LIGHT AND ELECTRON MICROSCOPIC EVALUATION OF TESTICULAR TISSUE AFTER EXPOSURE TO METHOTREXATE

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Abstract

This study was designed to evaluate the toxic effects of chronic exposure to methotrexate on testicular structure in adult male albino rat judged by light and electron microscope (EM). Group I: served as control group (n = 5) and Group II: injected subcutaneously by methotrexate in a dose of 12.5 microgram /kg body weight (n = 10), which is equally divided into two subgroups; IIa (that were daily for one week) (5rats) and IIb (that were daily for two week) (5 rats). At the end of experimental period, all of the rats were scarified and both testes were prepared for histological and ultrastructural study. In methotrexate one-week treated animals, there were several pathological changes include degenerated seminiferous tubules and distortion in spermatogenic cells. In animals treated with methotrexate for two-weeks, seminiferous tubules showed disorganized spermatogenic cells with reduced number of spermatids. EM evaluation of methotrexate -induced changes revealed significant thickening in basal lamina, structural changes in mitochondria, endoplasmic reticulum and cytoplasm of Sertoli cell, decrease in number of microtubules of centriole in sperms and loss of the tight junctions due to widening of intracellular space between spermatogenic cells. It could be concluded that exposure to anticancer drugs has a deleterious effect on testicular function as regards spermatogenesis with proved ultrastructural changes.

Keywords: Methotrexate, Testes, Ultrastructure, Anticancer, histopathology.

Introduction

Advance in chemotherapy and radiotherapy have had a profound effect on the outcome of certain cancers. Effects of antineoplastic therapy on hematological, renal, hepatic, and gastrointestinal systems are common and thoroughly chronicled. Adverse effects of cancer therapy on the endocrine system are not less important but, except for gonadotoxic effects; they have not been thoroughly organized (1).

The use of chemotherapeutics has known to cause toxic effects in multiorgan systems (2). Methotrexate (MTX), formerly known as amethopterin is a folic acid antagonist agent used for chemotherapeutic purposes in malign tumors (acute lymphoblastic leukemia, non-Hodgkin’s and

lymphoma, breast cancer, malignancies of the head and neck, among others) and nonneoplastic diseases (particularly rheumatoid arthritis). Past investigations have reported damage in the seminiferous tubules of the testis, a decrease in sperm numbers, and sperm DNA damage following administration of MTX (3, 4).

Spermatogensis occurs in a continuous cycle of division (mitosis, meiosis, differentiation, and maturation) resulting in the production of approximately 2 million sperm per day from puberty to old age. The high rate of cell division makes these germ cells particularly sensitive to cytotoxic agents, in contrast to the Leydig or Sertoli cells. Among the germ cells, spermatogonia were the most sensitive to cytotoxic effects, followed in order by stem cells, maturing spermatocytes, spermatids, and spermatozoa (4, 5). Cytotoxic chemotherapeutic agents are common causes of gonadal dysfunction, as either infertility or hypogonadism, in cancer survivors. There are several risk factors for development of gonadal toxicity after treatment with cytotoxic drugs including the specific chemotherapeutic combination, the dose intensity, and the age and sex of the patient (6).

The seminiferous epithelium within a tubule contains several types of cells, Sertoli cells are supportive cells each with a large, pale nucleus containing a large nucleolus. The Sertoli nucleus has a rounded, triangular or an oval shape with deep infoldings that can be seen in EM. The cytoplasm of a Sertoli cell extends from the basal lamina to the tubular lumen. The spermatogonia are in contact with the basal lamina in the basal compartment. These germ cells are cuboidal in shape with either a slightly flattened or a spherical nucleus and are adjacent to the basement membrane (7). Methotrexate effect on the diameter of seminiferous tubule followed by increasing of interstitial space. Also the size of various stages of primary, secondary spermatocytes, and spermatids were reduced. Methotrexate induced cytotoxicity on the proliferation of cellular contents of seminiferous tubules elucidating the mechanism of dose-dependent drug induced testicular damage during spermatogenesis (8, 9).

The present study was designed to evaluate the toxic effects imposed on the testicular structure by low-dose chronic exposure to methotrexate judged by light and electron microscopy.

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 injected subcutaneously by methotrexate in a dose of 12.5 microgram/kg body weight daily for one week and two weeks (10).

Animals and experimental design

The study was performed on 15 adult male albino rats (*Rattus norvegicus*) weighing from 80-100 gm. Rats were purchased from the laboratories of ministry of agriculture, and kept under standard conditions, temperature 20 ^{o}C, humidity 60% and 12-hr day/night cycle, and maintained on standard diet and free water supply till the start of study regimens. The animals were divided into 2 groups each in a separate cage: Group I: represented the experimental control group and did not receive additional treatment other than standard care and housing

(n = 5) and Group II: injected subcutaneously by methotrexate in a dose of 12.5 microgram /kg body weight (n = 10), which is equally divided into two subgroups; IIa (that were daily for one week) (5rats) and IIb (that were daily for two week) (5 rats). At 24h after the last injection , the rats were sacrificed by using chloroform inhalation and both testes were prepared for histological & ultrastructure examination.

Histological studies

After fixation in 10% neutral formalin, testes were processed to prepare 5μm paraffin sections. Finally, sections were stained with haematoxylin and eosin (11). Microscopic examination of the stained sections was then carried out by Olympus Light Microscope to determine possible cytoarchitectural changes.

Ultrastructure studies

For electron microscopic analysis, two control and 4 methotrexate –treated rats (2 from each group) were perfused through the heart by saline to obtain clear circulatory system then fixed with 2.5% glutaraldehyde in 0.1 m cacodylate buffer, then postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, then embedded in epon araldite (12). One micrometer semithin sections were prepared by LEICA Ultra microtome and were mounted on glass slide 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 100SX (JEOL, Japan) transmission electron microscope.

Results

Light microscopic examination of the specimens harvested from control animals showed normal spermatogenic 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. Some of spermatogonia A population differentiate to become type B cells. The other part of that daughter cells persists as stem cells. Furthermore, type B spermatogonia undergo mitosis to from large ovoid cells known as primary spermatocytes. The lumen was full of many sperms (Fig.1A & 2A). In methotrexate one-week treated animals, there was hyaline material containing necrotic debris and degenerated cells with dark nuclei in the lumen of the seminiferous tubules. In addition to disorganization and exfoliation of some spermatogenic cells into the lumen of the tubules (Fig.1B & 2B). In animals treated with methotrexate for 2-weeks, showed several pathological features. The most prominent pathological alternations were distortion of seminiferous tubules and degeneration of Sertoli cells. Most of seminiferous tubules detached from the surrounding interstitial tissue and large empty spaces between the spermatogenic cells. Few numbers of spermatogonia were detached from the basal lamina. In addition, basal insertion of sperms, vacuolated spermatocyte and absence of different stages of spermatogenic cells. There were decreases in number of sperms within the lumen of seminiferous tubules. (Fig.1C &2C).

Ultrastructure examination of control rat testis showed large and oval nucleus of Sertoli cell with prominent nucleolus. In addition, their cytoplasm contains abundant smooth endoplasmic reticulum, ovoid golgi apparatus and numerous mitochondria with spherical or cylinder shape. Primary spermatocytes contain large spherical nucleus with faint granular chromatin and spherical mitochondria that aggregates peripherally (Fig.3A &3B). 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.3C &3D).There were two types of spermatogonia; type A and type B. Type A spermatogonia characterized by large pale rounded nuclei containing fine light and non-condensed chromatin, scantly, homogenous and granular cytoplasm with poor rough endoplasmic reticulum. The mitochondria are abundant with spherical or ovoid shaped. Type B spermatogonia were slightly smaller and contain rounded nuclei with more electron dense nucleoplasmic matrix than type A and numerous chromatin clumps. The cytoplasmic organelles were similar to those described in the type A (Fig. 4A). Moreover, the lumen contained many transverse sections of normal sperm (Fig. 4B).

EM evaluation of methotrexate one-week treated animals showed Sertoli cell with indented nucleus and mild degeneration of mitochondria (Fig. 5A). Type B spermatogonia has pyknotic nucleus, dilated RER and swollen degenerated mitochondria. Vacuolated spermatogonia type A with interrupted nuclear membrane (Fig. 5B). Moreover, there was degeneration of early spermatid with rarified cytoplasm (Fig. 5C). Degenerated and vacuolated cells in the lumen were seen (Fig. 6A). Figure (6B) showed distortion and malformation of sperms in the lumen of the seminiferous tubules. In animals treated with methotrexate for 2-weeks, showed basement membrane that appeared thicker than normal, Sertoli cell having indented nucleus and disappearance of tight junction due to widening of intracellular space between sertoli cell and primary spermatocyte (Fig. 7A). Type B Spermatogonia showed dilated in perinuclear membrane and swollen degenerated mitochondria. While spermatogonia type (A) was destroyed with rarified cytoplasm (Fig. 7B). Distortion and vacuolation of early spermatid were seen (Fig 8A& 8B). Figure (8C) showed abnormal sperms with decrease in number of microtubules of centriole.

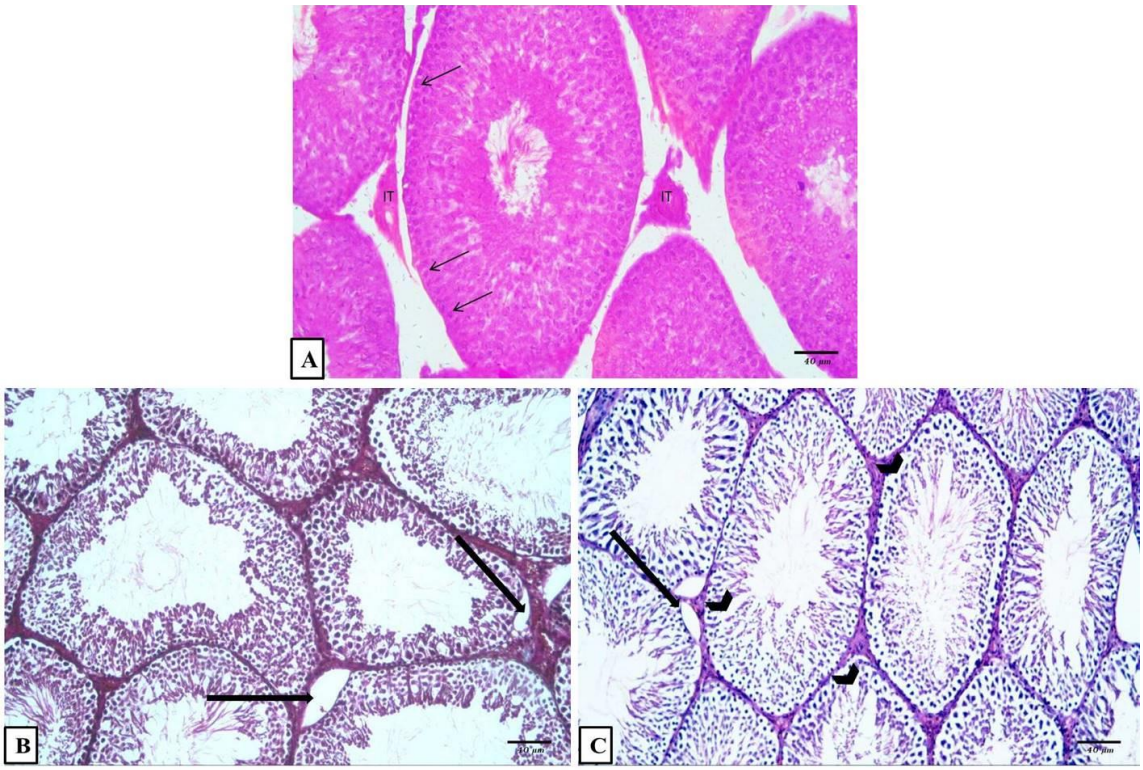

Fig (1): A photomicrograph of testis showing: **A)** Normal appearance of seminiferous tubules lined with different stages of spermatogenic cells, spermatogonia (arrow) and rounded spermatid. Interstitial tissue (IT) can be seen inbetween the seminiferous tubules (group I). **B)** Marked detachment of the spermatogenic epithelium from one side of the tubule (arrow) (group IIa). **C)** Variable irregular shaped tubules that detached from the surrounding interstitial tissue (arrow), disorganized spermatogenic cells and large empty spaces between the spermatogemic cells (arrow head) (group IIb). (H&E, x250).

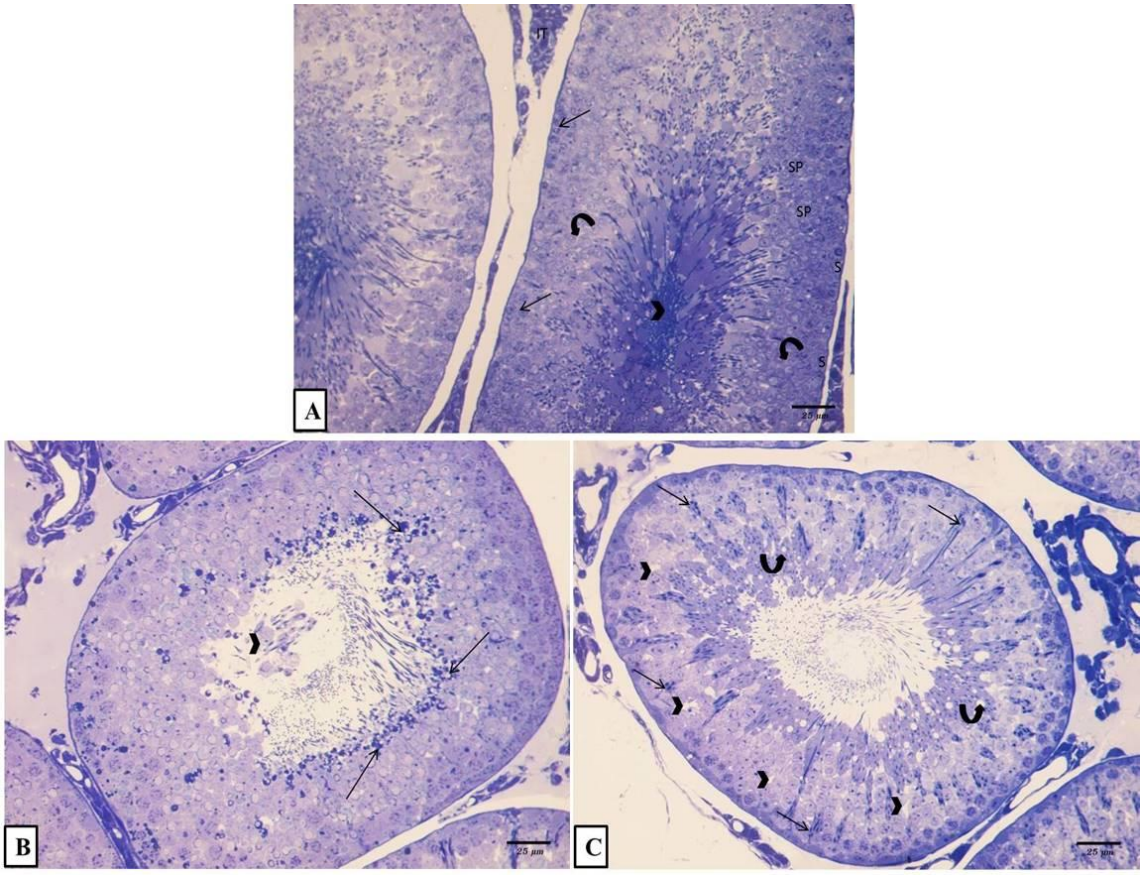

Fig (2): A photomicrograph of testis showing: **A)** Normal spermatogenic cell layers; spermatogonia (arrow), primary spermatocytes (curved arrow), early spermatids (SP), late spermatids with condensed nuclei and sperms (arrow head). Sertoli cells (S) present between the germ cells. Notice leydig cells with normal blood vessels can be seen in the interstitial tissue(IT) (group I). **B)** Hyaline material containg necrotic debris and degenerated cells with dark nuclei (arrow) in the lumen of the tubule, some spermatogenic cells disorganized and exfoliated into the lumen of the tubules (arrow head) (group IIa). **C)** Basal insertion of sperms (arrow), vacuolated degenerated spermatocyte (curved arrow) and wide intercellular spaces between spermatogenic cells (arrow head) (group IIb). (Toluidine blue x 400)

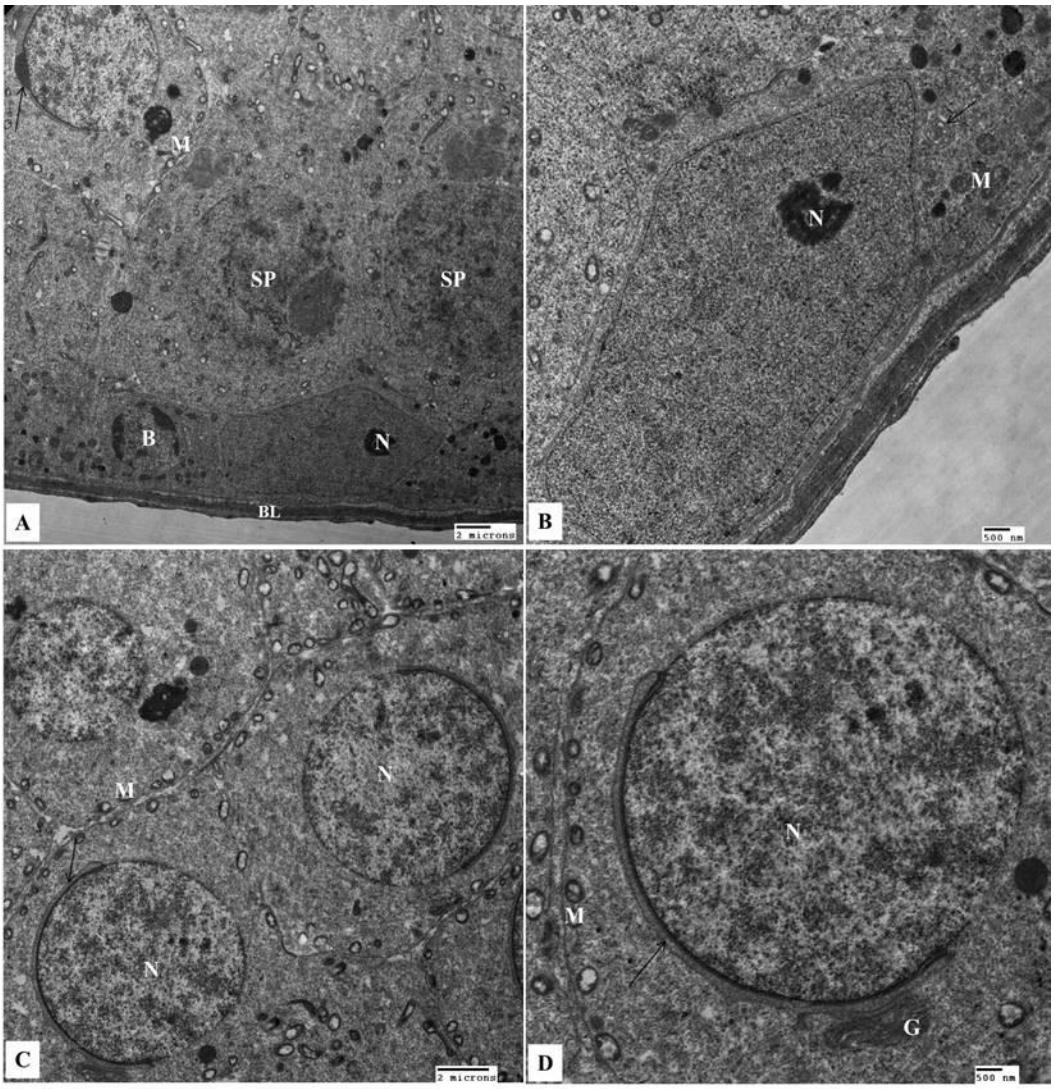

Fig (3): An electron micrograph of testis of Group I showing: **A)** The basal part of normal sertoli cell containing a large nucleus with a prominent nucleolus (N), spermatogonium type B (B) resting on the basal lamina (BL) with rounded nucleus, normal spermatocyte with rounded nucleus (sp) and normal early round spermatid with rounded nucleus, acrosomal cap (arrow) and peripherally located mitochondria (M). **B)** The basal part of normal sertoli cell containing a large nucleus with a prominent nucleolus (N), cytoplasm, mitochondria (M) and Golgi apparatus (arrow) are most probably normal. **C)** Normal early round spermatid with rounded nucleus (N), acrosomal cap (arrow) and peripherally located mitochondria (M). **D)** The higher magnification of normal early round spermatid with rounded nucleus (N), acrosomal cap (arrow) and peripherally located mitochondria (M). notice normal appearance of Golgi apparatus (G).

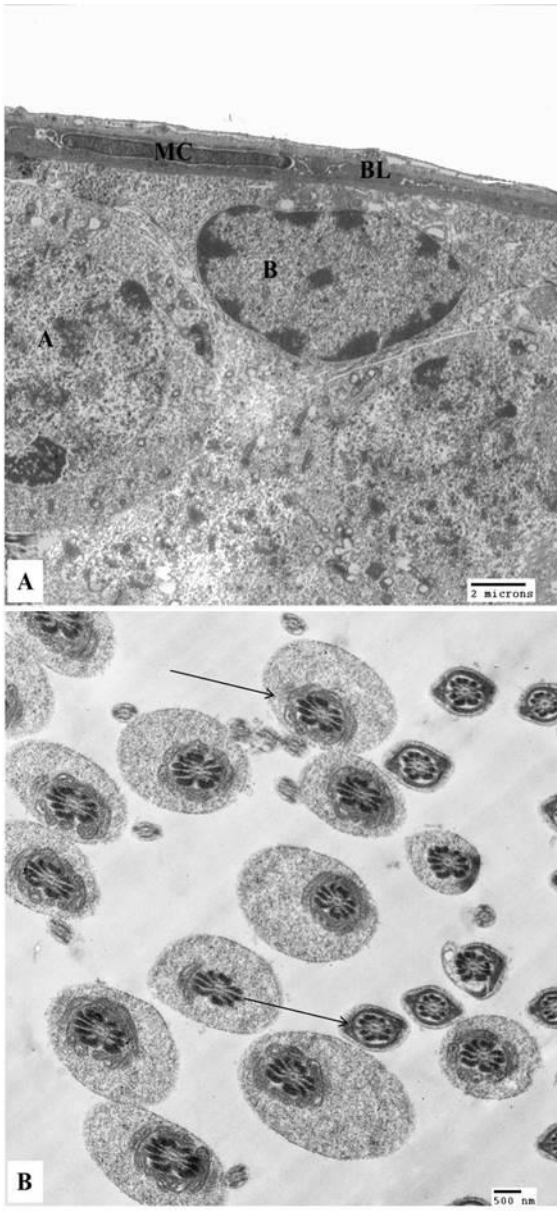

Fig (4): An electron micrograph of testis of Group I showing: **A)** Normal basal lamina (BL) with normal myoid cell (MC), normal appearance for spermatogonia type A (A) and type B (B). **B)** Transverse section of of normal sperms (arrow) in the lumen.

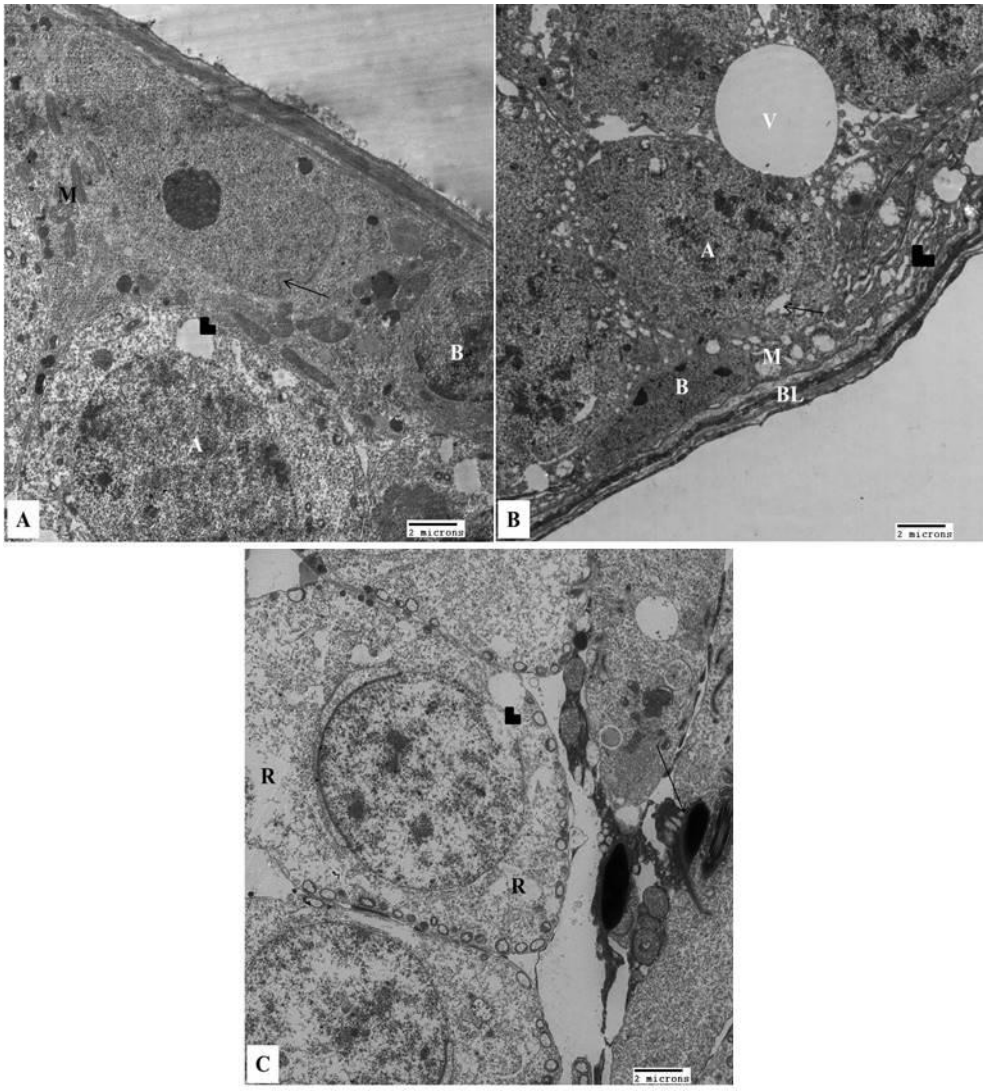

Fig (5): An electron micrograph of testis of Group IIa showing: **A)** Sertoli cell with indented nucleus (arrow) and mild degenerated mitochondria (M). normal spermatogonium type B (B) with rounded nucleus, while spermatogonium type A (A) with slightly rarified cytoplasm (arrow head) and disrupted mitochondria. **B)** Irregular basal lamina (BL) , spermatogonia type B (B) with pynotic nucleus, dilated RER (arrow head) and swollen degenerated mitochondria (M). Spermatogonia type A (A) with interrupted nuclear membrane (arrow). Notice large vacuole (V) between . Spermatogonia type A and primary spermatocye. **C)** Accumulated heads (arrow) of sperm inbetween degenerated early spermatid with rarified cytoplasm (R) and interrupted nuclear membrane (arrow head).

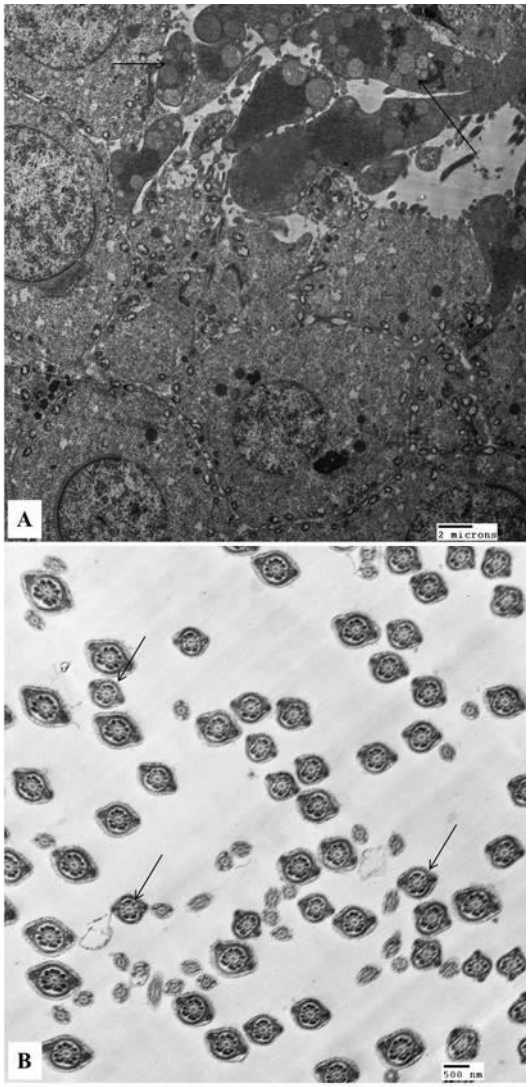

Fig (6): An electron micrograph of testis of Group IIa showing: **A)** Degenerated cells (arrow) in the lumen. Notice presence of normal spermatid. **B)** Transverse section of abnormal sperms (arrow) in the lumen.

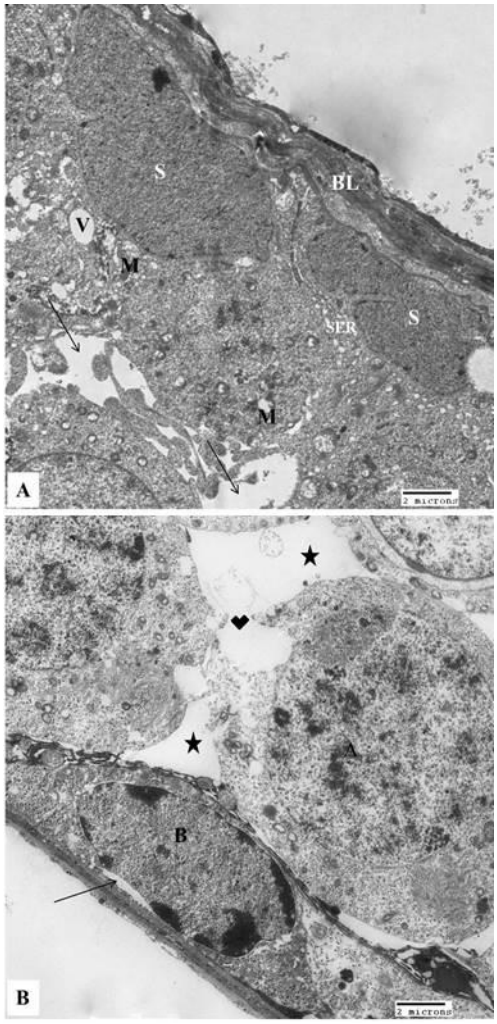

Fig (7): An electron micrograph of testis of Group IIb showing: **A)** Thick basal lamina (BL), two indented sertoli cells (S) with cytoplasmic vacuolation (V), dilated smooth endoplasmic reticulum (SER) and swollen degenerated mitochondria (M).notice loss of tight junction due to widening of intracellular space between sertoli cell and primary spermatocyte (arrow). **B)** Two type of spermatogonia. Spermatogonia type (B) rested on the basal lamina with dilated in perinuclear membrane (arrow) and swollen degenerated mitochondria. While spermatogonia type (A) was destroyed (head arrow) with rarified cytoplasm. notice widening in the intercellular space between the cells (star).

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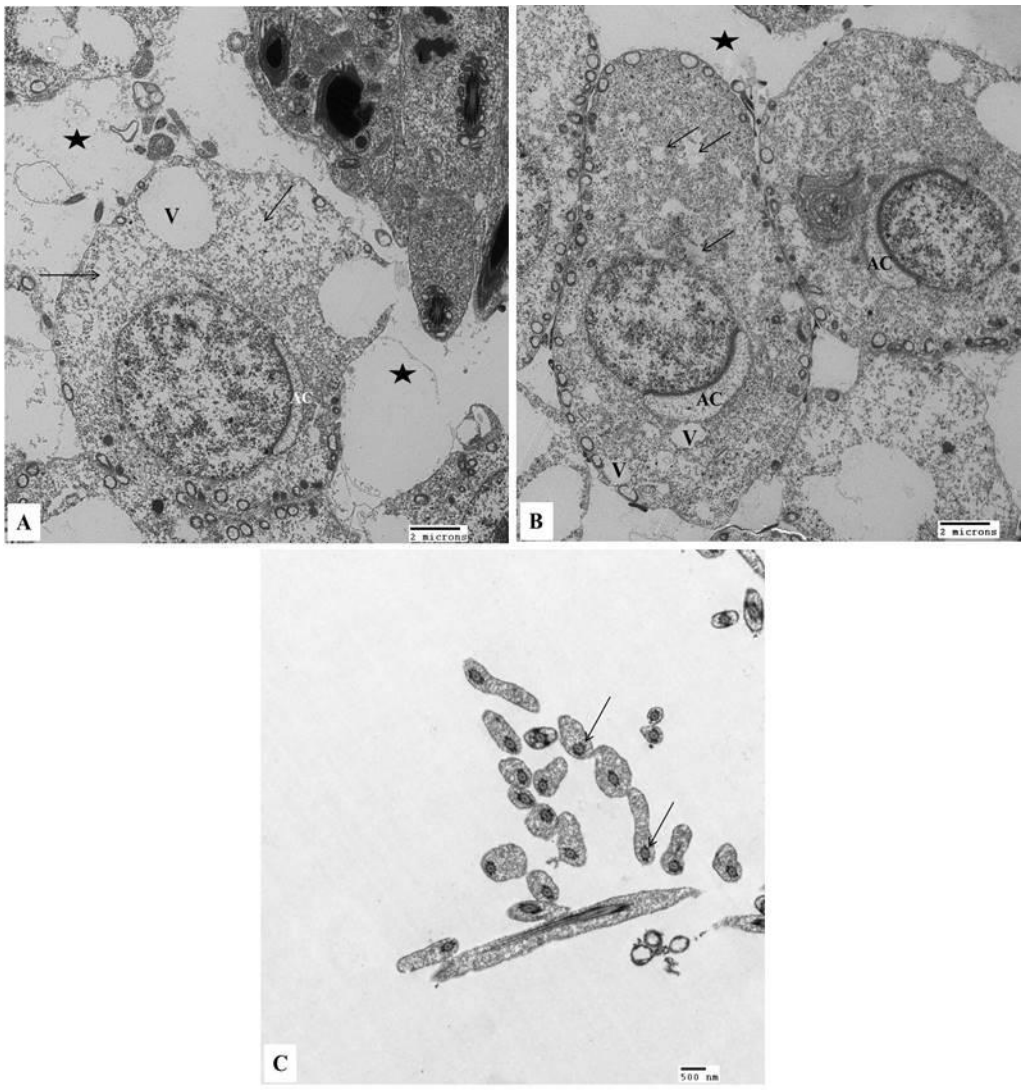

Fig (8): An electron micrograph of testis of Group IIb showing: **A)** Distorted early spermatid with large cytoplasmic vacuolation (V) and rarified cytoplasm (arrow) surrounded with large lytic area (L). notice distorted head of sperm (arrow head). **B)** Spermatid giant cells with rarified(arrow) and vaculated (V) cytoplasm. Its nuclei with abnormal acrosomal cap (AC). Notice the widening of intracellular space between the cells (star). **C)** Transverse and longitudinal section of abnormal sperms with decrease in number of microtubules of centriole (arrow).

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. In 1981, Shamberger *et al.* (13) 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 (14).

In 1993, Lee *et al.* (15) reviewed the coincidence of spontaneous testicular atrophy and its morphological changes in male rats exposed to toxins and reported an incidence of testicular degeneration of 2.5% in rats used for oral toxicity studies and 9.4% in rats used for inhalation studies. This signified that male reproductive orangs are vulnerable to toxicity even if not the target organ of the study. This picture is highly amplified with the use of cytotoxic and antimetabolic drugs as chemotherapeutic drugs (16).

The present study proved the occurrence of testicular changes evidenced by light and electron microscopic examination after methotrexate subcutaneous injection in dose of 12.5µg/kg/day for one week and these changed were worsened after administration for another week. These results illustrated the correlation between continuity of administration of cytotoxic drugs and occurrence of testicular changes leading to infertility. These results go in hand with Matsui *et al.* (17) who evaluated the testicular toxicity of cyclophosphamide in rats after administration of 100mg/kg single oral dose and were sacrificed at 7, 14 and 21 thereafter and reported decreased preleptotene spermatocytes at day 7, decreased zygotene spermatocytes at Day 14, and decreased pachytene spermatocytes at Day 21 and concluded that testicular toxicity can be detected from Day seven after a single administration of cyclophosphamide.

Animals treated with methotrexate (12.5 µg/kg body weight) daily for one week revealed intensive pathological changes including degeneration of seminiferous tubules, detachment of germinal epithelium. Moreover, exfoliation and disorganization of spermatogenic cells into 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 (18, 19).

On the other hand, Animals treated with methotrexate (12.5 µg/kg body weight) daily for two week confirmed sever alteration, distortion of seminiferous tubules and decrease in number of sperm in the lumen of seminiferous tubules. Histopathological lesions of testes after MXT treatment were in accordance with Sheikhbahaei *et al.* (20) who found that MXT caused a destructive effect on testicular germinal cells, apoptosis and decrease in sperm number.

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 for one week revealed changes in spermatogonia, spermatids, Sertoli cells and sperms. Ultrastructure findings that observed in MXT group include; dilatation of smooth endoplasmic reticulum, degenerated mitochondria and some lytic regions appeared due to widening of the intracellular space between the spermatogenic cells. Similar ultrastructure results were observed by Yuncu *et al.* (21) 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 methotrexate daily for two week proved thicknening of basement membrane, severe changes in Sertoli and spermatogonia type A and type B. also distortion and vacuolation of early spermatid and decrease in number of microtubules of centriole in sperm. These results agreed with Spano *et al.,* (22), who reported eosinophilic globular bodies , sequestered necrotic spermatids , and the germ cell degeneration was associated with cytoplasmic vacuolation of sertoli cells and markedly decreased maturing spermatids. Furthermore, similar electron microscopic changes were detected by Daggulli *et al.,* (23).

It could be concluded exposure to anticancer drugs has a deleterious effect on testicular function as regards spermatogenesis with proved ultrastructural changes.

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