

Protective Role of Thyme Leave Extract on Methotrexate-Induced Histological and Immunohistochemical Changes in Testes of Rats

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ABSTRACT

Background: Methotrexate (MTX) is an anticancer drug that induces testicular abnormalities but, thyme is antioxidant compound, which scavenges oxidative damage.

Objective: this study aimed to investigate the protective role of thyme leave extract on the histological and immunohistochemical testicular changes induced by MTX.

Material and Methods: forty adult male rats were used and categorized into 4 equal groups (10 rats/each); **Group I:** control rats group without treatment. **Group II:** control rats administrated thyme only for a month, **Group III:** rats injected (i.p) with MTX for four weeks, **Group IV:** rats injected with MTX and administrated thyme extract.

Results: the control rat testes stained with H&E showed normal histological structure of testes; rats administrated thyme demonstrated normal structure similar to the normal control. Rats injected with MTX showed damaged testicular tissue but, rats co-treated with MTX and thyme demonstrated improvement in the testicular structure. The caspase-3 immunostain was expressed in the control and the thyme group as weak reaction in the spermatogonic cells. However, MTX group showed dense reaction for caspase-3 and group of MTX and thyme detected obviously reduction of caspase -3. Normal intense immunoreactive of PCNA was realized in the control and thyme groups. But, MTX group showed an obvious decrement of immunoreactivity and MTX and thyme group expressed more reduction in the immunoreactivity. **Conclusion:** thyme extract had the ability to ameliorate the abnormalities occurred in the testes of rats after MTX treatment.

Keywords: testis rat, MTX, thyme, histological study, H&E, immunohistochemical, caspase-3 and PCNA.

INTRODUCTION

The testis is the male gonad in animals. The testicles are components of both the reproductive and the endocrine systems. The primary functions of the testicles are to produce sperm (spermatogenesis) and to produce androgens, especially testosterone. Gonadotropin hormones produced by the anterior pituitary gland affect both testes functions. LH results in the release of testosterone. Both testosterone and FSH are needed to support spermatogenesis ⁽¹⁾. The testicle obtains its oval shape from tissues known as lobules and consists of seminal tubes, which are coiled tubes that contain cells and tissues responsible for formation of spermatozoa, which is the process of spermatogenesis and Leydig cells are responsible for production of male hormones, such as testosterone and other androgens ⁽²⁾.

Methotrexate (MTX) is an anticancer drug. The mechanisms by which MTX has anti-inflammatory action might differ greatly from the mechanisms used to target malignant diseases ⁽³⁾. MTX caused increased malondialdehyde level and number of the apoptotic cells. MTX gives rise to serious damage in the testes. MTX is frequently used in the treatment of several diseases including cancers, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus and dermatomyositis ⁽⁴⁾. Previously, chemotherapeutic agents have been reported to cause permanent azoospermia and infertility in men. In addition, methotrexate has been shown to damage the

seminiferous tubules of the testicles, lower the sperm count and cause genetic mutations in DNA of sperms. MTX caused severe tissue destruction in testicles by increasing e formation of the free oxygen radicals ⁽⁵⁾.

Anti-oxidants are agents that help to decrease many oxidation reactions created by free radicals, thereby delaying or preventing the cells and tissues damage. There is evidence in confirming the role of anti-oxidants, which may control our body against confirmed cases. Herbs are normally wealthy in bioactive plant products with nourishment incentive to keep vitality adjustment in the body and considerable restorative incentive in a few sicknesses ⁽⁶⁾.

Thyme plant (*Thymus vulgaris*) has essential oils and more than 60 anti-oxidative phenolic compounds that have anti-oxidant properties and anti-microbial activity. Thyme was utilized as a part of human nourishment as a zest and sustenance season and as cell reinforcement for treatment of various infections. It has been also used in animal feed and in poultry as an anti-oxidant and growth stimulant agent ⁽⁷⁾.

The present study aimed to investigate the protective role of thyme leaves extract on testes of rats injected with MTX.

MATERIAL and METHODS

Animal selection and care:

Forty adult male rats 300 ± 50 g were used in the present investigation and were obtained from Vacsera (51 Wezaret El Zeraa St. Agouza, Giza, Egypt). The



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animals were housed in plastic cages (2 per cage) for one week for acclimatization under the same conditions of temperature and natural dark-light cycle. Food and tap water were freely available to the animals throughout the experiment.

Ethical approval: all care and procedures adopted for the present investigation were in accordance with the approval of the Institution Animal Ethics Committee of National Research Center and in accordance with recommendations of the proper care and use of laboratory animals.

Chemicals:

MTX was purchased from Ebewe Pharma Company (Egypt) and was dissolved in 0.9% saline. Dried leaves of thyme (*Thymus vulgaris*) were purchased from local market and were identified by the Egyptian Institute for Herbs. The thyme leaves were grounded into powder using an electrical grinder. One hundred grams of the fine-powder were mixed with 200 ml of boiling distilled water in a covered flask and left for 30 minutes. After that, the extract was cooled and filtered by filter paper to remove the particulate material. The filtrate was dried in a vacuum. The required dose then weighted and reconstituted in 5 ml of distilled water a minute before oral administration.

Experimental design:

Forty rats were categorized into 4 equal groups housed in cages. All were kept under the same conditions and received the same diet. **Group I:** the control rats received 0.2 ml of saline (0.9% NaCl) four weeks.

Group II: rats administered thyme only (orally) at a dose 500 mg/kg b.w/d for four weeks. **Group III:** rats injected (i.p) with MTX dissolved in saline (1mg/kg b.w/ week) for four weeks to induce testicular changes. **Group IV:** rats injected with 1mg/kg b.w per week MTX and administered thyme extract (orally) at a dose of 500mg/kg/bw/d for four weeks.

The histological study:

At the end of each experimental period, rats were sacrificed. The testicular tissues were collected and washed with saline, cut into small pieces and then specimens were fixed in 10% neutral formalin. The fixed specimens were sliced, processed and embedded in paraffin blocks. The blocks were cut into 5 µm thick paraffin sections by a rotary microtome. The sections were stained with Hematoxylin and Eosin (H&E)⁽⁸⁾ for the histological examination.

For immunohistochemical preparation:

The sections were fixed in 10% neutral buffer formalin then embedded in paraffin wax. Monoclonal antibody against PCNA and polyclonal antibody against caspase-3 were used by applying avidin-biotin complex (ABC) technique. Caspase-3 (Biocare Polymer Detection Biosciences, San Diego, CA, USA) was used as a marker against apoptotic cells resulting from the testicular changes. PCNA (Thermo Fisher Scientific Industries, Waltham, MA, USA) was used as

a marker for vascular endothelial cells and expression of hematopoietic progenitor stem cells⁽⁹⁾. The ABC method, a biotinylated secondary antibody reacts with peroxidase conjugated streptavidin molecules. Colour reaction was developed by using diaminobenzidine (DAB) that gave a brown colour. Haematoxylin was used as a counter stain⁽¹⁰⁾. The sections were deparaffinized in xylene, and rehydrated in alcohol series. Sections were washed in phosphate buffered saline (PBS). PBS was used for all the subsequent washes and for dilution of the antibodies. Tissue sections were heated in a microwave oven twice for 5 minutes and were subsequently rinsed in 3 % H₂O₂ to block endogenous peroxidase. Sections were washed by using PBS at pH 7.6 three times for 15 minutes. The excess of PBS was dried by using absorbent paper and then placed in humid chamber. Sections were incubated for 1 hour at room temperature with either the primary monoclonal antibody (anti-caspase-3) or polyclonal antibody (anti-PCNA). The slides were washed 3 times with PBS for 5 minutes per each. Sections were incubated with the appropriate secondary antibody (anti-rabbit peroxidase) for 30 minutes at room temperature. The slides were washed with PBS 3 times for 5 minutes per each. Diaminobenzidine (DAB) was the final chromogen and the hematoxylin was used for nuclear counter staining and washed in running tap water for 30 minutes until desired intensity of blue colour. All samples were processed under the same conditions and the staining sections were examined by light microscopy. Finally, caspase-3 immunoreactivity was expressed as a brown colour in the apoptotic cells in seminiferous tubule cells resulting from testicular changes. PCNA immunoreactivity was expressed as a brown colour in the nuclei.

RESULTS

1- Histological examination:

a) Haematoxylin & Eosin (H&E):

The control rats testes stained with H&E consist of normal seminiferous tubules with regular arrangement of spermatogenic cells spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and sperms on thin basement membrane (**Fig. 1**). Rats administered with thyme leaf extract only showed normal seminiferous tubules with normal spermatogenic cells and spermatozoa similar to the normal control (**Fig. 2**). Rats injected with MTX only showed severe damage in the seminiferous tubules, debris of damaged spermatogenic cells, obvious decrement of spermatozoa, degeneration of inflammatory & phagocytic cells in the interstitial tissues and thickness of basement membranes (**Fig.3**). Rats injected with MTX and administered thyme leaf extract showed somewhat normal seminiferous

tubules with normal arrangement of spermatogonia on the thin basement membranes, normal spermatogenic cells and normal spermatozoa. Enhancement of inflammatory and phagocytic cells in the interstitial tissues was seen (Fig.4).

2- Immunohistochemical examination:

a) Caspase-3 immunostain expression in the testes:

Caspase-3 marker is a polyclonal antibody against apoptotic spermatogenic cells. The normal control testis of rats (group I) showed weak caspase-3 reaction in the spermatogenic cells (Figs.5&6) as well as that observed in the testes of rats administered thyme leaves extract (Group II) (Figs.7&8). The abnormal testicular sections of rats treated with MTX (Group III) expressed intense caspase-3 immunoreactivity in the spermatogenic cells in the form of brown colour (Figs.9&10). The rats administered thyme leaves extract and injected with MTX (Group IV) expressed an obvious improvement and a reduction of caspase-3 immunostain in spermatogenic cells and most of them appeared somewhat similar to the normal ones (figs.11&12).

b) PCNA immune stain results:

The normal control testes of rats (Group I) showed normal intense immune positive reaction in the nuclei of most of the basal spermatogenic cells in the seminiferous tubules (Figs. 13&14). Group of rats administered thyme leaves extract only (Group II) showed normal immune positive reaction in nuclei of the basal spermatogenic cells in the seminiferous tubules similar to the normal group (Figs. 15&16). In group of testicular abnormalities induced by MTX only (Group III) showed an obvious decrement of immunoreactivity in nuclei of the basal spermatogenic cells of the seminiferous tubules (Figs. 17&18). Rats injected with methotrexate (i.p) and administered thyme extract expressed more reduction in the immunoreactivity in nuclei of the basal spermatogenic cells of the seminiferous tubules (Figs. 19&20). In brief, treatment of rats with thyme leaves extract showed obvious improvement against methotrexate treatment and regained the seminiferous tubules and spermatogenic cells to the normal architecture.

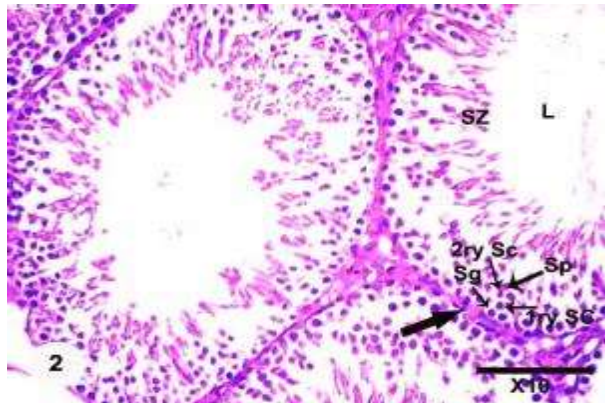
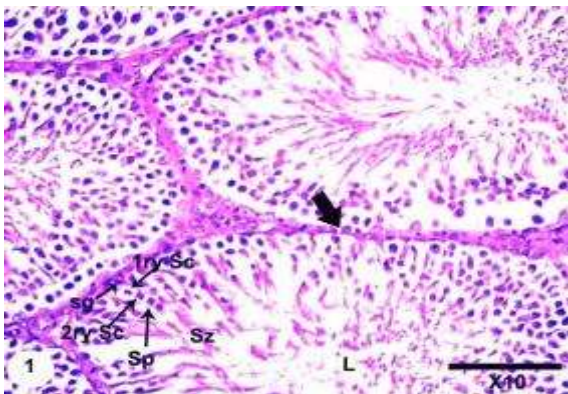


Fig.1: a photomicrograph of a section of the testis of the normal control testis of rat showing normal seminiferous tubules with regular arrangement of spermatogenic cells on a thin basement membrane (thick arrows) as; spermatogonia (Sg), primary spermatocytes (1ry Sc), secondary spermatocytes (2ry Sc), spermatids (Sp) and a lot of spermatozoa (Sz) in the lumen (L). H&E, Bar =6.25µm.

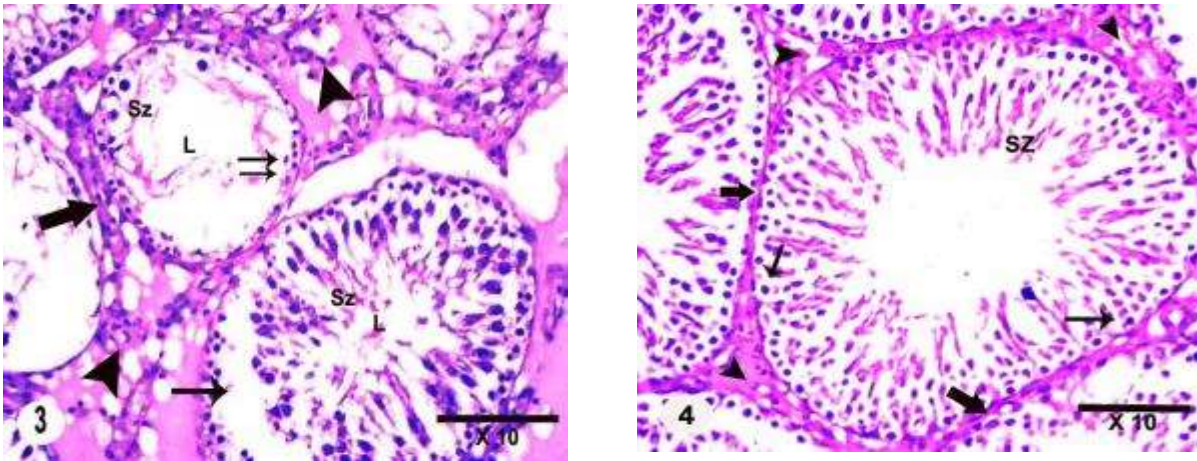
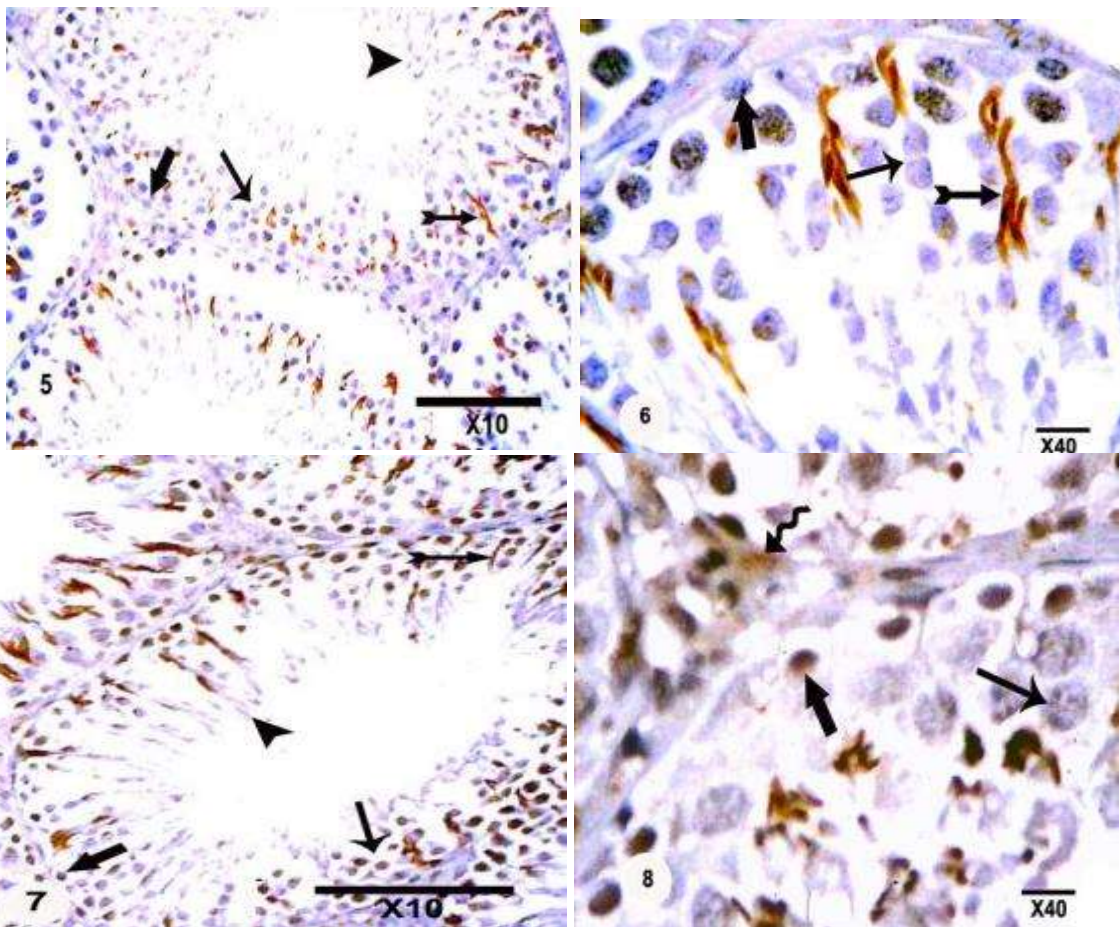


Fig. 2: section of rat testis administrated with thyme leaves extract only showing normal seminiferous tubules with regular arrangement of spermatogenic cells on the thin basement membrane (thick arrows) as; spermatogonia (Sg), primary spermatocytes (1ry Sc), secondary spermatocytes (2ry Sc), spermatids (Sp) and a lot of spermatozoa (Sz) in the lumen (L) similar to the normal control .H&E, Bar =6.25µm.

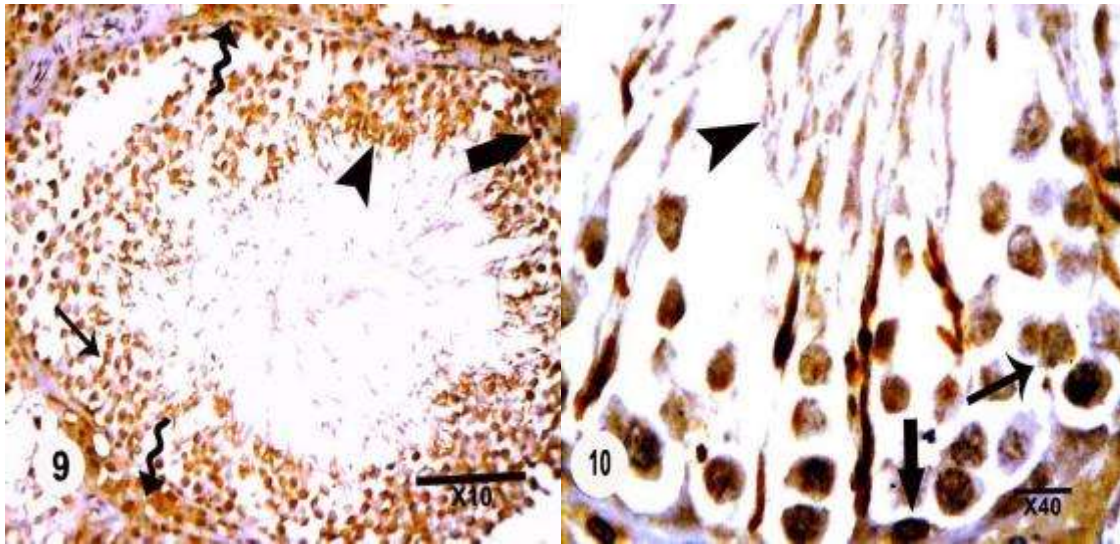
Fig. 3: photomicrograph of section of the testis of rat injected (i.p) with MTX only showing degeneration of spermatogenic cells (thin arrows), debris of damaged spermatogenic cells in others (double arrows) and reduction of the spermatozoa in the lumen with thick of basement membrane (thick arrow). Inflammatory and phagocytic cells in the interstitial cells are clearly seen (arrowhead).H&E, Bar =6.25µm.

Fig.4: photomicrograph of section of the testis of rat injected (i.p) with MTX and administrated thyme leaves extract showing a seminiferous tubule with normal spermatogenic cells (thin arrow) , normal spermatozoa in lumen and normal arrangement of spermatogonia with thin basement membrane. Enhancement of inflammatory and phagocytic cells (arrowhead) in the interstitial tissues.H&E, Bar=6.25 µ m.

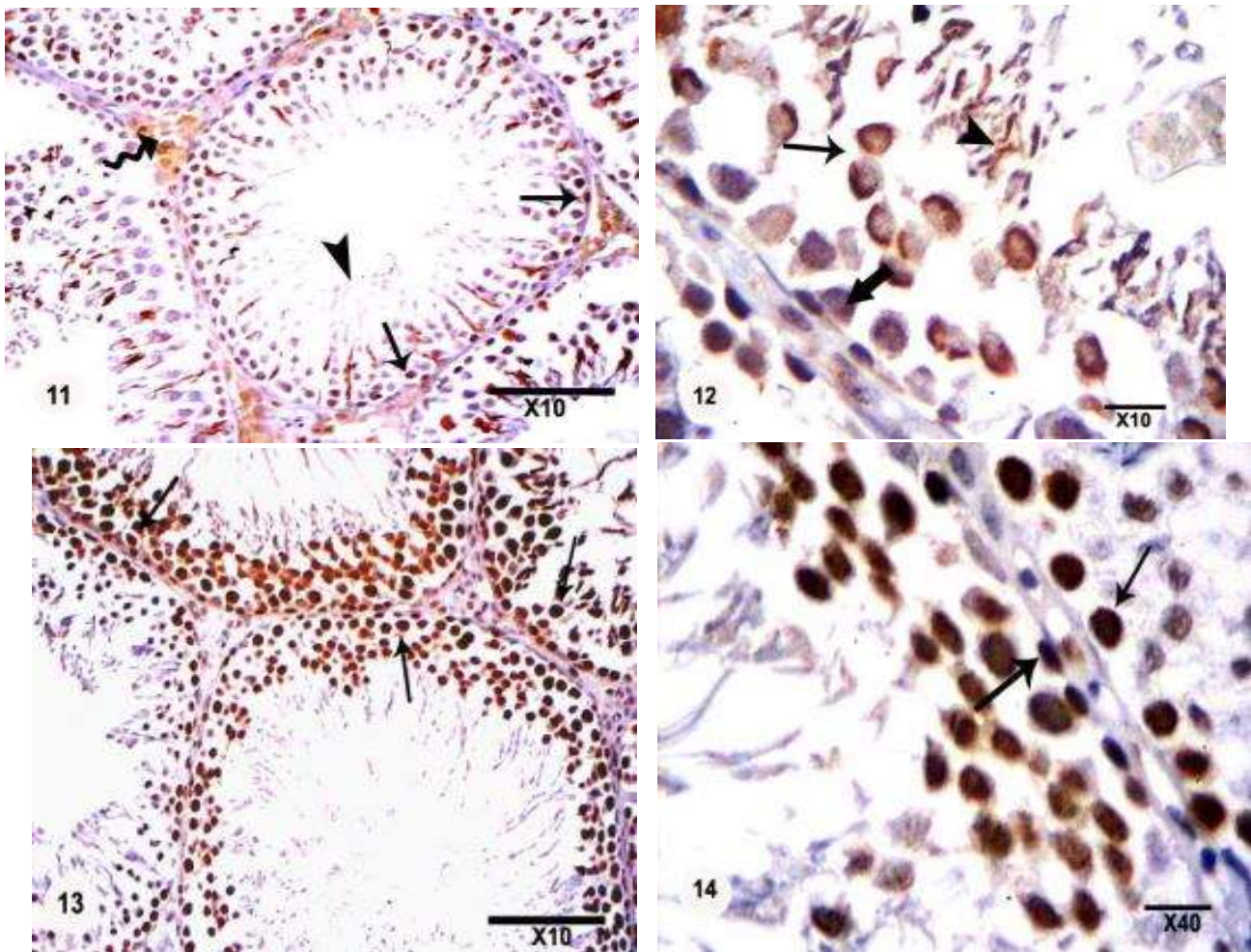
Figs. 5&6: photomicrographs of sections of the testis of a control rat showing normal appearance spermatogonia (thick arrow), spermatocytes (thin arrow) and spermatozoa (arrowhead) and a few scattered apoptotic cells in the Sertoli cells Double head arrow). Caspase -3 immunostain, Bar = 25 & 6.25 µm, respectively.



Figs. 7&8: photomicrographs of sections of the testis of rat administrated thyme leaves extract only showing normal appearance spermatogonia (thick arrow), spermatocytes (thin arrow) and spermatozoa (arrowhead) and a few apoptotic cells near the basement membrane (zigzage arrow).Caspase -3 immunostain, Bar = 25 & 6.25 µm, respectively.

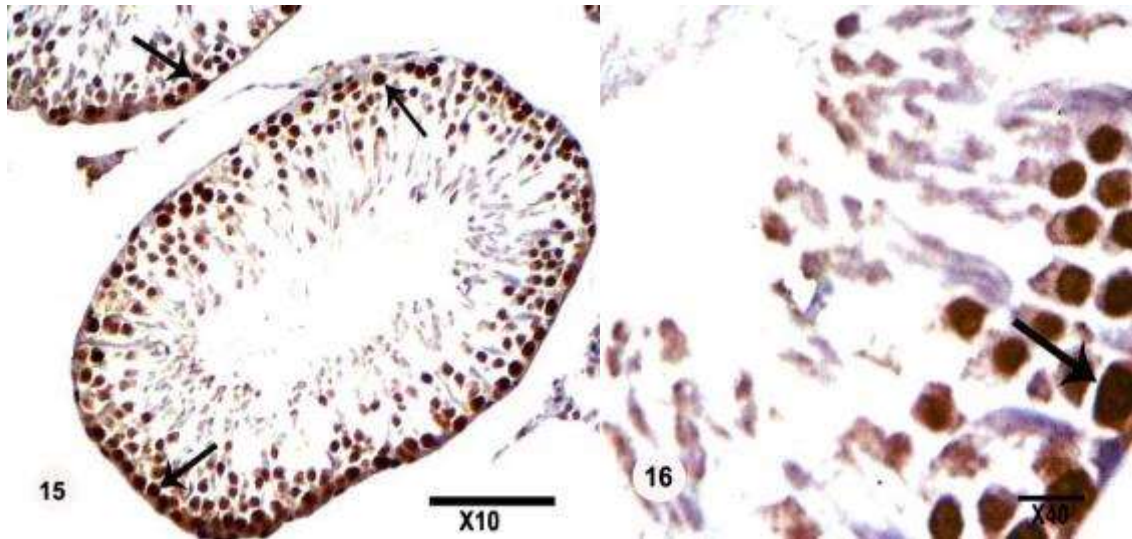


Figs.9-10: photomicrographs of sections of the testis of rat treated with MTX detecting clearly apoptosis in spermatogonia (Thick arrow), spermatocytes (Thin arrow) and spermatozoa (arrowhead) and basement membrane (Zigzage arrow).Caspase -3 immunostain, Bar = 25 & 6.25 μ m, respectively.

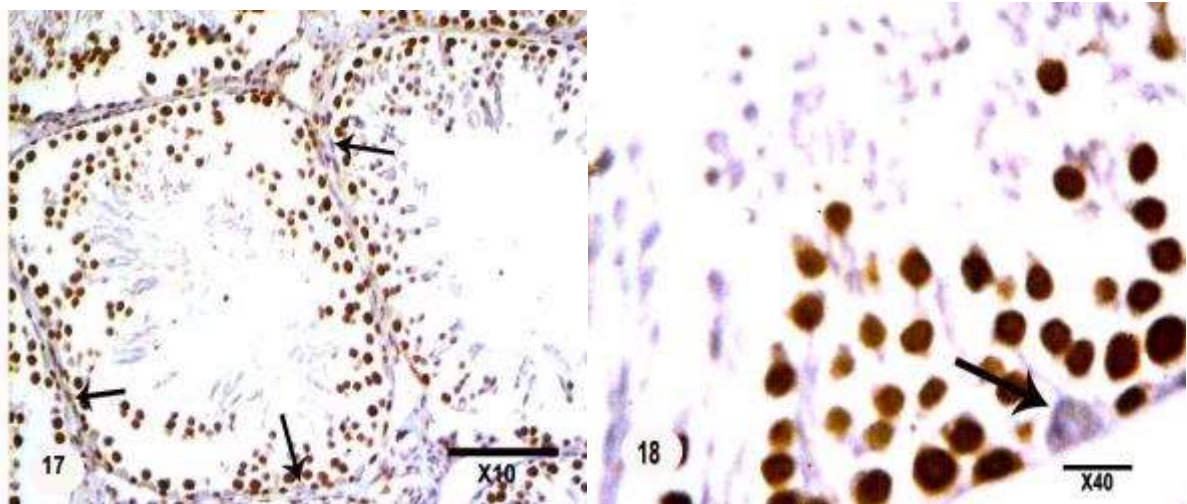


Figs.11-12: photomicrographs of sections of the testis of rat injected with MTX and administrated thyme leaves extract detecting obvious reduction of caspase 3 immuno-reaction in spermatogonia (thick arrow), spermatocytes (thin arrow) and spermatozoa (arrowhead) and basement membrane (Zigzage arrow).Caspase -3 immunostain, Bar = 25 & 6.25 μ m, respectively.

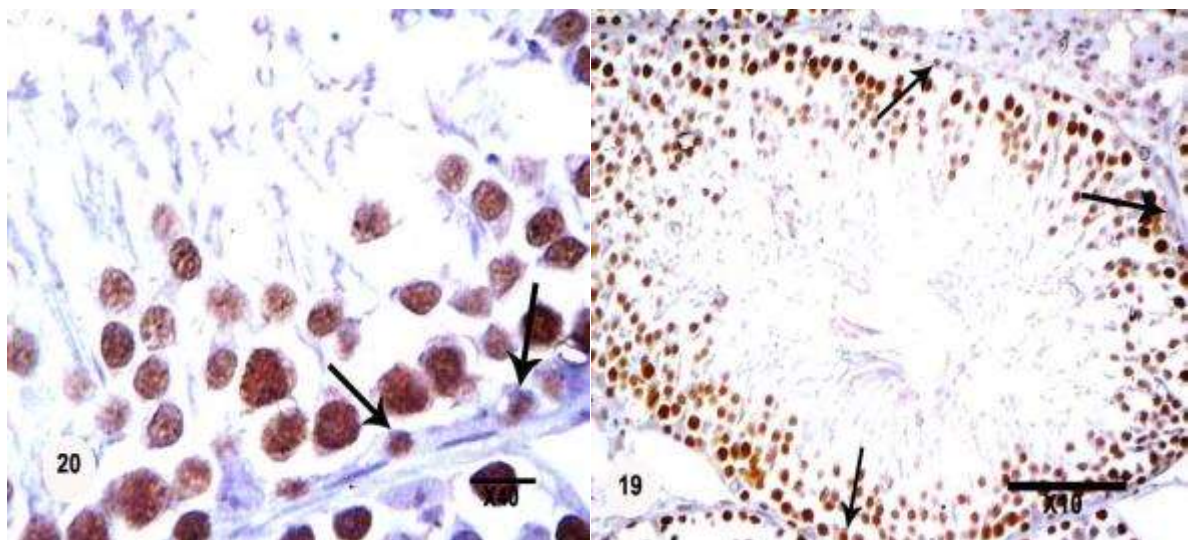
Figs. 13-14: photomicrographs of sections of testis of a control rat expressing intense immune positive reaction in nuclei (Brown nuclear reaction) of most of the basal spermatogenic cells in the seminiferous tubules (arrows). PCNA, Bar = 25 & 6.25 μ m, respectively.



Figs. 15-16: photomicrographs of sections of rat administrated thyme leaves extract only showing normal positive immune reaction in nuclei (Brown nuclear reaction) of most of the basal spermatogenic cells in the seminiferous tubules (Arrows) similar to the normal control. PCNA, Bar = 25 & 6.25 μ m, respectively.



Figs. 17-18: photomicrographs of sections of rat injected with MTX expressing an obvious decrement of immunoreactivity in nuclei of the basal spermatogenic cells (arrows) of the seminiferous tubules.



Figs. 19-20: photomicrographs of sections of rat injected with MTX and administrated thyme leaves extract expressing a reduced immunoreactivity in nuclei of the basal spermatogenic cells in the seminiferous tubules (arrows). PCNA, Bar = 25 & 6.25 μ m, respectively.

DISCUSSION

Today, there are many different kinds of chemotherapy that are used as anticancer drugs. It is important to search for therapies, which can reduce the side effects of anticancer treatments without altering their efficacy or increasing toxicity or damage in target organs such as testis⁽¹¹⁾. The results of the present study indicated that MTX led to oxidative tissue damage by increasing cellular lipid peroxidase activity that led to oxidative stress.

The present work showed that MTX induced testis abnormalities to rats at a dose 1mg/kg b.w/d once per week for four weeks. There are many changes in seminiferous tubules and spermatogenic cells after injection of MTX. Damage in the seminiferous tubules included degenerated spermatogenic cells, reduced layers of the spermatogenic cells, obvious decrement of spermatozoa, inflammatory degeneration, phagocytic cells in the interstitial tissues with thickened basement membranes. A previous recorded that injection of MTX had similar effects on rat testis⁽¹²⁾. They a revealed decrease in diameter of the seminiferous tubules, increased interstitial spaces as well as distortion of morphology of Leydig cells⁽¹²⁾. Additionally, the status of oxidative stress during treatment with MTX accompanied by increasing lipid peroxidation in various tissues is responsible for sperm motility. The effects of intra-peritoneal injection of MTX on testis of rats showed impaired spermatogenesis and increase in germ cell apoptosis⁽¹³⁾. In a previous study, the therapeutic dose of MTX was capable of producing histopathological changes in the testis. Use of MTX affected the testes and caused inflammatory changes⁽¹⁴⁾.

The present work showed that administration of thyme leaves extract at a dose 500 mg/kg b.w/d for a month with injection of MTX at a dose 1mg/kg b.w. once per week for four weeks improved testicular changes such as improvement of architecture of the seminiferous tubules, spermatogenic cells, spermatozoa and basement membranes. In addition, inflammatory phagocytic cells were enhanced. Similar results declared that thyme is a lipophilic molecule that is able to prevent lipid peroxidation through Fenton reaction⁽¹⁵⁾ Moreover, the polyphenolic compounds inhibit the enzymes oxidation, which prevent e formation of free radicals and improve reproductive performance⁽¹⁶⁾.

Thym vulgaris extract containing anthocyanin and flavonoid compounds that improved the structure of the seminiferous tubules after the induced abnormality Furthermore, thyme reduces the effects of the oxidative stress induced under various conditions and improves cells to cope with these conditions by preventing the reduction of glutathione and increasing antioxidant capacity⁽¹⁷⁾. *Thyme vulgaris* scavenges the oxidation agents and can be used for subinfertile men because it has antioxidants that enhance testis structure and reproductive parameters⁽¹⁸⁾. The current results

expressed intense caspase-3 immunoreactivity in the spermatogonia, spermatocytes, spermatozoa and basement membranes that led to apoptosis in testis of rats treated by MTX comparable to the control rats, which showed a few scattered apoptotic Sertoli cells with normal appearance of other testicular cells. Rats group treated with MTX plus thyme leaves together expressed obviously reduction of caspase-3 immunoreactivity in the testicular cells comparable to abnormal testicular rat group. Caspases are cysteine proteases that are activated in response to pro-apoptotic signals and trigger a cascade of proteolytic cleavage of cellular substrates that lead to cell death⁽¹³⁾. Caspase-3 is a key caspase involved in The execution phase of apoptosis needs proteolytic cleavage of caspase-3 through both extrinsic and intrinsic signaling pathways⁽¹⁹⁾. MTX induced testicular toxicity due to its direct toxic effect via dihydrofolate reductase enzyme inhibition preventing DNA synthesis with cell cycle arrest⁽²⁰⁾ and caused DNA damage. A study detected the important role of apoptotic cell death in the pathogenesis of MTX-induced testicular damage. The testicular apoptotic cell death is highly associated with oxidative stress and changes in caspase-3 expression may play an important role in this process⁽²¹⁾. Oxidative stress attacks cellular enzymes decreasing the activity of these enzymes and facilitates the process of apoptosis and cellular damage⁽²²⁾. The oxidative damage particles in rat's testes increased the caspase-3 and apoptosis in spermatogonia⁽²³⁾. The significantly increased testicular caspase-3 expression in MTX group was consistent with increased mRNA expression⁽²⁴⁾.

MTX-induced an increase in the testicular caspase-3 immunostaining was comparable to the control group⁽²⁵⁾. Similarly, *Thymus vulgaris* extract decreased the apoptotic cell death as evidenced by increased caspase-3/7 activity⁽²⁶⁾. Antioxidants inhibited oxidative stress through scavenging apoptotic activity and decreased caspase-3. The anticancer mechanism of antioxidant actions of thyme extract against proapoptotic effects caused by inactivation of caspase-3⁽²⁷⁾. *Thyme vulgaris* extract reduced the caspase-3 expression and ultimately protected the cells from oxidative stress-induced apoptosis⁽²⁸⁾. However, the results showed that treatment with antioxidants capable of decreasing the elevated caspase-3 expression in the testicular tissue of rats after oxidative stress induction⁽²⁹⁾. The present results expressed normal intense immune positive reaction to nuclei of most of the basal spermatogenic cells in the seminiferous tubules in the normal control group. Group of rats administrated thyme leaves extract only expressed normal immune positive reaction. Group of testicular abnormalities induced by MTX showed an obvious decrement of immunoreactivity in the nuclei of the basal spermatogenic cells of the seminiferous tubules. Rats injected with MTX plus thyme expressed more

reduction in the immunoreactivity in the nuclei of the basal spermatogenic cells of the seminiferous tubules. Similar results showed weak positive immunostaining with PCNA in some spermatogonia and negative immunostaining in other spermatogonia in group of rats with testicular abnormalities after treatment with MTX⁽³⁰⁾. Moreover, MTX inhibited spermatogenesis through its impact on cell multiplication and differentiation, as it decreased the protein expression of PCNA in the spermatogonia, which is essential for DNA replication and for subsequent cell growth and proliferation⁽³¹⁾. Furthermore, the antioxidants regulate PCNA and lead to the promotion of cell cycle progression and the reduction of apoptosis. Herbal compounds that have antioxidants are implicated in modulating a variety of cellular activities leading to cell proliferation and survival⁽³²⁾. Treatment with resveratrol, which is a natural strong antioxidant and polyphenolic compound enhanced and increased PCNA expression by scavenging oxidative stress. The antioxidants protect drug-induced oxidative stress and prevent DNA damage⁽³³⁾.

CONCLUSION

Thyme leaves extract has effective role in improving the histological structure of testis and decreasing caspase-3 expression by preventing apoptosis in the testicular cells as well as increasing PCNA expression resulting in increasing proliferation of the testicular cells after the damaging effects induced by MTX.

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