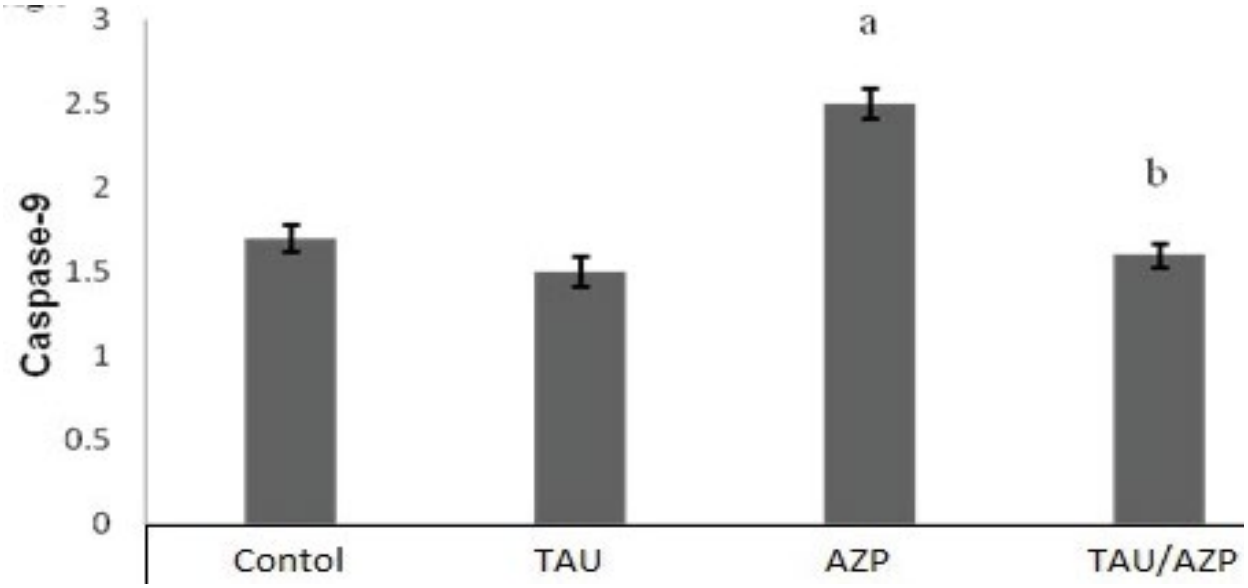

Figure 3: Transcriptional expression of Caspase 9 measured by real-time qPCR. Data were expressed as means ± SEM (n=10), (a) Significant difference from control; (b) Significant difference from AZA group; all at P<0.05.

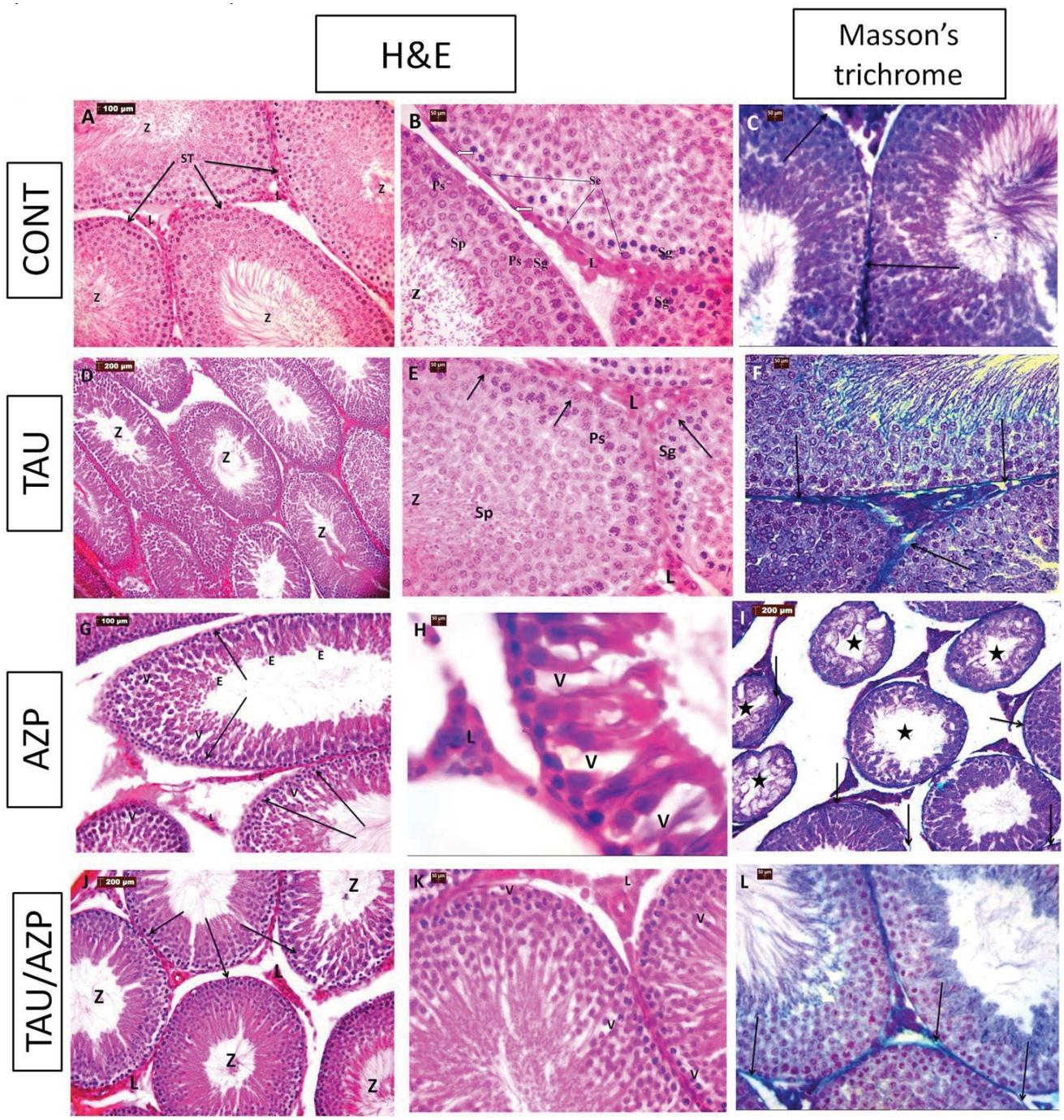

Figure 4: Photomicrographs of testis sections from all experimental groups. Control group (A-C): (A) the normal pattern of seminiferous tubules (STs) full of spermatozoa (Z) with clusters of Leydig cells (L) between the tubules. **(B):** STs lined by spermatogenic cells, including spermatogonia (Sg), primary spermatocytes (Ps), spermatids (Sp), spermatozoa (Z), and Sertoli cells (Se) (black arrows). Surrounded by myoid cells (white arrows) with clusters of Leydig cells in-between (L). **(C):** A thin layer of collagen surrounds the STs (arrows). **TAU group (D-F):** the normal pattern of STs appears as the control. **AZP group (G-I): (G)** widely separated STs, some showing loss of normal architecture, with the presence of many vacuolated cells (v) and disorganized spermatogenic cells. (H): the lumen contains some exfoliated, degenerate spermatogenic cells (E). The pyknotic nuclei in the basal part of the tubules and in the Leydig cells are noticeable. (I): Normal amount of collagen fibers (arrows) around the empty (*) STs. **TAU & AZP group (J-L): (J)**.

rats when compared with control rats. These reductions were improved by TAU treatment to reach nearly the normal levels on comparison to AZP treated rats, data highlighted in Table 3.

Effect of AZP and TAU on percentage of DNA fragmentation, caspase-9 and Bcl2 expression

A significant increase in the DNA fragmentation was noticed after

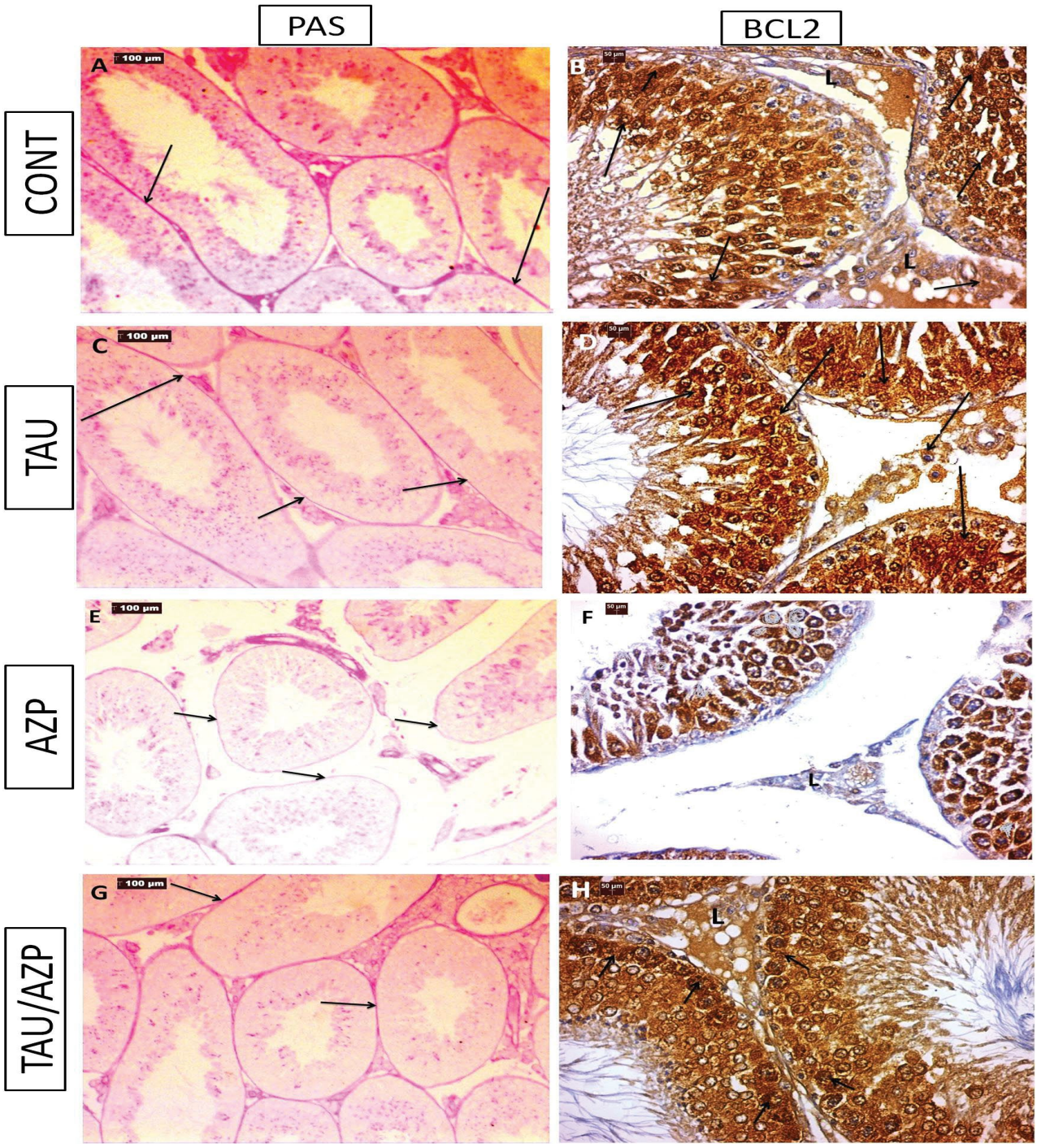

Figure 5: PAS reaction of the basement membrane of seminiferous tubules and Bcl2 immunohistochemistry in the different groups. Control group (A&B): (A) the normal pattern of PAS-positive reaction of the basement membrane of STs (arrows). **(B):** Most of the spermatogenic and Leydig cells show a strong Bcl2-positive reaction (arrows). **TAU group (C&D):** similar results as the control are noticed. **AZP group (E&F): (E)** a weak PAS reaction in the basement membrane of STs (arrows). **(F):** Only some spermatogenic cells have a weak Bcl2-positive reaction (arrows), and Bcl2 was negative in Leydig cells (L). **TAU & AZP group (G&H): (G)** nearly normal pattern of PAS-positive reaction of the basement membrane of STs (arrows). **(H):** Most of the basal spermatogenic cells have equivocally positive BCL2 staining (arrows), with weakly positive staining of Leydig cells (L).

AZP treatment from 70.55 ± 6 to 91.27 ± 10.5. Administration of TAU prior to AZP administration decreased the latter level significantly to 68.19 ± 4.8, when compared to AZP treated group (Figure 1).The overall activity of Bcl2 (Figure 2) and caspase-9 (Figure 3) in the homogenates

of AZP treated rats' testes were significantly reduced from 1.7 ± 0.07 and 1.3 ± 0.08 to 1 ± 0.13 and 0.7 ± 0.09, respectively when compared to the control rats. While TAU supplementation for six weeks revealed a significant increase from 1 ± 0.13 and 0.7 ± 0.09 to 1.6 ± 0.1 and 1.2 ± 0.07, respectively when compared to AZP treated rats. Collectively, these results showed that the Bcl2-capase-9 pathway was activated in the testis of AZP treated rats, which is corrected by TAU treatment for six weeks.

Histological Results

Light microscopic examination

Light microscopic examination of sections of adult rat testis from the control group (Figure 4A-C) showed that testis is formed by seminiferous tubules (STs) which are lined by spermatogenic cells and Sertoli cells. Leydig (interstitial) cells were present in the interstitial space between STs and surrounded by blood vessels as seen by H and E stain. In Masson's trichrome stained sections the STs were surrounded with, thin collagenous fibers. Testicular sections in the TAU group revealed a high similarity to the normal ST pattern (Figure 4D-4F).

Histopathological changes were observed in the testis of rats in the AZP group (Figure 4G-I), including widening of interstitial spaces, disruption and atrophy of the STs, disorganization of the spermatogonia, few spermatogenic cells and spermatozoa, numerous cells with pyknotic nuclei and eosinophilic or vacuolated cytoplasm. The majority of the affected germ cells were spermatogonia and spermatocytes. Some Leydig cells appeared atrophied with pyknotic nuclei. No obvious changes in collagen fibers in-between STs could be detected with Masson's trichrome stain.

In the TAU/AZP-treated group (Figure 4J-4L) the histology appeared more or less similar to that of the control, with ameliorated changes, in spite of presence of some vacuolated cells. Normal condensation of collagen in-between STs was seen with Masson's trichrome stain.

Histochemical and Immunohistochemical Results

In Figure 5, PAS reaction for detection of mucopolysaccharides in the basement membrane of the STs and Bcl2 immunohistochemistry, which is an oncoprotein that inhibits apoptosis, was used to assess the different groups. In the control group, the basement membrane of the STs gave a strong positive PAS reaction (Figure 5A). Bcl2 immunohistochemistry revealed that most of spermatogonia were strongly positive, with the appearance of dark brown granules in their cytoplasm (Figure 5B) Examination of testis from the TAU-treated groups gave similar results too (Figures 5C and 5D).

Conversely, in the AZP group (Figure 5E) a weak PAS reaction was observed in the basement membranes of STs. There were few Bcl2-positive cells; these positive cells were situated on the basal side, while the remaining cells had many vacuoles and were negative for Bcl2 (Figure 5F). A strong positive PAS reaction was detected in the TAU / AZP group, similar to that in the control group (Figure 5G) and most of the basal spermatogenic cells have equivalent positive BCL2 staining with weakly positive staining of Leydig cells (Figure 5H).

Histomorphometrical results

Significant decreases in the seminiferous tubule diameter (STD), epithelial height (EH), tubular differentiation index (TDI), repopulation index (RI), spermiogenesis index (SPI) and PAS density in the AZP group were noted. While a non-significant increase in the collagen fiber area percentage was observed in the AZP group compared to the control group in Masson's trichrome staining. These changes were significantly improved in the TAU/AZP-treated group compared to the group treated with AZP alone (Table 4).

Quantitative image analysis-based evaluation of BCL2

Immunohistochemical stain revealed significant decreases in the expression of Bcl2 in the cytoplasm of spermatogenic cells and Sertoli cells in the AZP group than in the other groups. The decrease in the expression of Bcl2 includes both number of stained cells and its optical density. These changes were significantly improved in the TAU/AZP-treated group compared to the AZP group (Table 5).

Discussion

Oral administration of AZP caused a significant decrease in body and testicular weights in addition to reduction in serum testosterone, LH and FSH levels; events that may be attributed to the oxidative stress induced by this drug testosterone. This agrees with the results of Sachin et al. [35] and Onanuga et al. [36] who noticed a decrease in body and testicular weight of mice treated with AZP and was rationalized by the decrease of the anabolic effect of testosterone. The study of Bairyk et al. [37], reported that oxidative stress reduced enzymatic and non-enzymatic level in Leydig cells and caused reduction of testosterone. Therefore, the AZP-induced oxidative stress in testicular tissue may rationalize the inhibition androgenesis by Leydig cells at the level of the anterior pituitary [38].

This postulation is consistent with that revealed by Duan et al. [39], who found marked decline in serum FSH and LH with oxidative stress induced by 4-Nonylphenol on spermatogenesis. Taurine supplementation, which is a free β-amino acid with remarkable antioxidant activity, for 6 weeks improved these changes significantly by improving the body and testicular weights [40] and increasing the levels of gonadotrophic hormones; testosterone, LH and FSH levels [21,22]. They stated that TAU increased the testosterone hormone release both in vivo and in vitro. On the other hand, Yang et al. [41], explained this increase in testosterone levels to the anti-oxidative stress and anti-apoptotic effects of TAU.

Inflammation is the process of responding to injury and tissue damage that is characterized by recruitment and activation of macrophages, lymphocytes and other cells, which trigger a coordinated action of proinflammatory cytokines. This stimulates an increased blood supply to the affected area, an increase in capillary permeability allowing larger serum molecules to enter the tissues and an increase in leukocyte migration into the tissue [42]. The most susceptible response to testicular inflammation is the inhibition of spermatogenesis, by damaging the seminiferous epithelium and promoting the apoptosis of spermatogenic cells in the reproductive system. Moreover, inflammation is also associated with oxidative stress which impairs sperm function [43]. This crosstalk between inflammation and spermatogenesis is evidenced by the current histological results. Thereby, we suggest a direct association between AZP supplementation and testicular inflammation evidenced by elevated testicular IL1-ß and TNF-α. This agrees with the study of Ramonda et al. [44], who reported an increase of TNF–α level in semen associated with reduced sperm count, motility and morphology. These disturbances were successfully ameliorated by Taurine, as evidenced by the study of Ahmed [45], who attributed these corrective effects to anti-inflammatory, antiapoptotic (intrinsic apoptotic pathway) and steroidegenic effects of TAU.

Parameters	Group I (Control)	Group II (TAU)	Group III (AZP)	Group IV (TAU & AZP)
STD(μm)	230.35 ± 10.1	232.14 ± 15.01	210.88^{a} ± 8.09	224.45 ± 9.43^{b}
EH(μm)	41.645 ± 2.12	43.132 ± 3.12	29.865^{a} ± 1.82	$36.665^{a,b}$ ± 2.76
TDI %	91 ± 6.87	94 ± 5.57	47^{a} ± 2.34	$60^{a,b}$ ± 5.54
RI %	82.74 ± 4.421	83.29 ± 6.457	75.34^{a} ± 5.81	79.45^{b} ± 5.12
SPI %	90.89 ± 4.12	91.37 ± 4.49	53.5^{a} ± 4.43	65.24^{b} ± 4.31
Collagen fiber area %	5.262 ± 0.35	5.345 ± 0.53	5.96^{a} ± 1.33	6.13^{b} ± 2.21
PAS density	0.354 ± 0.01	0.432 ± 0.05	0.23^{a} ± 0.11	0.32^{b} ± 0.012
Data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc multiple comparison test (n=10). Difference between groups was considered statistically significant when $P \leq 0.05$.				
(a) Significant values versus group control,				
(b) Significant values versus AZP group.				
STD: Diameters of seminiferous tubules ; EH: Epithelial height , TDI: Tubular differentiation index ; RI: Repopulation index; SPI: Spermiogenesis index; PAS: Periodic acid Schiff reaction				

Table 4: Effects of TUA, AZP, and their combination on spermatogenesis indices in the seminiferous tubules.

Groups	Group I	Group II	Group III	Group IV
Parameters	**(Control)**	**(TAU)**	**(AZP)**	**(TAU & AZP)**
Apoptotic index (ratio of apoptotic cells to normal cells)	0.078 ± 0.015	0.075 ± 0.013	1.1^{a} ± 0.12	0.6^{b} ± 0.05
BCL2 optical density	137 ± 16	140 ± 8	48^{a} ± 6.5	110^{b} ± 9
Data were expressed as Mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni post hoc multiple comparison test (n=10). Difference between groups was considered statistically significant when $P \leq 0.05$. (a) Significant values versus group control, (b) Significant values versus AZP group				

Table 5: Effects of TAU, AZP, and their combination on the Bcl2 expression in the cytoplasm of spermatogenic and sertoli cells.

Any inflammatory damage on the male genital tract leads to the increased generation of reactive oxygen species (ROS). Oxidative stress arises when excess free radicals exceed the antioxidant defence of the male reproductive tract, thereby damaging male reproductive tract. Superoxide, hydroxyl and hydrogen hydroxide radicals are the major ROS present in seminal plasma [46]. Various oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins [47], mutations that are major causes of cancer, and can be reversed by DNA repair mechanism [48]. AZP is one of these oxidants that caused a marked DNA fragmentation of the testicular tissue in rats of the present study.

AZP treatment for four weeks in the present study induced testicular inflammation and hence significant reduction of SOD and CAT levels; this decrease was corrected by pre-treatment of TAU for 6 weeks. Sperm quality and quantity are critical factors to the male fertility [49]. There is a positive correlation between abnormal and immature sperms with oxidative stress [50]. Consistent with these findings [51], reported that ROS promoted apoptosis which may lead to a decrease in sperm viability and density. In the present study, we demonstrated that the percentage of abnormal sperms was significantly increased in the AZP group as well as an increase in the percentage of immature, immotile and dead sperms associated with decreased in the levels of SOD and CAT in testicular tissues [52].

TAU was also reported to have the ability to scavenge reactive oxygen species (ROS) and attenuate lipid peroxidation and, consequently, stabilizes biological membranes [53]. The anti-inflammatory action of TAU, evidenced in the current study, confirms previous findings of [54], who demonstrated the effective role of TAU as an anti-inflammatory supplement when given to alleviate the inflammation induced in the kidney by chronic ethanol ingestion.

The potent antioxidant properties of TAU are additionally associated with increased antioxidant enzyme activity, SOD, catalase and GSH which are the key cellular antioxidant enzymes. The antioxidant effects attributed to TAU may be associated with its sulfur moiety, and the modulation of GSH and GSH levels by TAU is critical in the cellular defence against oxidative stress. Consistent with the antioxidant properties of taurine observed in the present study [55], also found protective role of TAU on hepatocytes subjected to iron-overload as a way of oxidative stress.

In the present study, treatment by AZP for four weeks induced activation of caspase-9 (an upstream protease of caspase-3 and downstream effectors of the Bcl2 mitochondrial apoptosis pathway) which indicates an increase in the apoptotic activity in the testicular tissue. Bcl2 is a prosurvival multidomain protein that regulates apoptosis by preventing the release of proapoptogenic factors from the mitochondria (e.g., cytochrome c) and subsequent caspase activation. In our study the anti-apoptotic protein BCL2 level was significantly reduced with AZP treatment. This dysregulation of the fine-tuned apoptotic pathway is considered one of the mechanisms of AZP-induced injury of the testicular function which might be the reason for decreased sperm viability and mobility. In alignment with our findings [56], documented an increase in the activity of Bcl2/Caspase-9 apoptosis pathway in the testis of mice subjected to apoptotic inducers.

The protective role of TAU is highlightened in the adjustment of caspase-9 activity and Bcl2 level towards the control values in order to properly control the proliferation and differentiation of germ cells during spermatogenesis. This anti-apoptotic effect of TAU was also demonstrated by Takatani et al. [57], who suggested that Taurine inhibits apoptosis by preventing formation of the Apaf-1/caspase-9 apoptosome. In addition, Zulli et al. [58] reported an anti-apoptotic effect of taurine, results that encourage the usage of Tau as a dietary supplement.

A sensitive and indispensable method for revealing disturbances in spermatogenesis is histopathological examination [59]. Our histological results revealed widening of the interstitial spaces, disruption and atrophy of many seminiferous tubules with scanty spermatogenic cells and spermatozoa in AZP group. These results agree with those of Akinlolu [60] and Padmanabhan, et al. [61], while

cytoplasmic vacuolations was further reported by Karawya and El-Nahas [62]. The current study noticed a decrease in the diameters of the seminiferous tubules which was proved by the morphometrical studies and agrees with the results of Shrestha et al. and Khayatnouri et al. [63,64]. Many cells appeared shrieked with pyknotic nuclei and deeply acidophilic cytoplasm, which may indicate apoptosis. Sun et al. [65], reported that early injury of the cells occurred mainly in the form of apoptotic cells and cellular apoptosis are associated with the release of cytochrome c and others apoptosis-promoting substances. Kumar et al. [66], described pyknosis as irreversible condensation of chromatin in the nucleus of a cell necrosis, while Kroemer et al. [67], referred pyknosis to apoptosis.

AZP induced reproductive disorders was confirmed by histomorphometrical, as it significantly decreased in the diameter and epithelial height of the seminiferous tubules due to cell loss from the epithelium and epithelial sloughing in some tubules in addition to Leydig cells atrophy. Moreover, the results of this study demonstrated a decrease in RI, TDI and SPI. As testosterone supports spermatogenesis, sperm maturation and sexual function, thus any disruption in testosterone biosynthesis can adversely affects male fertility [68]. In another study of El-Sharaky et al. [69], the use of a Leydig cell toxicant resulted in decrease of testosterone level in rats, resulting in increased germ cell apoptosis. In addition, testosterone can affect Sertoli cells function and germinal cell degeneration; thus, dislocation could take place due Sertoli cells dysfunction and decreased testosterone level, the latter enhance premature detachment of epithelial cells [70,71]. Atrophy of Leydig cells can be responsible for the reduction in serum testosterone level. Therefore, the changes in the seminiferous tubules, observed in the current histopathological findings, may be a result of hormonal effect and not a consequence of a direct effect.

Considering the fact that the normal spermatogenesis is directly associated with reduction of oxidative stress and increasing endocrine activity by Leydig and Sertoli cells, TAU proved to protect spermatogenesis and decrease tubular atrophy. This may be partly by down-regulating oxidative stress enzymes and as well by improving testosterone biosynthesis. These results were confirmed by the histopathological finding that showed a remarkably higher seminiferous tubules diameter as well as germinal epithelium height in the testes in the TAU and AZP group, compared to AZP group. Taurine not only improved the morphological and histomorphometrical damage, but also the apoptotic cells number and morphology, which confirms Taurine's ability to decrease the toxic effects of AZP. Previous studies reported that Tau treatment prevented significantly the morphological damage, and the amount of apoptotic cells through suppressing the increased oxidative stress in diabetes-induced testicular dysfunction in the rat [72], through its antioxidant effect [73,74].

Conclusion

Conclusively, the present study suggests that AZP plays a destructive impact on reproductive system functions in male rats, while TAU supplementation has beneficial effects on the induced inflammation, testicular function and apoptosis. Taurine's benefit in reducing the oxidative stress and inflammatory response, hence testicular hypofunction, induced by AZP treatment, would encourage its supplementation as an add-on therapy when the use of AZP is mandatory. This could open new horizon to its usage as an adjunctive, complementary approach to chemotherapy supportive care.

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