Physiological and Histological Study of the Therapeutic Effect of Silibinin on Testicular Alteration in Adult Male Albino Rats

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Melebary *et al.*: Physiological and Histological effect of Silibinin on Testicular Alteration

The male reproductive system is vulnerable to oxidative stress, which is linked to several disorders. Silibinin, a natural polyphenolic flavonoid, possesses antioxidant, anti-inflammation and anticancer properties. Considering silibinin's wide range of biological and metabolic roles (silibinin) plays, the current study was conducted to evaluate the biochemical, histological, immunohistochemical and morphometric changes that may develop in the rat testis exposed to oxidative stress and the possible protective role of silibinin. Thirty adult rats were used in the current study. Rats were equally divided into three groups. G1 negative control group, G2 was given thioacetamide (200 mg/kg body weight, G3 received silibinin at (100 mg/kg body weight) for 4 w concurrently with thioacetamide. Serum levels of sexual hormones and testicular oxidative stress markers were measured. Testicular sections were stained with haematoxylin and eosin, and immunohistochemical stains for Vimentin and p53 subjected to morphometric and statistical analysis. Testicular histological architecture and biochemical and morphometric markers all declined in the thioacetamide group. When rats were given silibinin, however, the normal biochemical and morphometric parameters were restored and the histological structure of their testicles was preserved. This investigation showed that silibinin might increase testosterone and gonadotropin levels and enhance several testicular indices (luteinizing hormone and follicle-stimulating hormone). The protective effects of silibinin on reproductive toxicity and oxidative stress were evidenced by the antioxidant activities and improvement of the histological structures of both Leydig and Sertoli cells. As a result, it can be used as a supplement to improve male reproductive function, particularly spermatogenesis.

Key words: Silibinin, antioxidant, oxidative stress, Sertoli and Leydig cells, histological, testes, rats

Free radical-induced oxidative stress has been linked to several reproductive health issues, notably those affecting testicular function. 30 % to 80 % of infertile patients have significant levels of oxidative stress due to Reactive Oxygen Species (ROS)[1]. Environmental contaminants, chemotherapeutic agents and other medications, tobacco, poisons, radiation and diseases, including many types of cancer, all share a common trait called oxidative stress, which can have a devastating effect on fertility[2,3]. Potentially harming free radical attacks can impede spermatogenesis and lead to vascular blockage and severe damage to reproductive system cells[4]. Suppose the testicular biological system cannot detoxify or repair the detrimental effects of free radicals. In that case, cells and tissue are severely harmed, making it imperative to strike a balance between the production and metabolism of free radicals for proper testicular cell function[5,6]. Because they neutralize free radicals and inhibit their production in testicular cells, antioxidants help protect against this type of injury.

Since a natural antioxidant can inactivate damaging ROS, much interest has been in measuring their antioxidant activity in recent years[7-9]. Furthermore, natural antioxidants are considered healthy, helpful and safe[10-12].

One of the structural isomers of the flavonoid silymarin, Silibinin is isolated from milk thistle and is the most physiologically active form (*Silybum marianum* L. Gaert., Asteraceae)[13]. It has been traditionally used as a folk medicine for hepatic ailments[14]. Silibinin (SBN) has metal-chelating and antioxidant properties[15]. Silibinin has gained popularity in the last decade as a potential herbal treatment for many illnesses. It has therapeutic advantages against liver problems, different malignancies and gynecological ailments due to its anti-inflammatory, antioxidant and anticancer qualities[16-18]. Silibinin's antioxidant and anti-inflammatory capabilities change cell cycle

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regulators and trigger apoptosis in cancer cells[19]. It has been shown that Silibinin has a hypoglycemic effect and increases insulin sensitivity[20]. Silibinin strongly protects the testes because of the sequelae of antioxidative processes and modulation of the expression of effector proapoptotic caspase 3 activity[21,22]. Silibinin is also effective against arsenic[23] and lead[24] toxicity. Considering Silibinin's wide range of biological and metabolic roles plays, the current study was conducted to evaluate the biochemical, histological, immunohistochemical and morphometric changes that may develop in the rat testis exposed to oxidative stress and the possible protective role of SBN.

MATERIALS AND METHODS

Drugs and chemicals:

Thioacetamide (TAA) and SBN were obtained from Sigma Chemical Co., Missouri, United States of America (USA). Enzyme-Linked Immunosorbent Assay (ELISA) kits (Cat.: MBS738685, MBS724319, MBS036924, MBS701908, MBS702057, MBS2018978 and MBS2021901; respectively) for the determination of oxidative stress biomarkers (Malondialdehyde (MDA), Reduced Glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT)) and sexual hormones (Testosterone Hormone (TH), Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH)) levels were obtained from MyBio Source, San Diego, California, US. A polyclonal rabbit anti-goat antibody to Vimentin, a marker for Sertoli cells, from Santa Cruz Biotechnology in Dallas, Texas, USA. P53, a marker for apoptosis Leydig cells, is a rabbit polyclonal antibody (Cat: ab131442, Abcam, Cambridge, UK).

Experimental animals:

A total of 30 adult male Wistar albino rats weighing between 180 and 250 g were purchased from the King Fahd animal house in Jeddah, Saudi Arabia. The animals were housed in plastic cages (10 animals for each) at a temperature of 25° and 50 %-70 % humidity, with 12 h light/dark cycles, and were fed a freely standard rodent diet and water. The experimental procedures were performed following the standard guidelines for the care and use of experimental animals by the committee for control and supervision of animal experiments and the national institutional animal care.

Experimental design:

Negative control group (G1): For 4 w, rats were given 1 ml/kg Body Weight (BW) of normal saline solution (0.9 % Sodium chloride (NaCl)) intraperitoneal (i.p.) three times a week at 24 h intervals, in addition to 3 ml/kg BW of normal saline solution orally once a day.

Positive control group (G2): Rats were injected i.p. with TAA at the dose of (200 mg/kg BW) 3 times weekly at 24 h intervals for 4 w to induce testicular toxicity[25].

TAA+SBN group (G3): Rats received silibinin (100 mg/kg BW, orally by gavage, daily) for 4 w[26] concurrently with i.p. injection of TAA in the same dose as G2.

Testicular weight measurements:

After 4 w of experimentation, the animals final body weights were recorded and they fasted for 12 h before being sacrificed. At the sampling time, the right and left testes were dissected from the epididymis and the relative testis weight was estimated as (testis weight/body weight)×100 weighed[27].

Sample collection:

Phenobarbital at 60 mg/kg doses is used to anesthetize rats by injection through the i.p. route[28]. Blood was collected from the retro-orbital sinus in which 3 cm of blood into a plain tube was taken and centrifuged at 3000 rpm at 4° for 5 min to get the serum for biochemical analysis of sexual hormones. The abdomen was opened and the testis and epididymis were carefully dissected. The right testes were prepared for histological and immunohistochemical analysis. The left testes were prepared for biochemical analysis of oxidative stress markers.

Biochemical analysis:

Assay of LH, FSH and testosterone concentrations: The separated sera were used for the estimation of serum levels of TH, LH and FSH. Rat ELISA kits identified serum levels according to the instructions of the manufacturer.

Determination of the testicular oxidative stress status and antioxidant enzymes: The left testis was homogenized from each rat in ice-cold (10 % w/v) Phosphate-Buffered Saline (PBS) (50 mM Dipotassium phosphate (K2HPO4), pH 7.4). The supernatant was separated by centrifugation at 1000 g for 20 min at 4°. Tissue MDA, GSH, SOD and CAT levels were determined by spectrophotometer using ELISA assay kits according to the manufacturer's instructions[29-32].

Histological and immunohistochemical study:
Right testes were processed for light microscopic studies. After 24 h at room temperature in Bouin's solution, the right testes were fixed. After that, ascending grades of alcohol (70 %, 95 % and 100 %) were used to dehydrate the specimens, cleared in xylene then embedded into paraffin wax. Paraffin blocks were cut at 5 μm thickness using a Leica rotator microtome (Germany). For evaluation of testicular changes with exposure to the following stains; Hematoxylin and Eosin (H & E) stain[33], Masson trichrome stain demonstrates collagen fibers[34]. Immunohistochemical staining using the avidin-biotin-peroxidase complex technique[35] for the detection of Sertoli cells with an anti-Vimentin antibody and Leydig cells with p53, a marker for apoptosis.

Deparaffinized sections (6 μm) were mounted on positively charged slides for immunohistochemical staining to detect Vimentin and p53 reaction in testicular tissue using anti-Vimentin and anti-p53 antibodies. They were soaked in PBS and pre-treated with 3 % hydrogen peroxide in distilled water to inactivate endogenous peroxidase activity. Primary antibodies were applied and the samples were incubated in a humidified environment for 60 min. Then the slides were stained with 3,3-diaminobenzidine kit (purchased from Boster Bio-Technology Co., Ltd) as the chromogenic and counterstained with Mayer's haematoxylin slide dehydrated in 95 % ethanol, cleared in xylene. Coverslips were mounted using two drops of dibutylphthalate polystyrene xylene mounting medium. The positively stained nuclei were brown. On the other hand, one of the testicular sections was used as a negative control bypassing the step of applying the primary antibody.

Johnsen's mean testicular biopsy score count H and E 40x: Johnsen's mean testicular biopsy score (Table 1) was used to evaluate testicular histological damage and spermatogenesis with light microscopy[36,37]. Each testis's 30 tubules were given a grade. Histological evaluation of testicular seminiferous tubules was performed by assigning a score between 1 and 10 to each tubule based on the presence or absence of several germ cell types such as spermatozoa, spermatids, spermatocytes, spermatogonia, germ cells and Sertoli cells. The Johnsen test reflects the health of spermatogenesis on a scale from 0 to 100, with higher scores indicating better health and lower scores indicating a more severe malfunction. With a score of 1, inactive tubules are not even evaluated for epithelial development. Alternatively, a score of 10 indicates that the tubules are functioning at their peak activity level and that the epithelium has fully matured.

Morphometric analysis:

Image analysis was performed with the image analyzer Olympus Image J, NIH, 1.41b, America Inc, Melville, New York, USA, on tissue samples from all rats in each group (n=10). Ten non-overlapping randomly chosen fields per section were quantified for the following morphometric measurements were determined; the height of seminiferous epithelium extending from the basement membrane of the seminiferous tubule towards the lumen. The mean area percentage (%) collagen fibers content in Masson trichrome stained sections (20x). The mean number of Vimentin-immunopositive Sertoli cells (20x). The mean number of p53-immunopositive Leydig cells (20x).

TABLE 1: CROSS-SECTIONAL HISTOLOGICAL ANALYSIS OF THE SEMINIFEROUS TUBULES UTILIZING THE JOHNSEN SCORING SYSTEM

Score	Description
10	Full maturation of the sperm cells results in many spermatozoa. The thickness of the germinal epithelium is uniformly structured, creating a patent lumen
9	The germinal epithelium is disordered and there is significant sloughing or obliteration of the lumen despite numerous spermatozoa
8	There were few spermatozoa in the section
7	There are no spermatozoa but plenty of spermatids
6	Neither spermatozoa nor even many spermatids may be found
5	No spermatozoa or spermatids are present; however, there are many spermatocytes
4	The sperm count is low, with few spermatocytes but no spermatids or spermatozoa
3	The only type of germ cell found is spermatogenic
2	Sertoli cells are present, but no germ cells can be seen
1	There are no cells in the tubule

Statistical analysis:

A one-way Analysis of Variance (ANOVA) was used to compare the means and Standard Deviations (SDs) of the quantitative data. The post hoc Tukey test was employed to determine which pairwise comparisons between groups explained any significant ANOVA results. Probability value $p<0.05$ indicated significant differences. Using Pearson's correlation coefficient, researchers analyzed the relationship between the average number of Leydig cells and blood testosterone level and the number of Sertoli cells. Calculations were made on Statistical Package of Social Science (SPSS) software version 26 (IBM Corp., Armonk, New York, USA). Data was represented graphically using GraphPad® Prism 9 (2022), a statistical package.

RESULTS AND DISCUSSION

Table 2 and fig. 1 show the effect of TAA and SBN on relative testis weight in negative control and experimental rats (positive control group and SBN treated group). A significant ($p<0.0001$) decrease in relative testis weights was noted in TAA-treated rats compared to the negative control. Significant ($p<0.0001$) changes were observed in relative testis weights between negative control and SBN-treated rats. However, all these changes induced by TAA intoxication were significantly ($p<0.0001$) improved on oral treatment with SBN.

After 4 w of the experiment, there was a highly significant decrease ($p<0.0001$, $p<0.0001$ and $p<0.0001$; respectively) in TAA (G2) compared to the negative control group (G1) regarding serum TH, LH and FSH levels (Table 3 and fig. 2). However, all these changes induced by TAA intoxication were significantly ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively) increased after oral treatment with SBN. G3 (TAA+SBN) treated rats showed a non-significant difference compared to the negative control group.

After 4 w of the experiment, there was a highly significant difference ($p<0.0001$, $p<0.0001$ and $p<0.0001$; respectively) in TAA (G2) compared to the negative control group (G1) regarding testicular MDA, GSH, SOD and CAT levels. However, all these changes induced by TAA intoxication were significantly ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively) improved after oral treatment with SBN. Otherwise, G3 (TAA+SBN) treated rats showed a non-significant difference compared to the negative control group (Table 4 and fig. 3).

Seminiferous tubules bordered by spermatogenic cells and Sertoli cells were seen near one another in the testicles of the G1 negative control group.

TABLE 2: RELATIVE TESTIS WEIGHT OF ALL EXPERIMENTAL GROUPS

Groups	Negative control group (G1)	Positive control group (G2)	TAA+SBN group (G3)	p value
n	10	10	10	
Testis relative weight (g/100 g BW)	1.19±0.05	1.098±0.03[a]***	1.20±0.04[a]ns[b]****	0.0002, 0.9434<0.0001

Note: Data were presented as mean±SD, (n): Number of rats/group, ([a]): Compared to negative control group (G1); ([b]): Compared to positive control group (G2) and (ns): non-significant, ***p<0.001 and ****p<0.0001
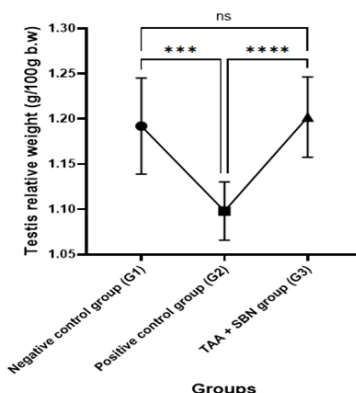

Fig. 1: Chart showing relative testis weight (g/100 g BW) among all experimental groups after the administration of TAA and SBN
Note: Data were presented as mean±SD; ns: non-significant, *** p<0.001 and ****p<0.0001

TABLE 3: COMPARISON BETWEEN ALL EXPERIMENTAL GROUPS ACCORDING TO THE LEVELS OF TS (NG/DL), LH (IU/L) AND FSH (IU/L)

Groups	Negative control group (G1)	Positive control group (G2)	TAA+SBN group (G3)	p value
n	10	10	10	
TH (ng\dl)	294.4±28.10	114.9±3.957[a]****	287.6±20.29[a]ns[b]****	<0.0001, 0.83<0.0001
LH (IU\L)	5.18±0.88	0.78±0.15[a]****	4.960±0.96[a]ns[b]****	<0.0001, 0.89<0.0001
FSH (IU\L)	5.76±0.82	0.95±0.34[a]****	5.52±0.79[a]ns[b]****	<0.0001, 0.82<0.0001

Note: Data were presented as mean±SD, (n): Number of rats/group, ([a]): Compared to negative control group (G1); ([b]): Compared to positive control group (G2) and (ns): non-significant, ****$p < 0.0001$
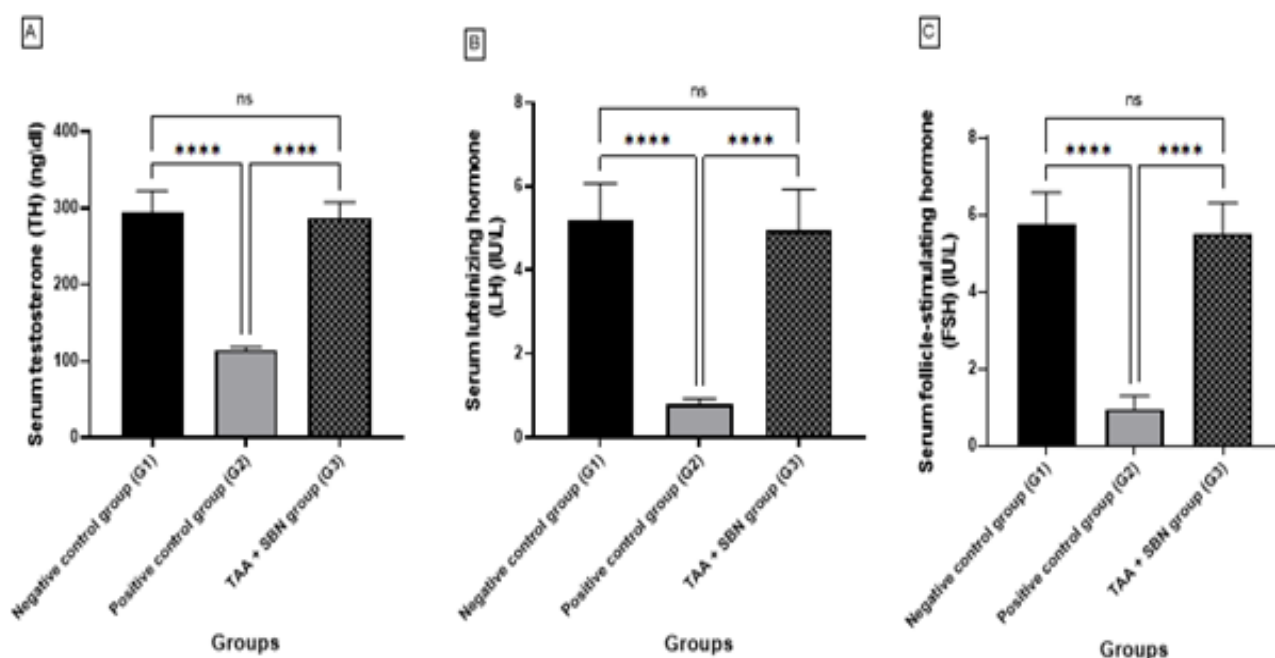

Fig. 2: (A): TS (ng\dl); (B): LH (IU\L) and (C): FSH (IU\L) among all experimental groups after the administration of TAA and SBN
Note: Data were presented as mean±SD; ns: non-significant and ****$p < 0.0001$

TABLE 4: COMPARISON BETWEEN ALL EXPERIMENTAL GROUPS ACCORDING TO THE LEVELS OF OXIDATIVE STRESS ENZYME MDA (NMOL\ML) AND THE ANTIOXIDANT ENZYMES SUCH AS REDUCED GSH (NG\ML), SOD (U\ML) AND CAT (MU\L) IN TESTIS TISSUE HOMOGENATE

Groups	Negative control group (G1)	Positive control group (G2)	TAA+SBN group (G3)	p value
n	10	10	10	
MDA (nmol\ml)	0.75±0.25	1.41±0.08[a]****	0.66±0.24[a]ns[b]****	<0.0001, 0.70<0.0001
GSH (ng\ml)	13.52±1.34	2.48±0.69[a]****	12.60±2.55[a]ns[b]****	<0.0001, 0.5631 <0.0001
SOD (u\ml)	167.40±8.30	84.50±16.97[a]****	159.0±9.47[a]ns[b]****	<0.0001, 0.99 <0.0001
CAT (Mu\l)	115.70±4.523	58.70±18.07[a]****	116.20±3.67[a]ns[b]****	<0.0001, 0.99 <0.0001

Note: Data were presented as mean±SD, (n): Number of rats/group, ([a]): Compared to negative control group (G1); ([b]): Compared to positive control group (G2) and (ns): non-significant, ***$p < 0.001$ and ****$p < 0.0001$
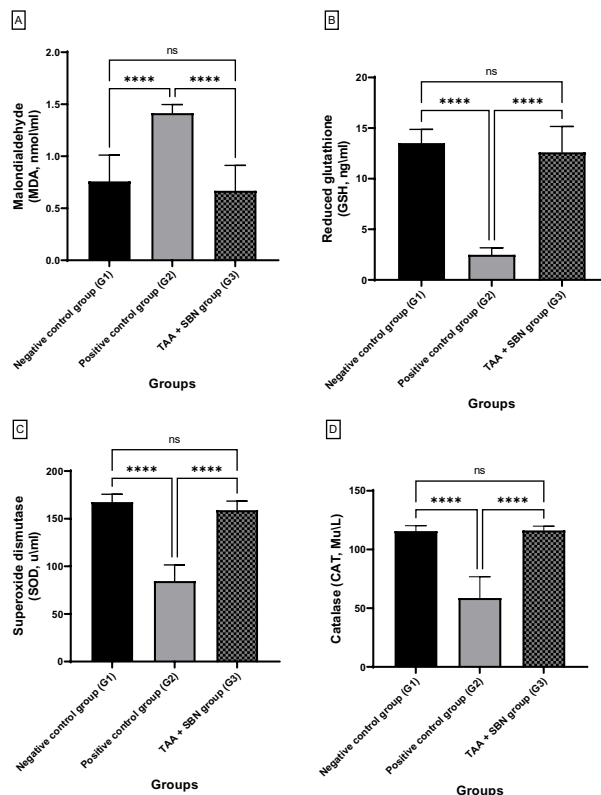
Fig. 3: (A): MDA (nmol/ml); (B): GSH (ng\ml); (C): SOD (u\ml) and (D): CAT (Mu/l) in testis tissue homogenate among all experimental groups after the administration of TAA and SBN
Note: Data were presented as mean±SD; ns: non-significant and ****$p < 0.0001$

Spermatogenic cells included spermatogonia, primary spermatocytes, spermatids and spermatozoa. Spermatogonia appeared as small rounded cells with rounded nuclei resting on the regular basement membrane. Primary spermatocytes were the largest spherical cells that had large rounded nuclei with partially condensed chromosomes. Spermatids appeared as small round cells with pale rounded nuclei and prominent nucleoli. Spermatozoa with deeply stained heads were also observed near the lumen of the tubules. Flattened, smooth muscle-like myoid cells were found external to the basal lamina of the seminiferous tubules. Sertoli cells appeared as tall cells lodged between spermatogenic cells with cytoplasmic extension. The interstitial tissue between the tubules showed Leydig's blood vessels and interstitial cells that appeared as large polygonal cells with vesicular nuclei and acidophilic cytoplasm containing small lipid droplets as shown in fig. 4.

In the positive control group (G2), examination of testicular sections demonstrated distorted histological architecture with widening interstitial spaces. Some of the seminiferous tubules had uneven basement membranes and the spermatogenic cells had been detached, leaving empty areas. Most spermatogenic cells showed apoptotic alterations, including pyknotic or irregular darkly pigmented nuclei and highly acidophilic cytoplasm. Other spermatogenic cells showed cytoplasmic vacuolation. In addition, some seminiferous tubules showed sloughed homogenous acidophilic necrotic material inside the lumen. Interstitial tissue contained most Leydig cells that appeared with darkly stained nuclei as shown in fig. 5.

Regarding TAA+SBN treated group (G3), testicular sections showed apparently normal-packed seminiferous tubules lined by several layers of spermatogenic cells and Sertoli cells resting on regular basement membranes surrounded by myoid cells with flattened nuclei. The spermatogenic epithelium still had a few gaps here and there. The interstitial gaps contained blood veins and polygonal Leydig cells with pale nuclei and acidophilic vacuolated cytoplasm. Yet, only a small percentage of cells displayed heavily stained nuclei as shown in fig. 6.

Sections from the negative control group (G1) revealed the normal distribution of bluish-stained collagen fibers in the testicular capsules (tunica

albuginea), basal laminae of the seminiferous tubules and interstitial tissue in-between (fig. 7a). However, TAA-treated rats (G2) showed widely separated collagen fibers (by edema) in the tunica albuginea and disrupted collagen fibers in the basal laminae of seminiferous tubules and interstitial tissue. Moreover, there was a marked increase in the collagen fibers in the testicular capsules, basal laminae of seminiferous tubules and interstitium (fig. 7b). TAA+SBN-treated rats showed an apparent normal distribution of collagen fibers compared to the control group as shown in fig. 7c.

In the negative control group, Vimentin filaments positive reaction within Sertoli cells was concentrated around the nucleus (perinuclear) in the basal part of the cell, then radiating apically toward the apical part of the cell (fig. 8a). In the positive control group (TAA), the number of Sertoli cells presenting positive Vimentin reaction was reduced with fragmented cytoplasmic extensions and marked irregularity of the basement membrane of the seminiferous tubule (fig. 8b and fig. 8c). These findings were improved in SBN-treated rats (G3), as many Sertoli cells showed normally distributed Vimentin filaments perinuclear and apical with cytoplasmic extensions. In addition, a regular basement membrane of the seminiferous tubule was detected (fig. 8d). Sections of the negative control group (G1) and TAA+SBN treated rats (G3) stained with anti-p53 showed positive brown nuclear immunoreaction in a few spermatogenic cells and Leydig cells. However, those of TAA-treated rats (G2) showed many immunoreactive spermatogenic and Leydig cells compared to G1. Regarding TAA+SBN

(G3) treated rats, some positive immunoreactive spermatogenic and Leydig cells were observed as shown in fig. 9.

The mean Johnsen score, the mean height of seminiferous epithelium, the mean number of Vimentin-immunopositive Sertoli cells and the mean number of p53-immunopositive Leydig cells values in the positive control group (TAA, G2) showed a statistically significant ($p<0.0001$, $p<0.0001$, $p<0.0001$ and $p<0.0001$; respectively) decrease as compared with the negative control group (G1) and SBN-treated group (G3). While the mean area percent (%) of collagen fibers content showed a statistically significant ($p<0.0001$) increase as compared with the negative control group (G1) and SBN-treated group (G3). The non-significant difference in all morphometric parameters was recorded in SBN-treated rats *vs.* the negative control group as shown in Table 5 and fig. 10.

Leydig cell number was negatively correlated with Sertoli cell number ($r=-0.8415$; $p<0.0001$) when data from rats in the negative control, positive control and SBN-treated groups were pooled. Therefore, the number of Sertoli cells generated in the experiment might predict the number of Leydig cells in the rat testis. When the number of Leydig cells was artificially decreased in experimental rats, a significant negative association was found between the serum TH level and the number of Leydig cells ($r=-0.9283$, $p<0.0001$) as shown in fig. 11.

It is widely believed that oxidative stress contributes significantly to sperm failure. Due to a high
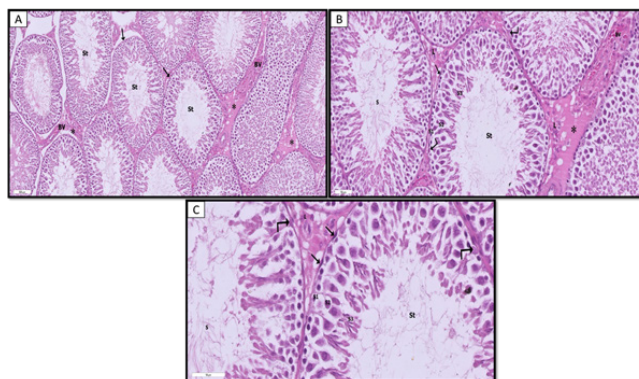

Fig. 4: Testicular sections of adult male rats in the negative control group (G1) showing, (A): Testicular section from the control group; (H & E stain 100x, scale bar 100 μm); (B): Each seminiferous tubule is lined by Sertoli cells (curved arrow) and stratified germinal epithelium. (H & E stain 20x, scale bar 50 μm) and (C): The seminiferous tubules surround by thin regular basal lamina and flattened myoid cells (↑) (H & E stain 40x, scale bar 50 μm)
Note: (St): Seminiferous tubules; (BV): Blood Vessels; (S1): Spermatogenic; (S2): Spermatocytes; (S3): Spermatids; (S4): Sperm flagella and (L): Clusters of Interstitial cells of Leydig
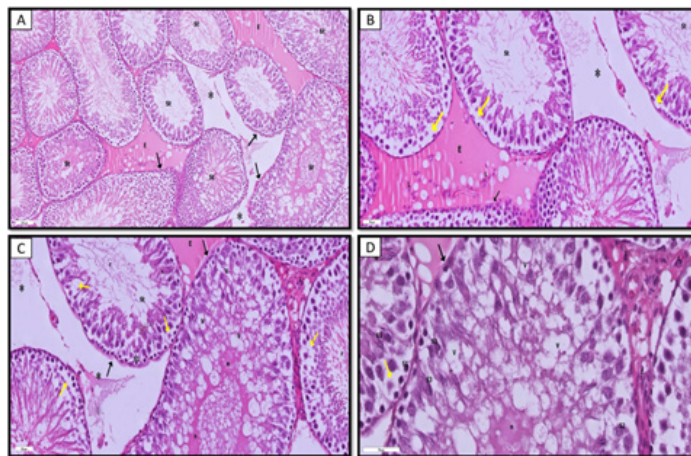
Fig. 5: Testicular sections of adult male rats in the positive control group (G2) shows, (A): Loss of normal architecture of testicular tissue with wide interstitial spaces (*) (H & E stain 100x, scale bar 100 µm); (B): Sloughing of the basal lamina of many seminiferous tubules (St) from lamina propria and wide interstitial spaces (H & E stain 20x, scale bar 50 µm); (C): Severely affected tubules with extensive loss of their germinal epithelium (yellow arrow) (H & E stain 20x, scale bar 50 µm) and (D): Distorted tubules with deeply stained pyknotic nuclei (H & E stain 40x, scale bar 50 µm)

Note: (E): Hyaline eosinophilic materials; (St): Seminiferous tubules; (r): remnant spermatogenic cells; (n): necrotic spermatogenic cells; (v): vacuoles; (L): Leydig cells; (S1): Spermatogonia; (S2): Spermatocytes; (S3): Spermatids and (S4): Sperm flagella
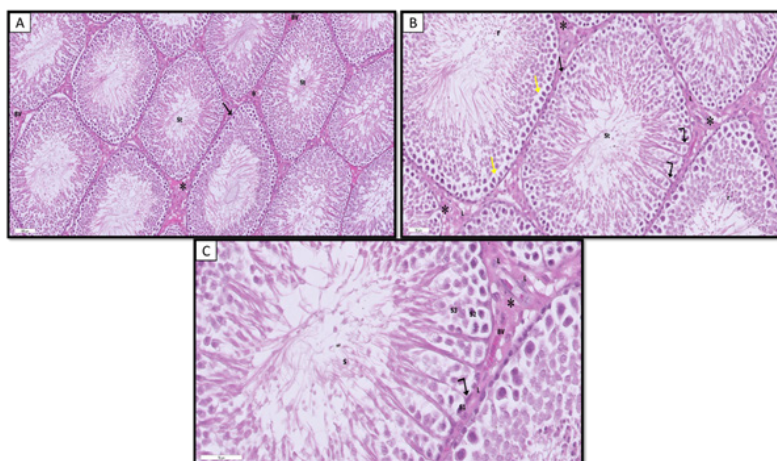

Fig. 6: Testicular sections of adult male rats in the TAA+SBN treated group (G3) showing, (A): Apparently, normal seminiferous tubules (St) with mostly intact germinal epithelium and regular outlines (↑) (H & E stain 100x, scale bar 100 µm); (B): The regular arrangement of closely packed seminiferous tubules (H & E stain 20x, scale bar 50 µm) and (C): Filled with sperm flagella (H & E stain 40x, scale bar 50 µm)

Note: (BV): Blood Vessels; (r): remnants of necrotic germinal epithelium; (L): Leydig cells; (S): Sperm flagella; (S1): Spermatogonium; (S2): Spermatocyte and (S3): Spermatids
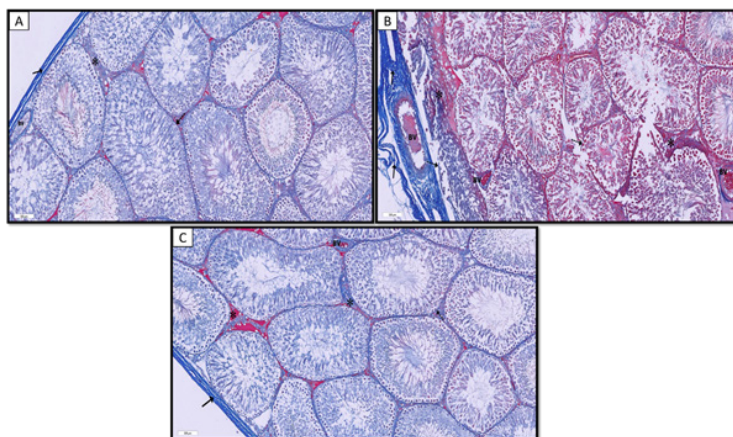

Fig. 7: Testicular sections of adult male rats in, (A): Negative control group (G1) shows a normal distribution of collagen fibers in tunica albuginea; (B): Positive control group (G2) shows a widely separated and marked increase in collagen fibers in the tunica albuginea (↑) and (C): TAA+SBN treated group (G3) shows an apparent normal distribution of collagen fibers in tunica albuginea (↑). Masson trichrome stain (100x, scale bar 100 µm)

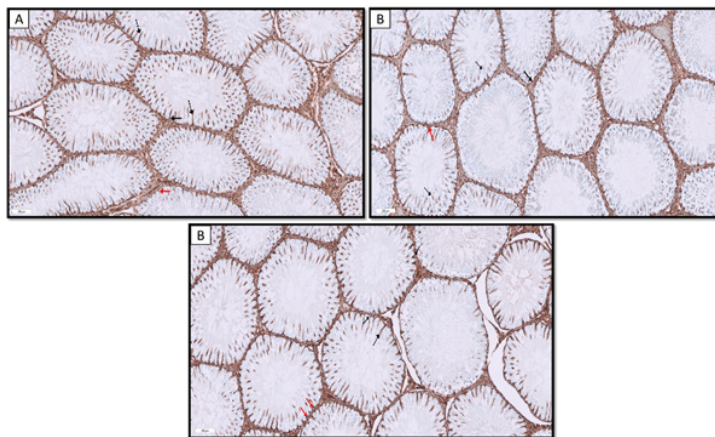Note: (BV): Blood Vessels and (*): Interstitial tissue

Fig. 8: Testicular sections of adult male rats in, (A): The negative control group (G1); (B): The positive control group (G2) and (C): TAA+SBN treated group (G3). Vimentin immunohistochemistry (100x, scale bar 100 μm)
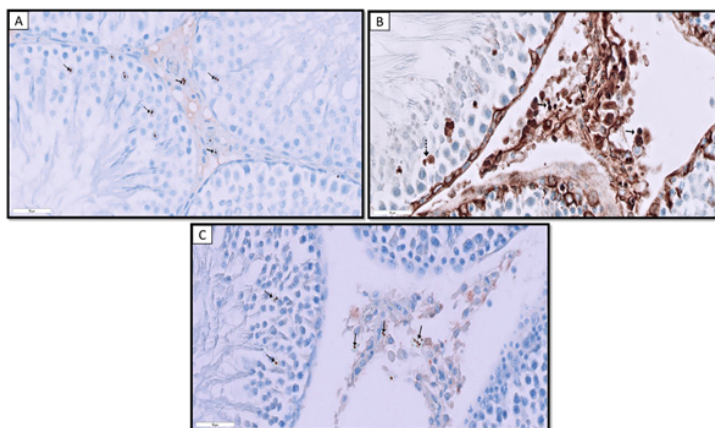

Fig. 9: Testicular sections of adult male rats in, (A): The negative control group (G1) shows few positive nuclear immunoreactions in spermatogenic cells (dot arrow) and Leydig cells (↑); (B): The positive control group (G2) shows many positive nuclear immunoreactive spermatogenic cells (dot arrow) and Leydig cells (↑) and (C): TAA+SBN treated group (G3) shows some positive nuclear immunoreactive spermatogenic cells (dot arrow) and Leydig cells (↑). P53 immunohistochemistry (100x, scale bar 100 μm)

TABLE 5: MORPHOMETRIC ANALYSIS IN ALL EXPERIMENTAL GROUPS ACCORDING TO THE MEAN DIAMETER OF SEMINIFEROUS TUBULES (MM), MEAN THICKNESS OF GERMINAL EPITHELIUM (μM), MEAN AREA % OF COLLAGEN FIBERS CONTENT IN MASSON TRICHROME STAINED SECTIONS (%), AND MEAN AREA % OF VIMENTIN-POSITIVE CELLS (%)

Groups	Negative control group (G1)	Positive control group (G2)	TAA+LUT group (G3)	p value
n	10	10	10	
The mean Johnsen score	9.200±0.6325	2.600±1.430[a]****	8.600±1.075[a]ns[b]****	<0.0001, 0.5457 <0.0001
The mean height of seminiferous epithelium (μm)	77.95±2.306	47.08±4.929[a]****	74.59±3.620[a]ns[b]****	<0.0001, 0.1595 <0.0001
The mean area % of collagen fibers content in Masson trichrome stained sections (%)	9.526 ±1.208	21.46±1.768[a]****	10.00±0.3034[a]ns[b]****	<0.0001, 0.7867 <0.0001
The mean number of Vimentin-immunopositively Sertoli cells	17.10±2.132	8.900±0.9944[a]****	16.66±0.9915[a]ns[b]****	<0.0001,0.8810 <0.0001
The mean number of p53-immunopositively Leydig cells	2.600±0.6992	8.300±1.337[a]****	3.100±0.9944[a]ns[b]****	<0.0001, 0.6473 <0.0001

Note: Data were presented as mean±SD, (n): Number of rats/group, ([a]): Compared to negative control group (G1); ([b]): Compared to positive control group (G2) and (ns): non-significant, ***p<0.001 and ****p<0.0001
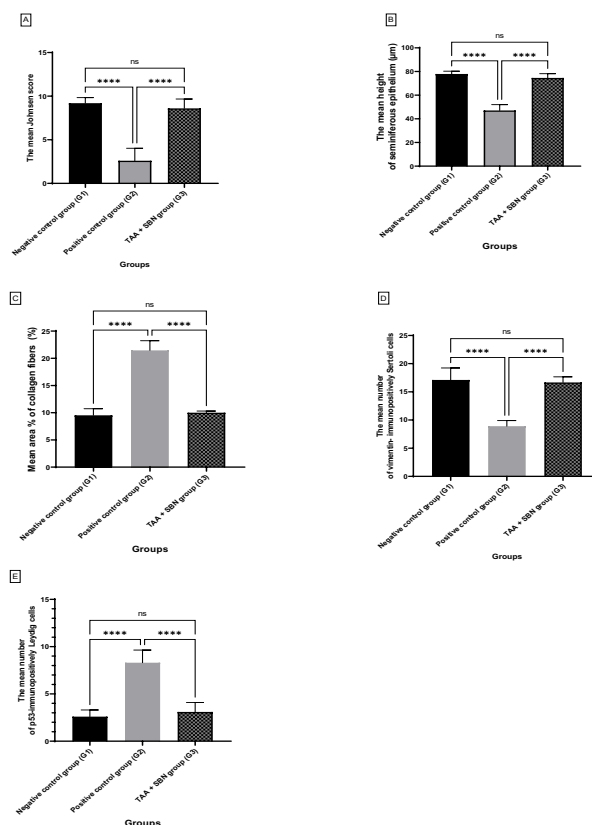
Fig. 10: (A): The mean Johnsen score; (B): The mean height of seminiferous epithelium (μm); (C): The mean area % of collagen fibers content in Masson trichrome stained sections (%); (D): The mean number of Vimentin- immunopositively Sertoli cells and (E): The mean number of p53-immunopositively Leydig cells
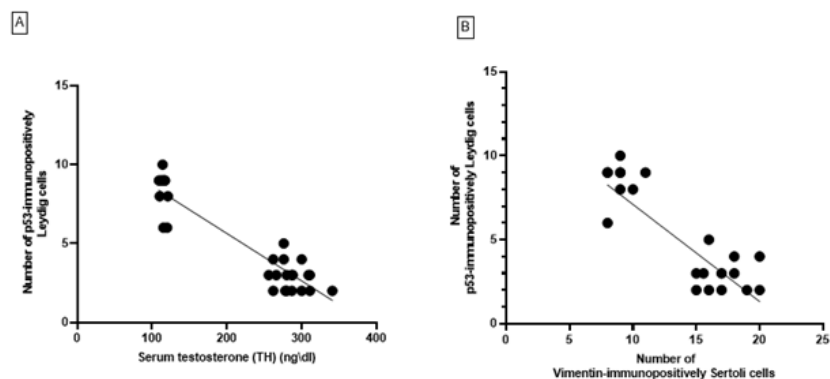**Note: Data were presented as mean±SD; ns: non-significant and ****$p<0.0001$**

Fig. 11: Pearson's Correlation between, (A): Leydig cell number and TS level and (B): Leydig cell number and Sertoli cell

concentration of Polyunsaturated Fatty Acids (PUFA) in their membranes, spermatocytes are particularly susceptible to the damaging effects of ROS[38]. Researchers from all over the world have concluded that an excessive amount of ROS in men's sperm is directly responsible for their inability to conceive. Because of a discrepancy between its synthesis and breakdown, ROS levels have risen in spermatozoa and seminal plasma. Diverse antioxidant scavengers in the cell are responsible for breaking ROS[39]. Healing ailments using plants has a long history, dating back to prehistoric times. The antioxidant and anti-inflammatory properties of SBN have made it a popular choice for treating hepatic diseases, with promising outcomes[24]. The present study investigated the biochemical, histological, immunohistochemical and morphometric changes that may develop in the rat testis exposed to oxidative stress and the possible protective role of SBN.

Oxidative stress is one of the most important causes of induction of different organ toxicity. In the present study, testicular homogenate content of GSH, SOD and CAT was significantly decreased in

the positive control group (TAA-treated rats). At the same time, MDA was significantly increased than other groups, indicating oxidative stress's role on testicular toxicity, therefore expecting these changes to be alleviated by the antioxidant treatment as in the present work. That supports a recent study by Celik et al.[11]. They reported that TAA exposure induced lipid peroxidation and decreased the efficacy of the antioxidant defense system via a reduction of CAT, SOD, GPx activity and GSH levels. Some studies have suggested that CAT and GSH were decreased with TAA[40]. The MDA is one of the most popular and reliable markers determining Overall Survival (OS) in clinical situations. It is produced by the peroxidation of PUFA. So, an increased MDA level is considered an important indicator of lipid peroxidation and subsequently, loss of membrane structures and functions. Testes, rich in PUFA and having high metabolism rates and cell replication and poor antioxidant defenses, are much more vulnerable to peroxidation injury than other tissues[41]. Increased MDA levels in rat testes following TAA exposure were similarly reported in previous studies[42]. Our results show that SBN protects rat testicular homogenate from the oxidative stress caused by TAA by boosting the animal's antioxidant defenses. SBN's cytoprotective impact may arise from several mechanisms, each of which acts on a different cellular level. Since SBN is a powerful antioxidant, ROS-based cell regulation mechanisms may be involved in some of its radical-scavenging activities[22]. Further, decreased height of lining spermatogenic epithelium and level of TH in the positive control group (TAA-treated rats) against the negative control may account for the loss of histological architecture of seminiferous tubules. The same characteristics have been noted before[25]. This explanation was enforced by the ability of MDA to react with multiple biomolecules, such as proteins or Deoxyribonucleic acid (DNA), leading to DNA damage, cell cycle arrest and apoptosis of germ cells. The high susceptibility of spermatozoa to DNA damage was attributed to the loss of their cytoplasm, which contains antioxidant enzymes and consequently, the loss of their capacity for DNA repair after spermiation[42].

Both endocrine and paracrine variables can affect testicular function. When it comes to the male reproductive system, the hypothalamic-pituitary-testicular axis is a major player. The hypothalamus regulates pituitary gonadotropin production by secreting gonadotropin-releasing hormone, which stimulates the production of the glycoprotein gonadotropins LH and FSH. These hormones then enter the bloodstream and travel to the testes, where they act directly on the somatic cells of the testis[43-45]. Leydig cells in the testicular interstitial tissue express LH receptor, whereas Sertoli cells bind FSH. Cholesterol in the Leydig cells is where LH triggers the creation of TH[46,47]. Androgens function in the testis to stimulate the development of both Leydig and Sertoli cells. Androgen has opposing effects on adult Leydig cells and adult Sertoli cells, partially suppressing steroid genesis in the former but boosting the latter's actions that are essential for spermatogenesis[48,49]. Serum TH reduction in the present study might be due to a disturbed oxidant antioxidant state[25]. Previously, it was suggested that necrosis of Leydig cells or diminished stimulation of the cells by interstitial cells stimulating hormone is the reason for testosterone reduction[50]. In the present study, treatment with 100 mg/kg SBN significantly restored sperm serum TH and the levels of gonadotrophins to near normal, which denotes the stimulation of spermatogenesis by SBN[51]. After 5 d of treatment with (50, 100, or 150) mg/kg/day SBN, it was observed that the mice's TH levels had increased in a dose-dependent manner relative to the control group[21]. They reported that SBN has an aromatase inhibitor property[21] which could explain the increase in the TH level.

Previous research has demonstrated the importance of Sertoli cells in the testis ability to differentiate, mature and function normally in adulthood. They regulate the maturation of Leydig cells, the activity of peritubular myoid cells and the survival and development of germ cells[52,53]. According to Vimentin immunoreactivity, in the negative control group, there was positive staining of Vimentin in Sertoli cells either located around the nucleus or extended to the apical region of Sertoli cells as its extension, which extended between spermatogonia and primary spermatocytes. In their study, ElGhamrawy et al. observed that Vimentin immunostaining was present in the middle and tips of Sertoli cell walls and neighboring germ cells and spermatozoa[54]. Rats administered TAA showed a decrease in immunoreactivity staining, but rats given SBN, in addition to TAA, showed an improvement. This decrease in vimentin expression in injured testicular tissue occurred because Vimentin filaments disintegrated away from the Sertoli cell membrane. Detached spermatogenic cells may then incur

apoptosis due to insufficient nourishment and support from nearby Sertoli cells[55]. This was represented in the present study by histopathological results, which showed close Sertoli cytoplasmic extensions in TAA and SBN groups but, conversely, loss of these relations in TAA-treated rats. Vimentin filaments in Sertoli cells, as determined by the research of ElGhamrawy *et al.*, were crucial in the upkeep of spermatogenesis, with their disruption or restoration linked to the repair and restoration of spermatogenesis[54].

Reduced serum TH levels in TAA-treated rats in this study could be attributed to the impairment of Leydig cells. This assumption was supported in the present work by the apoptotic appearance and positive expression of p53 of many Leydig cells in G2 (TAA-treated rats). That was furthermore established by a significant increase in the number of p53-positive Leydig cells decrease and serum TH. The current study also found that the number of Sertoli and Leydig cells was significantly lower in the TAA group. The research also uncovered an essential connection between Leydig cell count, Sertoli cell count and TH levels. Rebourcet *et al.* found that the size of the Sertoli cell population is predictive of the final testicular cell composition across all ages, which may explain this phenomenon. A decrease in Sertoli cell quantity or proliferation at any age will result in a corresponding fall in germ cell and Leydig cell populations, potentially negatively impacting fertility and health[49]. Results from this study corroborate those of Oufi *et al.*[22]. They found a positive association between TH level and the mean number of Leydig cells after administering SBN at a dose of 100 mg/kg intraperitoneally to rats[22].

Our study detected that TAA caused a marked decline in the testicular, body and testis relative weights compared to the negative control group. These results agree with prior studies that documented the same finding confirming the gonadotoxicity of TAA and the testicular atrophy results from the suppression of spermatogenesis[40]. However, TAA+SBN treatment raised testicular, body and testis relative weights compared to TAA-treated rats. Similar findings were reported by Muthumani *et al.*, who reported that all changes induced by arsenic intoxication significantly improved the oral administration of SBN (75 mg/kg[39]. Our histological studies also coincided with the biochemical and morphometric findings. TAA-induced injurious testicular changes. Similar findings were reported by Karabulut *et al.*[27].

They described a washed-out appearance of the seminiferous tubules, reflecting the degeneration of germinal epithelium and extensive cell loss after TAA administration.

Apoptosis, caused by oxidative stress, which permeabilizes the mitochondrial outer membrane and releases apoptotic proteins for diffusion into the cytosol, may explain the presence of small dark nuclei in the spermatogonia and the spermatids, which had shrunken nuclei and abnormally distributed mitochondria[57]. Rats given TAA exhibited severely damaged spermatogenic germ epithelium, as measured by the Johnsen score. The germinal epithelium lining the seminiferous tubules was patchy and atrophy was present in some of the tubules in this sample of testis tissue. Vascular congestion, edema and vacuolization have been observed in seminiferous tubules near the capsule[25]. We observed an improvement in testicular histology in the groups treated with TAA+SBN. This improvement was more statistically significant in TAA+SBN than in TAA treated group. This result was clarified by other authors who stated that the antioxidant properties of SBN afforded significant protection and restored the normal architecture of the testis[22]. Examination of Masson's trichrome-stained sections of the TAA+SBN treated rats showed that SBN administration improved the previous effect in the TAA-treated group as decreased collagen fibers distribution was much less in the capsule and around the blood vessels. Ezhilarasan *et al.*, who looked into the preventive role of SBN against liver fibrosis, came to similar conclusions. At a dose of 100 mg/kg/day, SBN was found to block the messenger RNA expression of collagen types 1 and 3, thereby lowering the enhanced collagen fibers deposition seen in N-nitrosodimethylamine-induced liver necrosis, inflammatory alterations and hepatic fibrosis[17].

In conclusion, this study showed that Silibinin might increase testosterone and gonadotropin levels and enhance several testicular indices (LH and FSH). Furthermore, the protective effects of SBN on reproductive toxicity and oxidative stress were evidenced by the antioxidant activities and improvement of the histological structures of both Leydig and Sertoli cells. As a result, it can be used as a supplement to improve male reproductive function, particularly spermatogenesis.

Conflict of interests:

The authors declared no conflict of interests.

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