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EFFECT OF GRAPE SEED EXTRACT, GERVITAL, AGAINST METHOTREXATE INDUCED HISTOLOGICAL AND ULTRASTRUCTURAL ALTERATIONS IN TESTES OF ALBINO RATS

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ABSTRACT

The present study was designed to investigate the effect of Gervital on testicular damage induced by the anticancer drug, methotrexate (MXT), in adult male albino rats. Forty male albino rats (*Rattus norvegicus*) were divided into five experimental groups (8 animals in each one) **Group 1**: served as control group. **Group 2**: animals of this group were orally administered grape seed extract only at a dose level of 150mg/kg body weight daily for 28 days. **Group 3**: animals of this group were intraperitoneally injected with a single dose of MXT (8mg/kg body weight) per week for 4 weeks. **Group 4**: animals of this group were intraperitoneally injected with a single dose of MXT followed by grape seed extract (GSE) daily for 28 days. **Group 5**: animals of this group were pretreated with GSE followed by MXT. At the end of the experimental period, all rats were sacrificed. Blood samples were collected for the biochemical study and testes were removed and prepared for histological and ultrastructural studies. **Results**: Methotrexate significantly reduced the final body and testes weight. **Histological observations**: several pathological changes were observed in the testicular tissues. These changes include degenerated seminiferous tubules, distorted spermatogenic cells and degenerated interstitial tissue with hemorrhage. **Ultrastructural examination** showed various severe degenerated features after MXT treatment including types A&B spermatogonia, round and elongated spermatids. On the other hand, GSE administration showed advanced degree of improvement in all the histopathological and ultrastructural changes; the testicular tissues appeared mostly normal. **Biochemical analysis** revealed that MXT injection significantly reduced serum testosterone level and superoxide dismutase (SOD) activity while, it significantly elevated malondialdehyde (MDA) level. Otherwise, administration of gervital restored the previous

biochemical changes to mostly normal level. The protective effect of gervital may be due its antioxidant and free radicals scavenging activities. The present study concluded that grape seed may improve the testicular toxicity induced by methotrexate.

KEYWORDS: gervital, methotrexate, testes, histopathological, ultrastructure, biochemical.

INTRODUCTION

Chemotherapy involves one or more antineoplastic drugs to inhibit cancer cell activity. Methotrexate (MXT) is a folic acid antimetabolite used as chemotherapeutic agent for many cancer types (leukemia, lung cancer, breast cancer, lymphoma, etc.) (Jahovic *et al*., 2004; Sener *et al*., 2006). Blumenfeld (2012) reported that the usage of MXT caused acute toxic side effects on tissues with high proliferation. These tissues also include bone marrow, liver. Lung, kidney, gut and central nervous system (Gibson *et al.*, 2011; Argyriou *et al.*, 2012). The genotoxic effect of MXT have been observed in both somatic and germ cells (Choudhury and Palo, 2004). In addition, there were several studies evaluated the effect of MXT on fertility. Shamberger *et al*. (1981) and Regheb and Sabanegh (2010) confirmed that MXT caused sterility in men during either long or short term toxicity. The most important side effect of MXT is testicular toxicity (Padmanabhan *et al.*, 2008; Nouri *et al.*, 2009). Moreover, MXT administration caused subsequent infertility by affecting the spermatogenesis process and showed degenerated seminiferous tubules, decrease in sperm number, damage of sperm DNA (Shrestha *et al.*, 2007; Padmanadhan *et al.*, 2008) and affect steroidogensis (Badri *et al.*, 2000).

Grape (*Vitis vinifera*) is one of the world's largest fruit crops and the most commonly consumed fruits in the world (Schamel, 2006; Percival, 2009). Dietary intake of foods (include grape seed) that rich in antioxidant constituents able to scavenge superoxide radicals in living cells and protect cells from severe diseases like cancer so, it considerd as powerful antioxidant compounds (Yilmaz and Teledo, 2004; Ahmed and Fatani, 2007). Grape seed extract (GSE) is a complex mixture of flavonoids, polyphenols containing dimers, trimmers, other oligomers (procyanidins) of catechin and epicatechin, proanthocyanidins and procyanidines (70-95%) (Raina *et al.*, 2007; Jia *et al.*, 2011). Many studies investigated that grape seed possess a broad spectrum of pharmacological properties including antitumor, anti-inflammatory (Li *et al.*, 2000; Nandakumar *et al.*, 2008), antibacterial, antiviral, anticarcinogenic (Carnesecchi *et al.*, 2002), anti-obesity by inhibiting lipid absorption from intestine which has been showed to occur partly via inhibition of lipase (Mereno *et al.*, 2003;

Ohyama *et at.*, 2011) and anti-allergic and consequently reduced the concentration of reactive oxygen species (Bagchi *et al.*, 1997). Hajizadeh *et al.* (2016) investigated the efficacy of GSE to prevent testicular tissue injury induced in mice. The aim of present study was to investigate the possible potential role of gervital to prevent testicular damage induced by methotrexate in adult male albino rats.

MATERIALS AND METHODS

Chemicals used

1. Methotrexate

Methotrexate (MXT) is an anticancer (anti-neoplastic) compound that is widely used as foliate antagonist. MXT is purchased from Mealan Company, Cairo, Egypt as odorless, fine yellow crystalline powder. Experimental animals were intraperitoneally injected with MXT at a dose level of 8 mg/kg body weight, once a week for 4 weeks (Padamanabhan *et al.*, 2009).

2. Gervital

Gervital is a trade name of grape seed extract (proanthocyanidins). Gervital is purchased from Amoun Pharmaceutical Company, Cairo, Egypt, as capsules (each contains 150mg grape seed extract). It was dissolved in distilled water and given orally to animals at a dose level of 150mg/kg body weight (Bagchi *et al* 2001) daily for 28 days.

Animals and experimental design

The study was performed on 40 adult male albino rats (*Rattus norvegicus*) weighing from 120-130g. Animals were obtained from National Research institute, Cairo, Egypt. All rats were housed under controlled laboratory conditions of 24±2°C and they were fed a standard pellet diet. Animals experiments were conducted accordance with the national ethical guidelines for the use and care of laboratory animals are approved by local ethical committee for animal experiments at Menoufia University protocol number (Approval No.MNSH1179).

Animals were randomly separated into 5 groups (8 in each). Group 1: represented the experimental control group and did not receive additional treatment other than standard care and housing. Group 2: animals of this group were orally administered grape seed extract (150mg/kg body weight) for 28 days. Group 3: animals of this group were intraperitoneally injected with a single dose of MXT (8mg/kg body weight) per week for 4 weeks. Group 4: animals were intraperitoneally injected with a single dose of MXT/week for 4 weeks followed by grape seed extract (150mg/kg body weight) daily for 28 days. Group 5: animals

received GSE (150mg/kg body weight) daily for 28 days followed by single dose of MXT (8mg/kg body weight) weekly for 4 weeks.

At the end of each treatment, animals of each group were sacrificed by using chloroform inhalation. After an abdominal incision, blood samples were collected for biochemical analysis and whole testes were removed and were prepared for histological & ultrastructure examination.

Morphometric measurements

After dissection testes were rapidly removed from animals and weighted by a delicate balance. The length, width and thickness of the two testes of each animal were measured by a ruler to obtain their volume according to the equation of Kadhim *et al.* (1988) and make comparison between the change in both weight and volumes of testes of each group.

Histological studies

After fixation in 10% neutral formalin, testes were processed to prepare 5µm paraffin sections. Finally, sections were stained with haematoxylin and mounted in DBX. Microscopic examination of the stained sections was then carried out by Olympus Light Microscope to determine possible cytoarchitectural changes.

Ultrastructure studies

For the electron microscope studies, small pieces of the testes were fixed in 5% glutaraldehyde and 1% osmium tetroxide, dehydrated in a graded series of ethanol, then embedded in Epon 812 resin. Semi-thin sections were prepared by Reichert Ultra microtome and were mounted on the grids then stained with toluidine blue. Ultra-thin sections were cut, then they stained with 8% uranyle acetate in 70 % ethanol and finally stained with lead citrate. The stained sections were analyzed and photographed with a JEM 1400 (JEOL, Japan) transmission electron microscope.

Biochemical analysis

Blood samples were collected from the heart puncture of control and treated groups. Sera were separated by centrifugation and stored at -20°C. All the biochemical analyses were carried out in Biochemistry lab, Liver Institute, Menuofia University. Malondialdyhde (MDA) was determined by the method of Ohkawa *et al.* (1979) and superoxide dismutase

was determined according to Rest and spitznagel (1977). Testosterone was determined according to Maruyama (1987).

Statistical analysis

Data were expressed as mean ± standard error (M±SE). Statistical analysis was performed by using student t test. Values were considered statistically significant when P ≤0.01, P ≤0.05 and P ≤0.001.

RESULTS

Morphometric results

Changes of body weight

Data in figure (1) showed an insignificant difference in body weight of animals of both control group (125.4±2.13) and grape seed extract group (130.8±4.58). Whereas animals treated with MTX showed a significant decrease (99.4±4.56) in body weight when compared with control group. Body weight returned to normality after treatment with gervital either after (124.00±2.42) or before (123.60±2.73) MTX treatment (a significant increase when compared with MTX group).

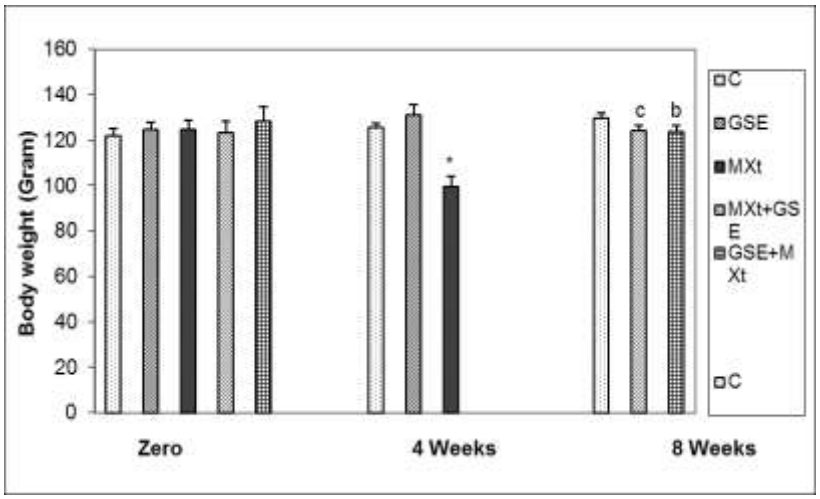

Figure 1: The change in body weight of rats of the different groups.

Weight and volume of testes

The values of weight and volume of testes are represented in figures (2 & 3). Oral administration of grape seed extract alone showed insignificant change in the weight (0.229±0.007) and volume (0.380±0.009) of testes when compared with control group (0.214±0.005) and (0.390±0.004), respectively. However, there is a significant decrease in both weight (0.159±0.008) and volume (0.281±0.015) of testes of animals treated with MXT

only, when compared with control group. Animals treated with MXT followed by grape seed extract showed a significant increase in both weight (0.237±0.010) and volume (0.370±0.014) of testes, when compared with MXT group.

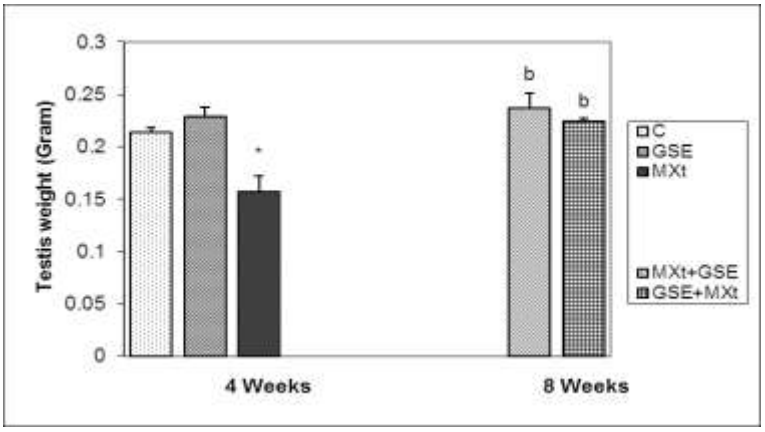

Figure 2: The change in the weight of testes of animals in the different groups.

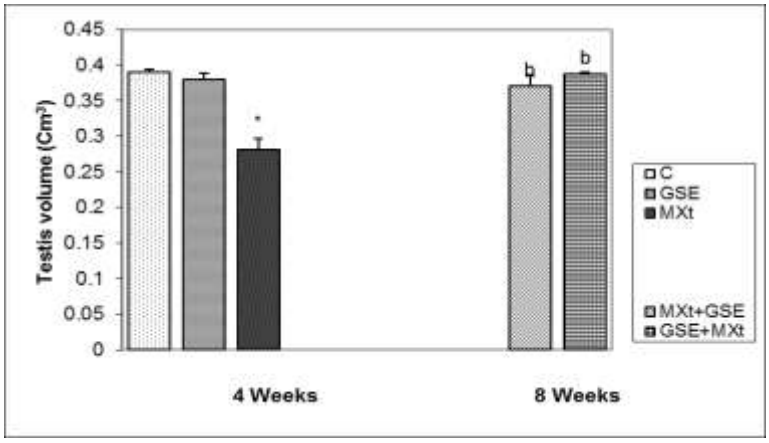

Figure 3: The change in the volume of testes of animals in the different groups.

Histological observation

Examination of testicular tissue of both control and gervital (150mg/kg body weight daily for 28 days) treated animals showed normal histological structure. There is a thick capsule of dense connective tissue, outer to seminiferous tubules known as tunica albuginea containing one or more loose layer of myoid cells. The wall of seminiferous tubules contains pyramidal shape cells known as Sertoli cells. Within the thick wall of the seminiferous tubules there were many different stages of germ cells. There were two types of spermatogonia; type A and type B spermatogonia. Half of spermatogonia A population differentiate to become type B cells. The other half of these daughter cells persists as stem cells. Furthermore, type B spermatogonia undergo mitosis to from large ovoid cells known as primary spermatocytes.

Secondary spermatocyte is smaller cell lying closer to the lumen. These cells are immediately undergo the second maturation division and so, they are seldom found (have short-lived). Round spermatids are rather small round cells with round nucleus. Then they elongate and the nucleus takes a peripheral position. These cells undergo a maturation process producing spermatozoa. There is an interstitial region between seminiferous tubules contains blood vessels and interstitial cells that are known as Leydig cells (Figs.4 &5).

Animals treated with single dose of MTX (8mg/kg body weight) per week for 4 weeks showed several pathological features. The most prominent pathological alternations were distorted seminiferous tubules and degenerated Sertoli cells. Few numbers of spermatogonia were detached from the basement membrane and appeared with cytoplasmic vacuolization and pyknotic nuclei. In addition, absence of different stages of spermatogenic cells and almost there is no sperms within the lumen of seminiferous tubules were appeared. Hemorrhage, congested blood vessels, cytoplasmic vacuolization and degenerated Leydig cells were seen in the interstitial tissue between seminiferous tubules (Fig.6). Also, there was a large number of mitotic figures (Fig.7).

Examination of testes of animals injected with MTX followed by grape seed extract (150mg/ kg body weight) daily for 28 days revealed that most of the seminiferous tubules restored their normal appearance. The spermatogenic cells were found in normally arranged layers. Hemorrhage and cytoplasmic vacuolization were rarely seen. Normal sperm distribution was appeared in most of seminiferous tubules (Fig.8).

Sections of testes of animals treated daily with gervital followed by MTX showed an obvious degree of protection. Most of seminiferous tubules appeared close to normal ones. There is no hemorrhage in interstitial tissue, normal spermatogenic cells, Sertoli cells and Leydig cell were seen. Although seminiferous tubules appeared mostly normal, little number of spermatogonia still appeared degenerated (Fig.9).

Ultrastructure observations

Normal testicular ultrastructure was observed in specimens of control animals. Normal lamina propria containing normal myoid cells with spindle-shaped nuclei and their delicate cytoplasmic processes, lying parallel to each other. The extracellular space contained few collagen fibers. Sertoli cells extended radially from the basement membrane to the lumen of the tubule. The nucleus of Sertoli cell appeared large, pale, oval and basal with enfolded

nuclear membrane, relatively homogenous chromatin material and one or more nucleoli. In addition, their cytoplasm contains abundant smooth endoplasmic reticulum, ovoid Golgi apparatus and numerous mitochondria with spherical or cylinder shape (Fig. 10). There are two types of spermatogonia; type A and type B. Type A spermatogonia are characterized by large pale ovoid nuclei containing fine light and non-condensed chromatin, scantly, homogenous and granular cytoplasm with poor rough endoplasmic reticulum. Golgi apparatus is simple and the mitochondria are abundant with spherical or ovoid shaped. Type B spermatogonia are slightly smaller and contain rounded nuclei with more electron dense nucleoplasmic matrix than type A and numerous chromatin clumps. The cytoplasmic organelles are similar to those described in the type A (Fig.11). The spermatocytes are differentiating into two types primary and secondary spermatocytes. Primary spermatocytes contain large spherical nucleus with faint granular chromatin and spherical mitochondria that aggregates peripherally (Fig.12). Secondary spermatocytes are rarely seen among germ cells and appeared smaller than primary spermatocytes (Fig.13).

Figure (14) showed the early spermatid that appeared round with large spherical nucleus contained chromatin clumps in a lightly stained cytoplasm. In addition, the mitochondria arranged at the periphery around the cell membrane. Round spermatid is large round cell with large spherical nucleus, normal mitochondria and Golgi apparatus. Also, they were seen in Golgi phase at different stages of acrosomal cap formation (Fig.15). Normal interstitial tissue was seen contained Leydig cells with large nucleus contained thin rim of chromatin and prominent nucleolus, rough endoplasmic reticulum, mitochondria and numerous lipid droplets (Fig.16).

Treatment with grape seed extract only (150 mg/kg body weight) for 28 days showed no ultrastructural changes when compared with control animals. Basement membrane appeared normal and seminiferous tubules contained mostly normal A and B spermatogonia, primary spermatocytes and round spermatids with normal acrosomal cap. In addition, junction complex between germ cells appeared mostly normal (Figs.17&18). Secondary spermatocytes were mostly normal and smaller in size than primary spermatocyte, with spherical nucleus and mitochondria aggregate periphery of cell membrane (Fig.19). Moreover, the lumen contained many transverse sections of normal sperm (Fig.20).

The ultrastructure observation of testicular tissues of animals treated with MXT (8mg/kg body weight once a week for 4 weeks) revealed different changes. These alternations include

basement membrane that appeared thicker than normal, contained distorted myoid cells with degenerated mitochondria. Moreover, there was increase in collagen fibers. Type A spermatogonia were severely affected and contained large number of degenerated mitochondria. The nucleus appeared degenerated with massive clumped chromatin (pyknotic nucleus). Type B spermatogonia appeared with abnormal nucleus with lysis of nucleoplasm. In addition, winding intracellular space between germ cells was appeared (Fig. 21). Sertoli cells appeared with severely affected nuclei, sometimes they were completely fragmented. Degenerated cytoplasm with some lytic areas, dilated smooth endoplasmic reticulum, degenerated mitochondria and different size of cytoplasmic vacuoles and lipid droplets were seen among the ultrastructural alterations in Sertoli cells (Figs. 22&23).

Secondary spermatocytes appeared with abnormal nuclei, vacuolated cytoplasm and degenerated mitochondria (Fig. 24). The same figure showed transvers sections of large number of distorted and malformed sperms in the lumen of the seminiferous tubules. Round spermatids appeared with degenerated mitochondria, vacuolated cytoplasm and winding intercellular spaces between germ cells (Fig. 25). Figure (26) showed abnormal interstitial tissue with degenerated Leydig cell contained abnormal nucleus with irregular nuclear envelope, clumped heterochromatin in addition to large number of vacuoles and many fat droplets.

Animals treated with MXT followed by GSE showed a marked degree of improvement in both seminiferous tubules and interstitial tissue except few degenerative features. Mostly normal basal lamina with myoid cells and tight junctions complex between germ cells were seen. Spermatogonia appeared mostly normal with few degenerated mitochondria and few dilated rough endoplasmic reticulum (Figs. 27&28). Moreover, early round spermatids and secondary spermatocytes appeared close to these of control group (winding spaces between germ cells sill appeared) (Figs. 29& 30). Leydig cells appeared mostly not differ from control group (Fig.31). Lumen of seminiferous tubules of animals in this group contained mostly normal transverse sections of sperms (Fig. 32).

The ultrastructure observation of testes of animals administered with grape seed extract before MXT showed mostly normal testicular tissues when compared with group that treated GSE after MXT toxicity. Sertoli cells and spermatogonia (types A & B) appeared close to normal (Fig.33). Normal secondary spermatocytes and round spermatids in different stages of acrosomal cap formation were observed (Figs. 34&35).

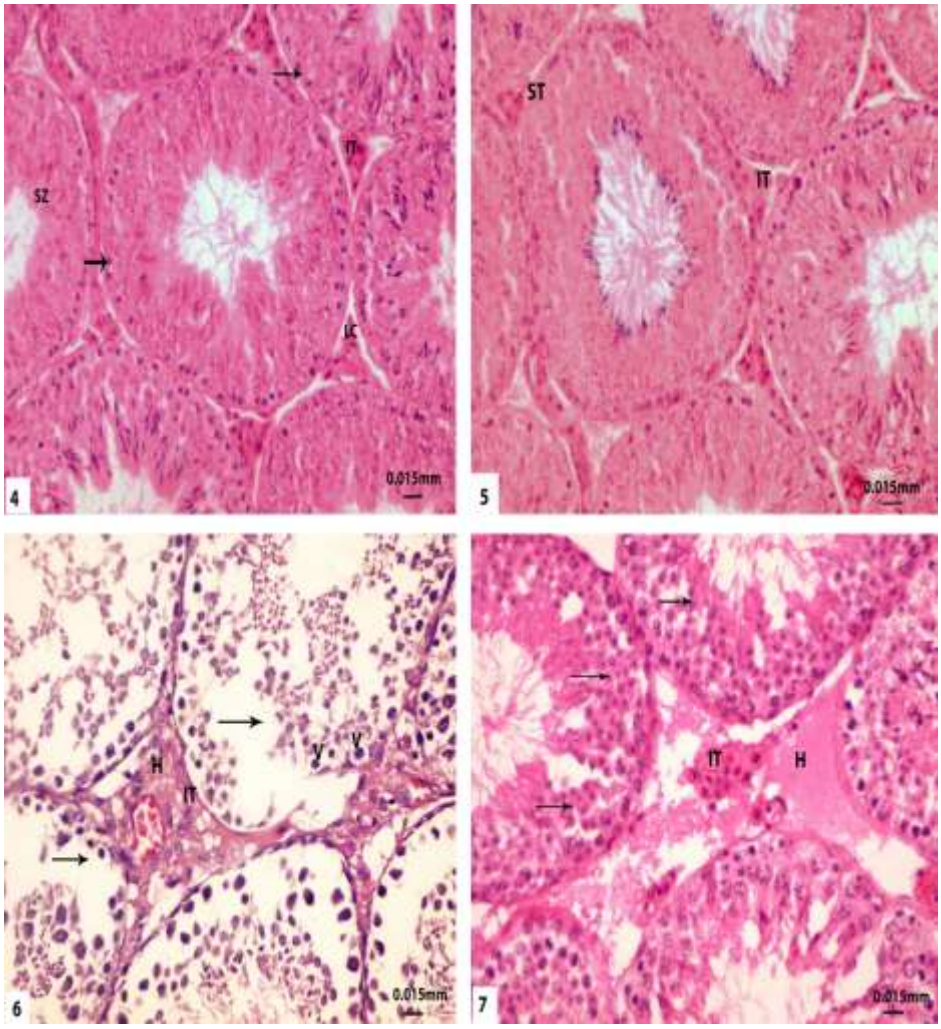

Fig. 4: A photomicrograph of testis of a control animal showing normal seminiferous tubules with different stages of spermatogenic cells, spermatogonia (arrows), round spermatids, spermatozoa (SZ) and interstitial tissue (IT) containing Leydig cell (LC).

Fig. 5: A photomicrograph of testis of animal daily treated with GSE only for 4 weeks showing mostly normal seminiferous tubule (ST) and interstitial tissue (IT).

Fig. 6: A photomicrograph of testis of animal treated with MXT once a week for 4 weeks showing degenerative seminiferous tubules, pyknotic spermatogenic cells with cytoplasmic vacuolization (V), degenerative interstitial tissue (IT) with hemorrhage(H) and sloughing of the germinal epithelium (arrows).

Fig. 7: A photomicrograph of testis of animal treated with MXT only showing degenerative interstitial tissue (IT) with hemorrhage (H), degenerated spermatogenic cells and some mitotic figures (arrows).

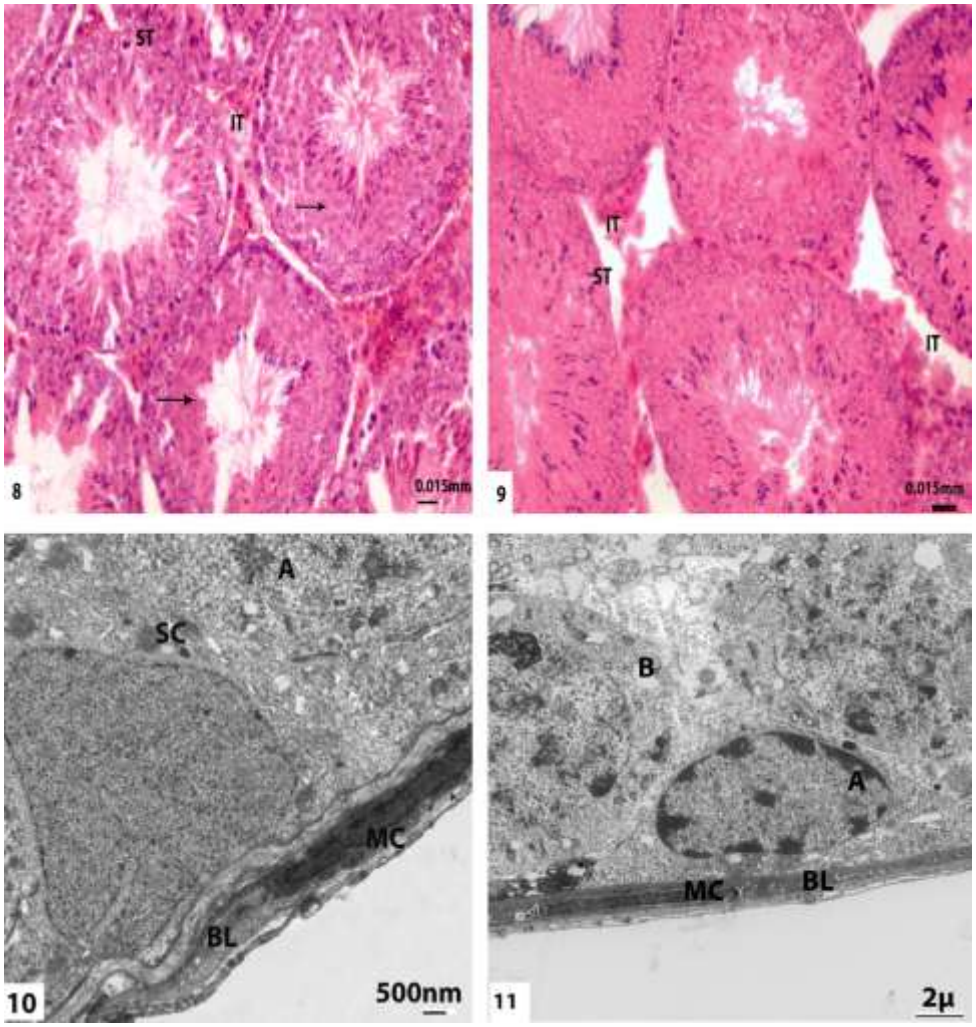

Fig. 8: A photomicrograph of testis of rat treated with MXT followed by GSE daily for 4 weeks showing, a marked improvement, seminiferous tubules (ST) are mostly normal, compact spermatogenic layers with few degenerative germ cells, mostly normal interstitial tissue (IT) and many mitotic figures (arrows).

Fig. 9: A photomicrograph of testis of rat treated with GSE followed by MXT showing obvious protective effect of GSE; seminiferous tubules (ST) mostly close to normal ones and normal interstitial tissue (IT).

Fig. 10: Electron micrograph of portion of seminiferous tubule of control animal showing normal basal lamina (BL), myoid cell (MC), type A spermatogonia (A), and normal Sertoli cell (SC).

Fig. 11: Electron micrograph of portion of seminiferous tubule of control animal showing normal basal lamina (BL), with normal myoid cell (MC), type A spermatogonia (A) and type B spermatogonia (B).

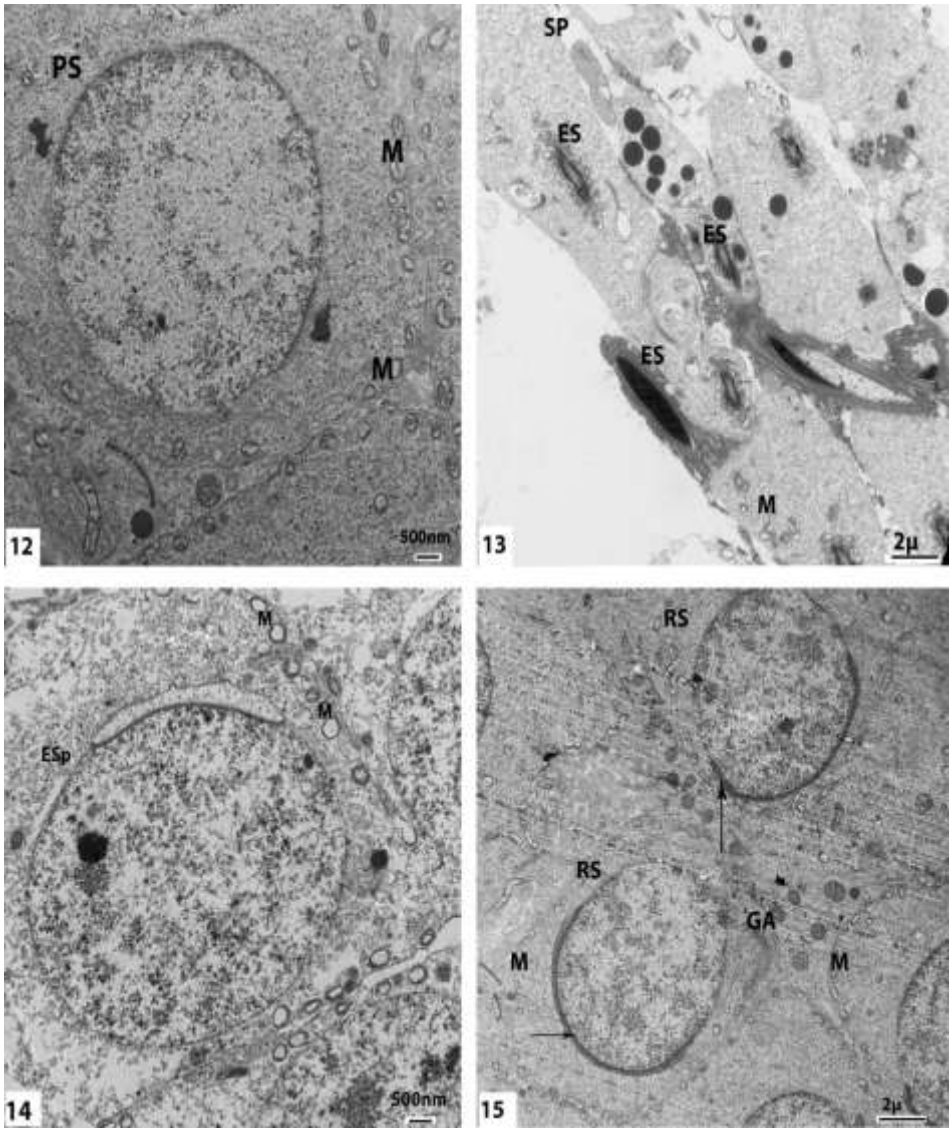

Fig. 12: Electron micrograph of portion of seminiferous tubule of control animal showing normal primary spermatocyte (PS) with peripherally arranged mitochondria (M).

Fig. 13: Electron micrograph of portion of seminiferous tubule of control animal showing normal secondary spermatocyte (SP), homogenous cytoplasm, normal mitochondria (M), and lumen contain elongated spermatids (ES).

Fig. 14: Electron micrograph of portion of seminiferous tubule of control animal showing early spermatid (ESp) and peripheral mitochondria (M).

Fig.15: Electron micrograph of portion of seminiferous tubule of control animal showing round spermatid (RS), normal mitochondria (M), Golgi apparatus(GA), and acrosomal cap (arrows).

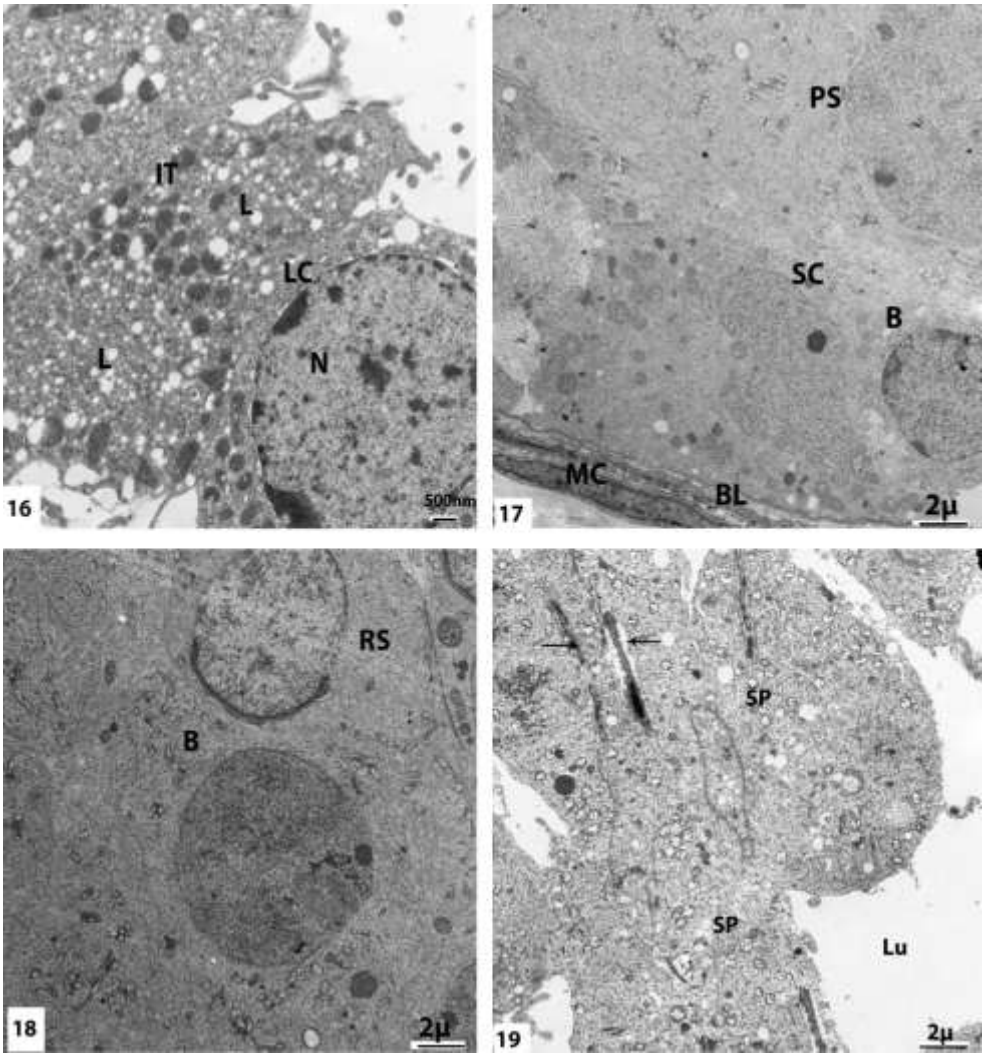

Fig. 16: Electron micrograph of portion of seminiferous tubule of control animal showing normal interstitial tissue (IT), contained Leydig cells (LC) with large nucleus (N), thin rim of chromatin, prominent nucleolus, rough endoplasmic reticulum, mitochondria and rich in lipid droplet (L).

Fig. 17: Electron micrograph of portion of seminiferous tubule of animal treated with GSE only showing normal basal lamina (BL), Sertoli cell (SC), type B spermatogonia (B) and primary spermatocyte (PS).

Fig. 18: Electron micrograph of portion of seminiferous tubule of animal treated with GSE only showing normal type B spermatogonia (B), and round spermatid (RS).

Fig. 19: Electron micrograph of portion of seminiferous tubule of animal treated with GSE only showing lumen of seminiferous tubule (Lu), normal secondary spermatocyte (SP) and elongated spermatids in stage of tail formation (arrows).

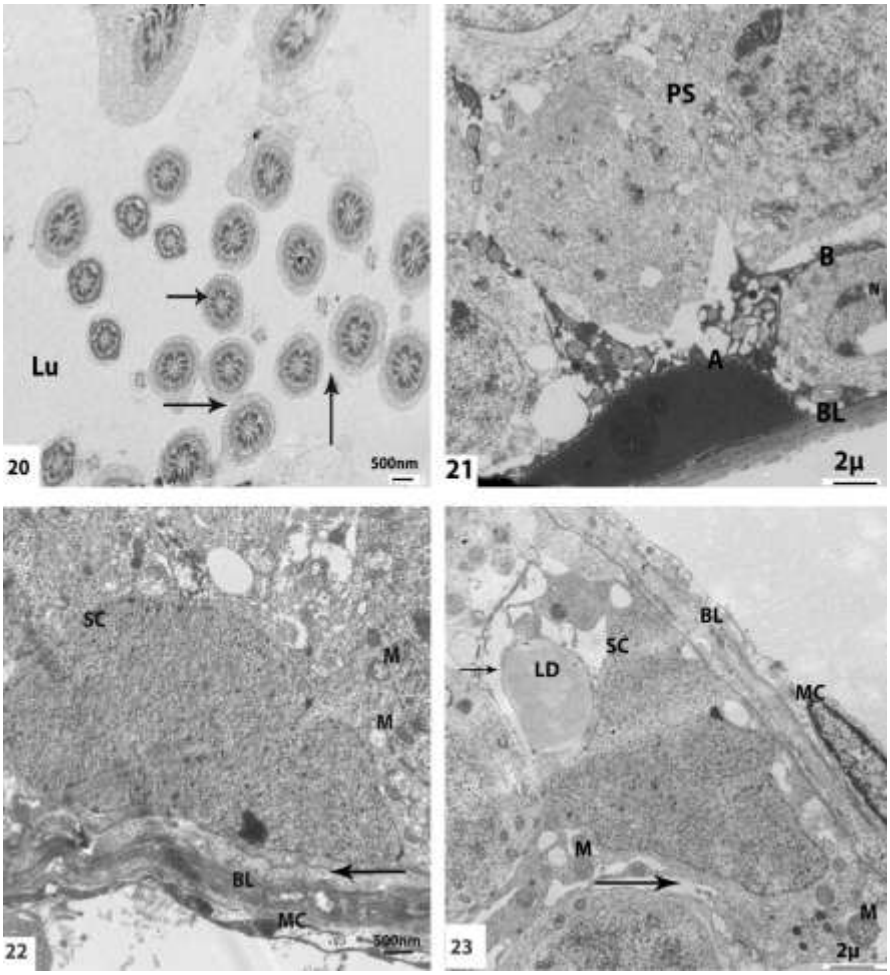

Fig. 20: Electron micrograph of portion of seminiferous tubule of animal treated with GSE only showing lumen (Lu) contains transverse sections of normal sperm (arrows).

Fig. 21: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing basal lamina (BL), distorted type A spermatogonia (A) with massive dark nucleus and nucleoleus, degenerated type B spermatogonia (B) with abnormal nucleus (N) and primary spermatocyte (PS).

Fig. 22: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing thick basal lamina (BL), large amount of collagen, degenerated (arrows), myoid cell (MC), Sertoli cell(SC) with fragmented nucleus degenerated mitochondria (M) and rarified cytoplasm.

Fig. 23: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing rupture basal lamina (BL), large amount of collagen,degenerated myoid cell (MC), Sertoli cell with several lipid droplets(LD) , vesicular mitochondria (M) and broken junction complexes between germ cells (arrows).

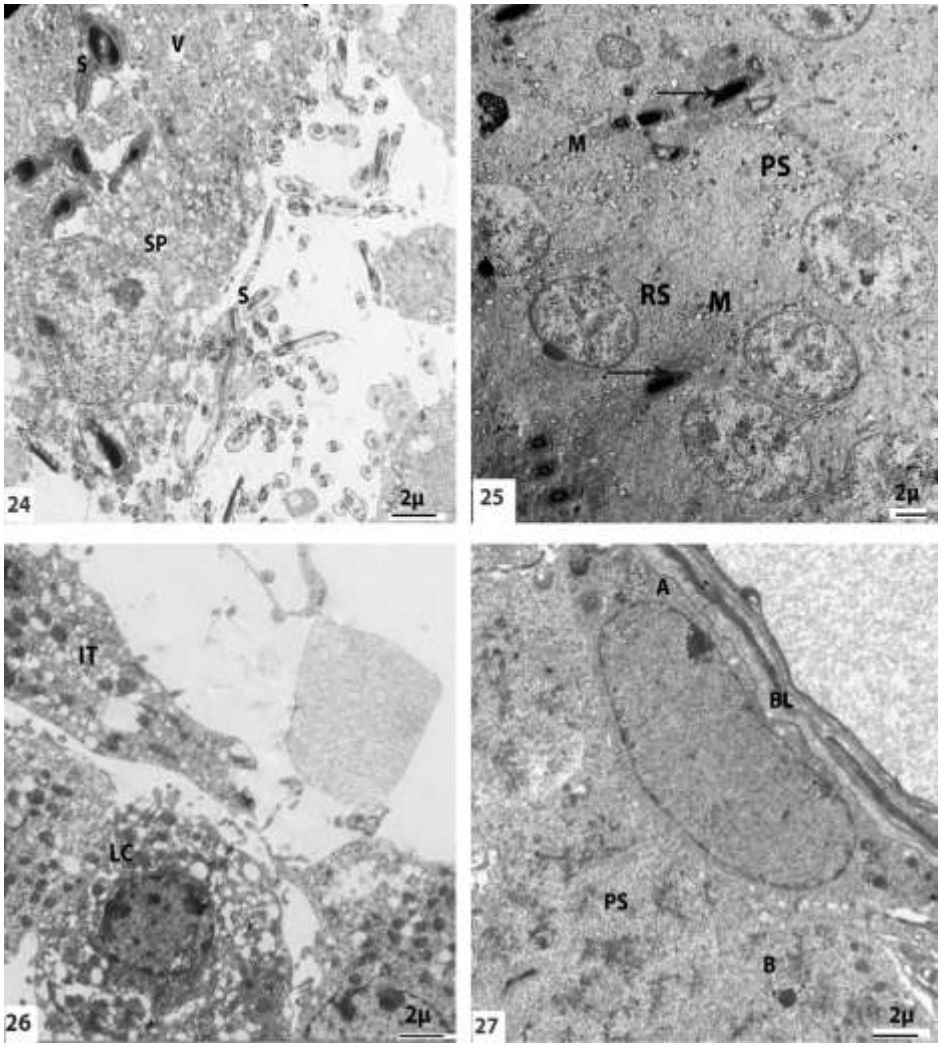

Fig. 24: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing abnormal secondary spermatocyte (SP) with vacuolated cytoplasm, degenerated mitochondria and the lumen contain malformed sperm (S).

Fig. 25: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing abnormal primary spermatocyte (PS) and round spermatid(RS) with vacuolated cytoplasm, degenerated mitochondria (M), abnormal acrosomal cap and disorganization of spermatid (arrows).

Fig. 26: Electron micrograph of portion of seminiferous tubule of animal treated with MXT only showing abnormal interstitial tissue (IT) with degenerated Leydig cell (LC).

Fig. 27: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal basal lamina (BL), type A spermatogonia (A), Type B spermatogonia (B) and primary spermatocyte (PS).

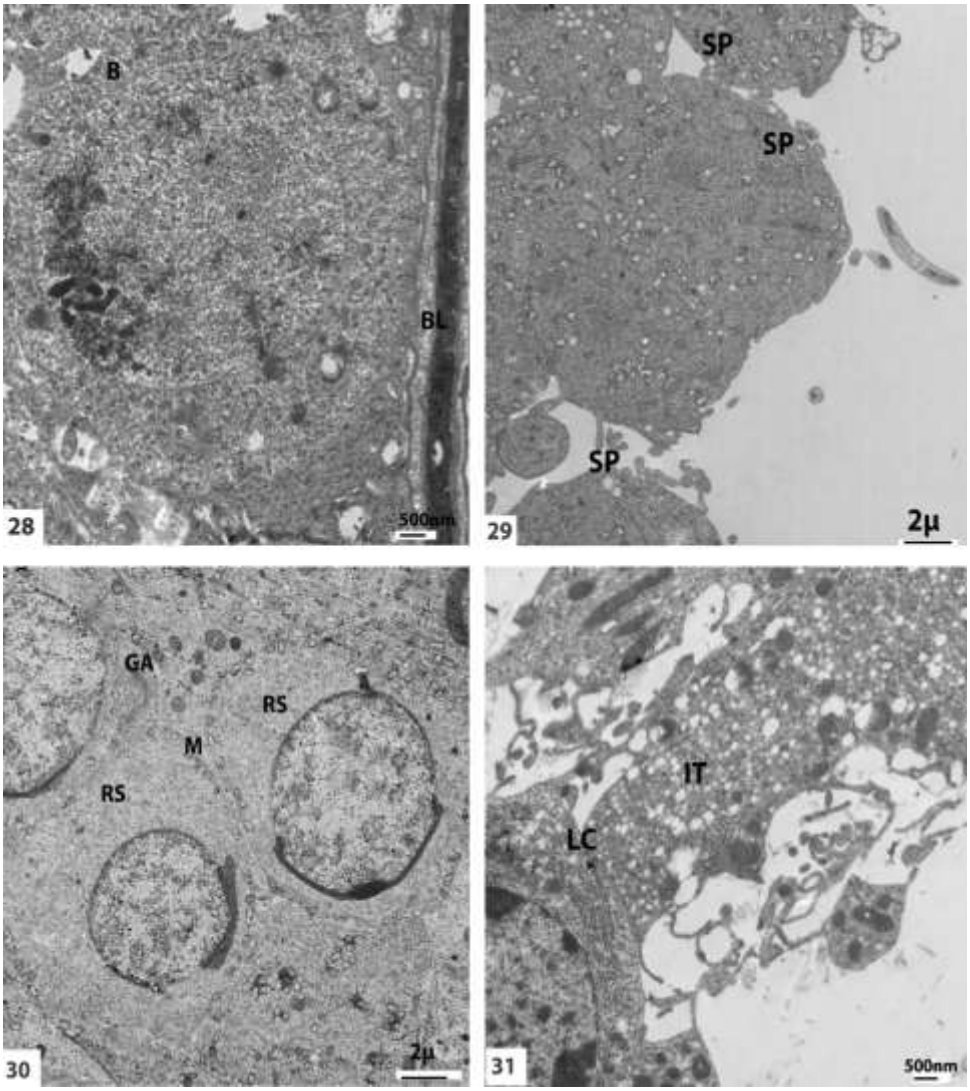

Fig. 28: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal basal lamina (BL) and type B spermatogonia (B).

Fig. 29: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal secondary spermatocytes (SP).

Fig. 30: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal round spermatid (RS), with homogenous cytoplasm, normal mitochondria (M) and Gologi appeatus (GA).

Fig. 31: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal interstitial tissue (IT) with normal Leydig cell (LC).

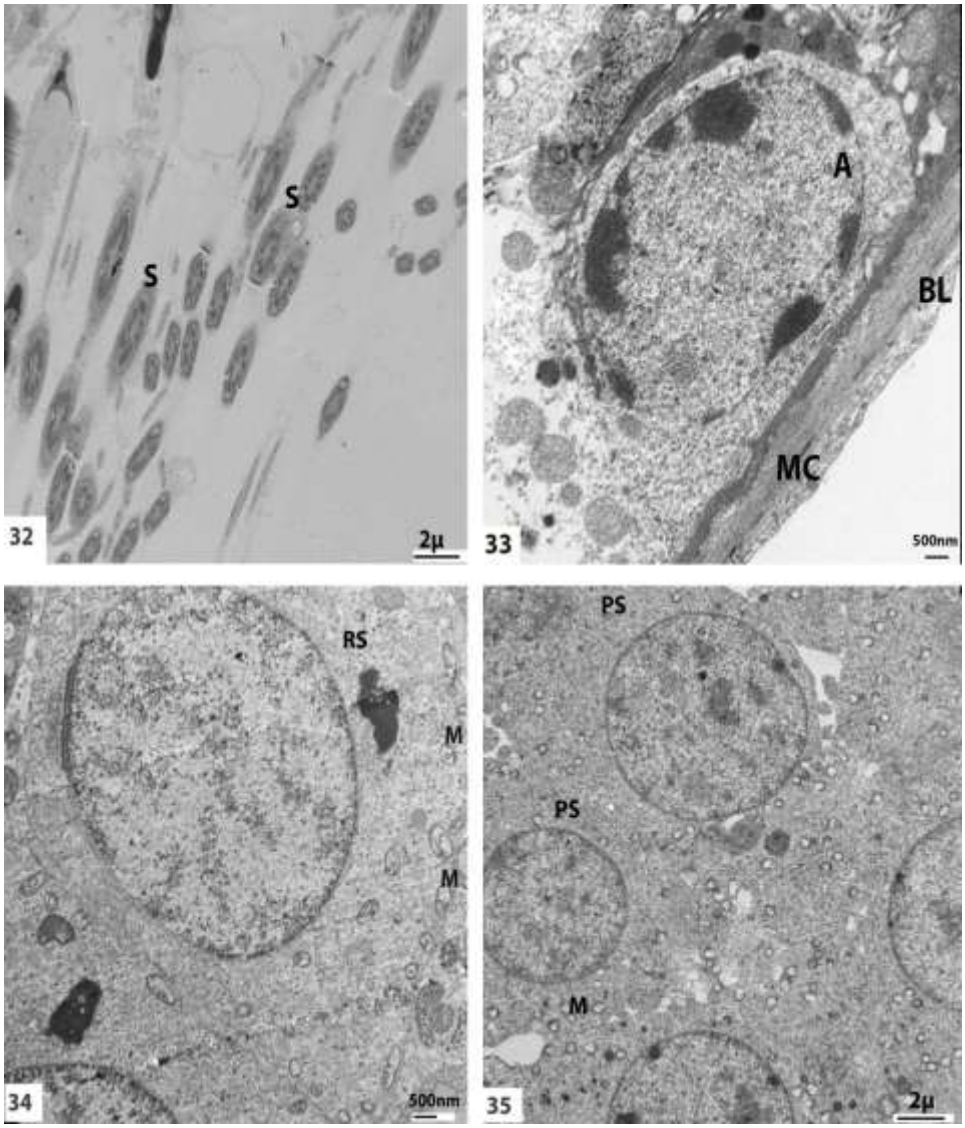

Fig. 32: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing lumen contains transverse sections of mostly normal sperms (S).

Fig. 33: Electron micrograph of portion of seminiferous tubule of animal treated with MXT followed by GSE showing mostly normal basal lamina (BL), with myoid cell (MC) and type A spermatogonia (A).

Fig. 34: Electron micrograph of portion of seminiferous tubule of animal treated with GSE followed by MXT showing closely to normal round spermatid (RS) with peripheral mitochondria (M).

Fig. 35: Electron micrograph of portion of seminiferous tubule of animal treated with GSE followed by MXT showing primary spermatocyte (PS) with normal nucleus and few degenerated mitochondria (M).

Biochemical analysis

Change in serum testosterone level

Data in figure (36) showed changes of serum testosterone level in the different experimental groups. There was insignificant change in the level of testosterone in animals treated with grape seed extract only (2.750±0.215) when compared with control group (2.460±0.124). On the other hand, treating animals with MXT showed a significant decrease (0.412±0.001) in testosterone level when compared with control group. When animals treated with grape seed extract (either before or after MXT treatment) restoration in the testosterone level to normality was recorded; (2.450±0.102) and (2.59±0.17) respectively.

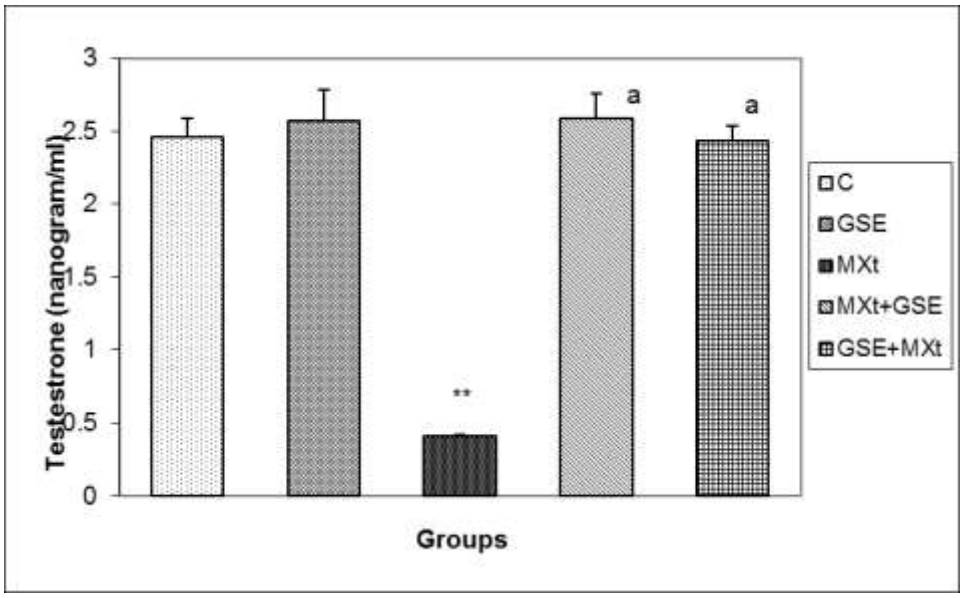

Fig. 36: Effect of the different treatments on serum testosterone level.

Change in serum malondialdehyde (MDA)

Figure (37) showed insignificant difference between MDA level of control animals and animals treated with grape seed extract only; (82.290±2.580) and (85.228±2.470) respectively. Moreover, significant increase in MDA level was recorded in animals treated with MTX (124.580±11.140) when compared with control group. When animals treated with GSE either before or after MXT treatment, significant decrease in MDA level (restored to normal level) was estimated when compared with animals treated with MTX only; (84.920±2.160), (87.760±2.360) and (124.580±11.140) respectively.

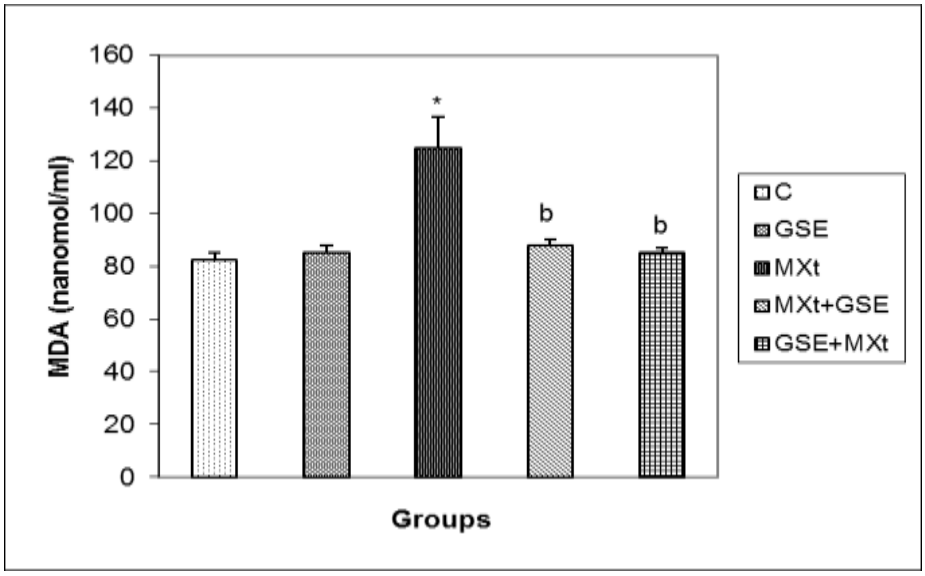

Fig. 37: Effect of the different treatments on serum MDA level.

Change in serum superoxide dismutase (SOD) activity

Data in figure (38) showed insignificant difference in serum SOD activity in animals treated with gervital only (21.550±0.737) when compared with control group (19.870±0.509). In spite of MTX administration, highly significant decrease in SOD activity (11.420±0.927) was recorded when compared with control group (19.870±0.509). There was significant increase in SOD activity in sera of animals treated with grape seed extract either before or after MTX; (20.200 ±0.562) or (20.300±0.480), when compared with MTX group (11.420±0.927).

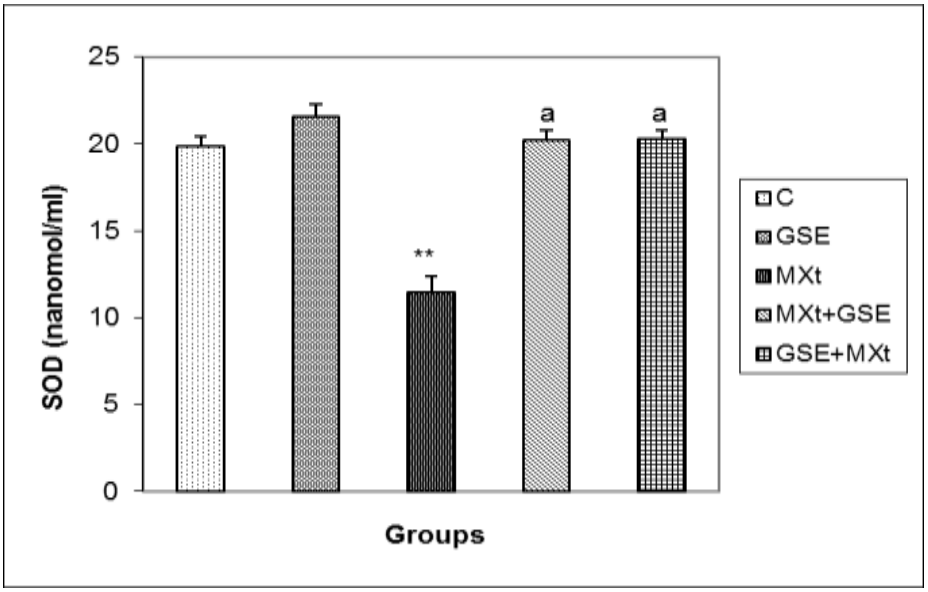

Fig. 38: Effect of the different treatments on serum SOD activity.

DISCUSSION

Methotrexate is an antineoplastic immunosuppressive drug. Although its wide use as anticancer drug, it causes drastic side effects in different body organs especially reproductive system. Shamberger *et al.* (1981) confirmed reversible sterility in men using MTX.

MTX is one of the most important widely used antimetabolic drugs that inhibit the enzyme dihydrofolic acid reductase which catalysis the conversion of folic acid into active form folinic acid by binding to it (Duran *et al.,* 2001). There are many recent studies try to found a way to reduce or prevent the MXT toxicity. There have been several intensive studies carried out using a great number of anti-oxidants trying to find a way to reduce or prevent MTX toxicity (Iraz *et al.*, 2006; Gokce *et al.*, 2011).

In the present study, there was a significant difference in both body weight and total weight of both testes in animals treated with MXT when compared with control group. Whereas GSE treatment resulted in a significant increase in both body and testes weight (reversed the effect of MXT). This agreed with the results that observed by Akinlolu *et al.* (2014) and Yuncu *et al.* (2015) who reported that MTX induced body and testes weight loss in rats injected intraperitoneally with MTX (20 mg/kg body weight). On the other hand, Hajizadeh *et al.* (2016) and Razmaraii *et al.* (2016) found that rats treated with grape seed extract after treatment with doxorubicin or fluoxetine significantly increased body weight. Khattab *et al.* (2010) who stated that oral administration of grape seed extract only has no effect on body weight and the weight of sex organs in rats. This confirmed that GSE is a widly dietary supplement and consider safe for human consumption (Cliflon, 2004).

Animals treated with methotrexate (8mg/kg body weight) once a week for 4 weeks revealed intensive pathological changes including severe degenerated seminiferous tubules, sloughing of germinal epithelium, cytoplasmic vacuolization and pyknotic spermatogenic cells nuclei. Moreover, interatubular haemorrhage, leucocytic infiltration, degenerated Sertoli cells and decrease of sperm number in the lumen of seminiferous tubules were observed. Similarly, previous studies have demonstrated that administration with different doses of MXT caused harmful effects on rat testes leading to infertility, atrophy of seminiferous tubules and sloughing of germ cells away from basal lamina (Eid *et al.,* 2002; Nouri *et al.*, 2009; Daggulli *et al.,* 2014; Patel *et al.,* 2014). Histopathological lesions of testes after MXT treatment were in accordance with Arash *et al.* (2007) and Sheikhbahaei *et al.* (2016) who found that MXT

caused a destructive effect on testicular germinal cells, apoptosis and decrease in sperm number.

In the present study, when animals pretreated with grape seed extract (150mg/kg body weight) for 28 days before MXT administration showed nearly normal structure of testes. Moreover, post-treatment with GSE revealed that the pathological lesions was not as great as MXT group. The testicular tissues appeared more similar to control one and restored nearly all spermatogenic layers, except detachment of few spermatogonia from the basement membrane in few seminiferous tubules. Animals treated with grape seeds extract (daily for 28 days) after MXT showed marked degree of improvement. Similarly, Cavusoglu *et al.* (2014) reported that GSE has stronge protective effect in rats at a dose level of 150mg/kg body weight. In addition, Hajizadeh *et al.* (2016) revealed that GSE promising protection and greatly improved the pathological lesions of testicular tissues (had normal featuring of seminiferous tubules) of fluoxetine treated mice.

It was emphasized that the testicular toxicity of MXT is associated with its ability to increase reactive oxygen species. There are various hypotheses that oxidative stress of MXT plays an important role in pathogenesis of it to induce testicular damage (Miketova *et al.*, 2005; Uzar *et al.*, 2006; Armagon *et al.*, 2008).

Concerning ultrastructure alternations observed in the present study, several drastic changes were observed in all germinal cells. Ultrastructure examination of testes of animals treated with MXT revealed severe changes in spermatogonia, spermatids, Sertoli cells and Leydig cells. Ultrastructure findings that observed in MXT group include; dilated rough endoplasmic reticulum, degenerated mitochondria, large number of lipid droplet (especially in Leydig cells) and some lytic regions in some seminiferous tubules. Similar ultrastructure results were observed by Yuncu *et al.* (2015) who found thickening and broken in basal lamina of seminiferous tubules, increase in spaces between Sertoli cell and its surrounding spermatogonia and other cellular organelles degeneration (rough endoplasmic reticulum, mitochondria and lysosomes) after MXT treatment.

Animals treated with GSE before or after MXT induced toxicity showed mostly normal ultrastructure features of testicular tissues. On the other hand, administration of GSE after MXT toxicity showed an obvious improvement in ultrastructure alternation when compared

with MXT group. Similarly, Karlen *et al.* (2014) showed that grape seed extract possess cytoprotective ultrastructure and antitoxic effects in hepatocytes during CCL_4 intoxication. Concerning the mechanism of MXT, Oktar *et al.* (2010) suggested that MXT increased the oxidative stress through the release of macrophages and neutrophils and finally causes elevation in the production of free radicals in tastes. Moreover, Jahovic *et al.* (2003) and Miyazono *et al.(* 2004) found that methotrexate induced toxicity (on testes and other organs) through its oxidative stress by producing free radicals that impaired the function of mitochondria resulted in enhanced lipid peroxidation, increase molondialdehyde (MDA) and decrease glutathione level in the blood. In this concept, Tripathi (2003) and Akinlolu *et al.* (2014) suggested that MXT inhibits synthesis of thymidylal, serine, and methionine which disrupt synthesis of DNA, RNA and protein leading to cell death. Therefor the oxidative stress developed as a result of an imbalance between reactive oxygen radicals system. Excessive amounts of reactive oxygen radicals induced production of abnormal sperms and fertility (Yulug *et al.*, 2013). Moreover, Tian and Cronstein (2007) reported that MXT restrict the synthesis of purines and pyrimidines necessary for DNA, RNA & ATP formation. Therefore, antioxidants may inhibit MXT toxicity and protect the testes tissues against its oxidative stress.

Statistical evolution of serum testosterone level in animals treated with MXT only showed a significant decrease when compared with control group. On the contrary, insignificant difference in testosterone level was observed in animals treated with grape seed extract before treatment with MXT (comparing with animals in both control and MXT groups). In this concern, Guyton and Hall (2011) revealed the same result in rats treated with MXT (20mg/kg body weight). Moreover, Akinlolu *et al.* (2014) contributed the effect of MXT on serum testosterone level to its enzymatic effect and impaired status of the hypothalmo-pituitary gonadal axis which regulated the synthesis and release of gonodoproteins and testosterone hormones.

On the other hand, treatment with grape seed extract after MXT induced testicular toxicity restored the activity of serum testosterone level to normal. Testosterone is a key hormone that regulates spermatogenesis. Similarly Khattab *et al.* (2010) reported that GSE was capable of restoring the activity of serum testosterone to normal level. This means that GSE increases the process of steroidogensis and hence testosterone production that improved sperm production and fertility process.

A significant increase in the level of oxidative parameter MDA and significant decrease in antioxidant enzyme (SOD) activity in serum were observed in animals treated with MXT. Similarly, Ahmed *et al.* (2015) and Yuncu *et al.* (2015) reported a significant increase in the level of MDA and decrease in activity of SOD in rats after treatment with MXT. If oxidative damage is involved in the beginning of pathology of the disease, a successful antioxidant therapy may prevent or delay the disease occurring.

Serum MDA level and SOD activity were returned mostly normal after treatment with grape seed extract. This agreed with Abd Elkader *et al.* (2011) who confirmed that GSE have a protective effect against lipid peroxidation as well as ameliorate the inhibition of SOD activity. Moreover, Orhan *et al.* (2007) and Rekha *et al.* (2013) demonstrated that oral administration of GSE exhibited a significant protective effect by lowering lipid peroxidation level with significant increase in SOD activity in rats.

All the above result depending on the scavenging activity of GSE that compete the free radicals-induced damage (result from MXT toxicity). Similarly, Shin and Moon (2010) indicated that GSE has antioxidant properties due to the presence of flavonoids, polyphenols, anthocyanin, pro-anthocyanidins, procyonidins and resveratrol. Several studies suggested that GSE has the ability to increase intracellular vitamin C level (free radical scavenge) and decrease capillary permeability and scavenge oxidant and free radicals (Li *et al*., 2001; Maier *et al.,* 2009). There was another mechanism of GSE via the induction of endogenous antioxidant enzymes. Enzyme system is responsible for scavenging free radicals which an important mechanism concerning the protective effect of GSE against many disorders.

CONCLUSION

The present study concluded that GSE (gervital) had an ameliorative effect against MXT induced testicular damage that confirmed through histological, ultrastructure and biochemical studies. The ameliorative effect of GSE may be attributed to its antioxidant potential against free radicals.

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