Original Article

Thymus vulgaris Attenuates Myleran-induced Reproductive Damage by Decreasing Oxidative Stress and Lipid Peroxidation in Male Rats

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Abstract

Context: *Thymus vulgaris* is an herbal with potent antioxidant and it has been shown to have beneficial effects during short-term administration. Myleran (MYL) is used for treatment of certain types of tumors. MYL produces free radicals and induces disturbance in sperm parameters. **Aims:** This study is designed to assess the effects of *T. vulgaris* against damage to the male rats' reproductive features induced by MYL. **Subjects and Methods:** Sixty-four male Wistar rats were randomly assigned into eight groups: control group; MYL (10 mg/kg) group; *T. vulgaris* groups (4.5, 9, and 18 mg/kg); and MYL (10 mg/kg) + *T. vulgaris* groups (4.5, 9, and 18 mg/kg; separately). Treatments were administered daily intraperitoneal injection for 60 days. Total antioxidant capacity, sperm factors, malondialdehyde (MDA), testosterone, and germinal layer height were analyzed. **Results:** Whole variables of MYL group decreased signifcantly compared to the control group ($P < 0.05$) except MDA level (which increased). The *T. vulgaris* and *T. vulgaris* + MYL treatments in all doses increased all parameters significantly except MDA level (which decreased) compared to the MYL group ($P < 0.05$). No significant modifications were observed in all *T. vulgaris* groups compared to the control group ($P > 0.05$). **Conclusions:** *T. vulgaris* reduces the poisonous properties of MYL on male reproductive factors.

Keywords: *Lipid peroxidation, Myleran, oxidative stress, reproductive parameters, Thymus vulgaris*

Introduction

One of the most common side effects of anticancer drugs is the interruption of spermatogenesis, which leads to sterility in numerous cases.[1] Myleran (MYL) is a DNA-destructive chemotherapy agent.[2] MYL stops cell division and has opposing effects on the cells with a high division rate, thus applying its highest influence on spermatogonial stem cells.[3] MYL also causes sperm chromosomal anomalies and lethal mutation mostly in sperms.[4] Boujrad *et al*. stated that the use of MYL induces incomplete performance of gonad in pregnant females and decreases somatic and germinal cells in the testis of newborns.[5] The disrupted spermatogenesis following treatment of MYL seems to be associated with the antioxidant properties of MYL and its major effect on spermatogonial stem cells.[6] MYL as an appropriate pharmaceutical method to evacuate seminiferous tubules has long been taken into consideration in order to study the performance of germinal stem cells.[7] MYL induces the cell death via free radical production[8] that impairs lipids, proteins, and nucleic acids.[9] The oxidative stress related to the free radicals decreases the antioxidant enzyme activity, increases reactive oxygen species (ROS) level, and induces lipid oxidation consequently.[10] This phenomenon, in turn, causes DNA breakdown and inactivation of specific proteins and consequently loss of biologic membranes.[11] Many plants with antioxidant properties exert protective effects

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against chemoprotective agents. One of these plants is *Nigella sativa* L., which has a medical and religious history.[12] *Nigella sativa* L., with white flowers that turn black in interaction with air, is native to Asia.[13,14] This herbal has been stated to have frequent pharmacologic effects such as attenuation of glucose, lipid, and hypertension; protection of kidney; and antimicrobial and antifungal effects due to its antioxidant and anti-inflammatory properties.[15,16] *Thymus vulgaris* by antioxidant properties can protect the male reproductive factors against MYL induced oxidative injury. Considering the antioxidant properties of *T. vulgaris*, it seems that extract of *T. vulgaris* is able to protect the male reproductive parameters contrary to MYL-induced oxidative stress. Furthermore, an evaluation of the collected works designated that there is no learning about the effects of *T. vulgaris* against MYL-induced oxidative stress on male reproductive factors of rats.

Subjects and Methods

Animals

This study was done on 64 male Wistar rats (9-week-old, 230–240 g) in the Kermanshah University of Medical Sciences. The study was lead conferring to the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines after due approval of the experimental protocol from the Institutional Animal Ethics Committee, Research Deputy of Kermanshah University of Medical Sciences. The rats were maintained on a regular diet and water *ad libitum* with a 12:12 h light/dark cycle at 23°C ± 2°C with a relative humidity of 50% ± 5%, in the animal home by considering 1-week adaptation before the experiments.[13]

Study groups and treatment of animals

The animals were randomly separated into eight groups: first group, the control group, which received intraperitoneal (IP) injection of saline equivalent to the amount of experimental groups; second group, the MYL group, in this crowd, the animals were given MYL (10 mg/kg, a single dose by IP injection) for 60 days; third to fifth groups, the *T. vulgaris* administration groups, in these groups, each animal, respectively, received doses of 4.5, 9, and 18 mg/kg of *T. vulgaris* IP injection for 60 days at 9 am; and sixth to eighth groups, *T. vulgaris* + MYL groups, in this group, every rats first received MYL (10 mg/kg) in order to make reproductive parameters destruction and then they respectively received diverse dose (4.5, 9, and 18 mg/kg, IP injection) of *T. vulgaris* for 60 days at 9 am.[3,11]

Animals' dissection and sampling

At the finale of the treatment dated, wholly rats were deeply anesthetized by IP injection of ketamine HCl (70 mg/kg) and xylazine (30 mg/kg). Blood sample was taken from the heart without cutting the chest. The samples were centrifuged at 255 g for 15 min. The blood serum was isolated and part of this was kept at −70°C. Then, the abdomen of the rats was cut. The epididymis tail was remote from the testes and placed in DMEMF12 medium. The left testis was fixed in a 10% formalin for morphometrical checks and the right testis for the malondialdehyde (MDA) level valuations.[10]

Sperm collection

Both cauda epididymides from each animal were crushed and conserved in a warmed Petri dish containing 10 ml Hank's Balanced Salt Solution at 37°C. The spermatozoa were allowed to disperse into the buffer. Fifteen minutes later, the cauda was removed and the suspension was slightly shaken to be normalized. It was then observed by a light microscope at a magnification of × 400.[17]

Motility

In this method, four degrees of sperm motility were studied based on the WHO methods, Class A: Progressive motility. Progressive motility of the sperm of each sample was examined by an optical microscope with a magnification of ×40 in ten fields of view. For this purpose, at first, about 50 µl of semen liquid culture medium was taken and placed on a slide culture that was previously cleaned and dried with alcohol. Then, the slide culture was placed on it and examined by a microscope. Sperm counting was performed through a cell count device, and about 100 sperms were counted in each sample. In all the experimental and control groups, sperm parameter assessment was done by two qualified expert persons to minimize subjectivity.[11]

Viability

In this method, eosin staining was used to recognize living sperms from dead sperms. The basis of this staining is the absorption of stain by the membrane of dead cells and its disposal by the membrane of living cells. At the end of the given time, about 10 µl of the medium containing semen fluid was collected from each dish and then mixed with an equal volume of eosin stain solution (about 10 µl). After 5 min, part of the mixture was poured on a Neubauer's slide culture. Then, living sperms lack stain and dead sperms become pink. The prepared slide culture was examined with the magnification of ×40. At least 100 sperms were calculated from each random sample from the 10 fields of imaging, and the percentage of live sperm cell was documented.[10]

Morphology

The normal sperm cell morphology was evaluated through the investigation of smears from the right

epididymis. An aliquot of the sample was used to make the smears to appraise the deformities in the spermatozoa. Eosin/nigrosin staining was used to estimate the normal morphology. One drop of eosin was added to the suspension. The slides were then observed by a light microscope at × 400 magnification. A total of 500 spermatozoa were studied on the respective slide (5000 cells in each group) for irregularities of the head and tail.[17]

Sperm calculation

To investigate the number of sperms, 500 µL of the sperm suspension was diluted through the formaldehyde fixative (Sigma, USA). Approximately 20 µL was removed from the diluted solution into a hemocytometer by a Pasteur pipette. The hemocytometer was located into a Petri dish with dampened filter paper and allowed to stand for 20 min. The stable sperms were counted and assessed per 250 small squares of the hemocytometer using a × 40 objective lens. The number of sperm per mm^3 equated the number of sperm counted × the dilution/number counted in mm^2 × the depth of the chamber.[10]

Morphometric examination

The nonparenchymal tissues (fat, fascia, and vessels) of the removed left testis were dissected, and paraffin-entrenched blocks were prepared by an automatic tissue processor. The steps of this process consequently included fixation with 10% formal saline (48 h), washing under running water, dehydrating by raised doses of ethanol, clearing by xylene (10 min for each one), and embedding in soft paraffin (three times). At this stage, 5-µm histological thin sections were cut from the blocks, undertaken by a microtome instrument (Leica RM 4327, Leica Microsystems Nussloch GmbH, Germany), and five sections. For the confederation of the section choice, the first slice was the 4^{th} and the last was the 24^{th} (5 sections interval), and finally, the routine protocol for H and E staining was implemented. At the end of tissue processing, the stained sections were mounted by Entellus glue and assessed by an Olympus BX-42D-76P80 research microscope connected to a DP14 Camera with a 5.45-million pixel resolution and Olysia Bio-software (Olympus Optical Co., Ltd., Tokyo, Japan).[11]

Testosterone

The collected blood sample was centrifuged (5000 g) at 23°C for 15 min to get the serum. The serum samples were then kept in a deep freezer (−180°C). The serum testosterone level was examined through ELISA (Abcam 108666, USA) technique.[11]

Testis malondialdehyde

MDA levels were assessed as a marker of lipid peroxidation. In this regard, standardizing of the samples were carried out by homogenization buffer containing 5.55% KCl solution and the specimens centrifuged at 1500 g for 10 min, respectively. Then, the homogenated subjects were added to a reaction mixture containing sodium dodecyl sulfate, acetic acid (pH 4.5), and thiobarbituric acid. Following boiling the mixture for 30 min at 85°C, the absorbency of the supernatant was measured by spectrophotometry at 450 nm.[18]

Total antioxidant capacity

To measure the total antioxidant capacity (TAC), an acquisition kit (Cat No: TAC-96A, ZellBio GmbH, Germany) was purchased. The kit contains one reagent ready to use, buffer (200×), dye powder, reaction suspension solution, and standard and a microplate of 96 wells. In this assay, the TAC was equivalent to some antioxidant in the sample that was compared with ascorbic acid as standard. The kit's sensitivity was equal to 0.2 mM and final absorbance was read at 540 nm, and unit conversion was performed.[18]

Statistical analysis

The data were analyzed by SPSS software (SPSS, New York: IBM, SPSS version 16.0) using one-way ANOVA postulation followed by Tukey's *post hoc* test, and $P < 0.05$ was considered statistically significant. The variables were represented as the mean ± standard error of mean.

Results

Motility and viability

MYL caused a significant decline in viability and progressive motility compared to the control group ($P < 0.05$). No significant variations were detected in the *T. vulgaris* groups in comparison with the control group ($P > 0.05$). Furthermore, viability and progressive motility in completely treated *T. vulgaris* and MYL + *T. vulgaris* groups improved significantly in comparison with the MYL group [$P < 0.05$, Table 1].

Count and morphology

The sperm count and morphological normality reduced significantly in the MYL group equated to the control group ($P < 0.05$). No significant deviances were realized in the *T. vulgaris* groups in comparison with the control group ($P > 0.05$). However, the sperm count and normal morphology were improved significantly in all treated *T. vulgaris* and MYL + *T. vulgaris* groups compared with the MYL group ($P < 0.05$) [Figure 1 and Table 1].

Seminiferous tubules

MYL caused a significant decline in the germinal layer of seminiferous tubule height in comparison with the control group ($P < 0.05$). No significant changes were observed in comparison with the control group ($P > 0.05$). Germinal layer of seminiferous tubule height in completely treated *T. vulgaris* and MYL + *T. vulgaris* groups improved significantly compared to the MYL group [$P < 0.05$, Figures 2 and 3].

Testosterone level

MYL affected a significant reduction in the testosterone hormone level compared to the control group ($P < 0.05$). No significant alterations were detected in the *T. vulgaris* groups in comparison with the control group ($P > 0.05$). In addition, the level of testosterone hormone in all treated *T. vulgaris* and MYL + *T. vulgaris* groups improved significantly compared to the MYL group [$P < 0.01$, Figure 4].

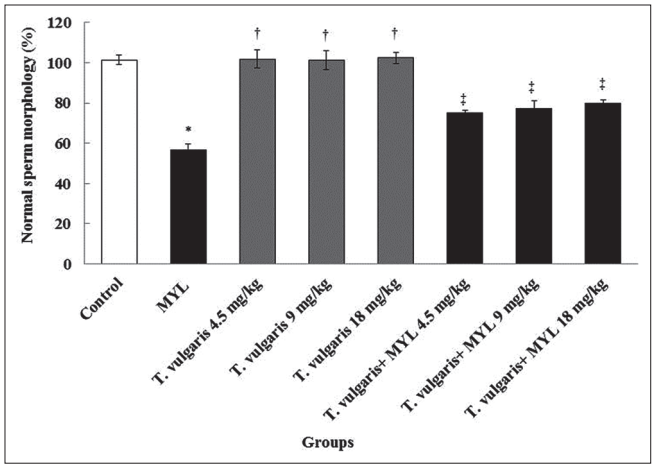

Figure 1: Comparison of normal sperm cell morphology in the treatment groups. *Significant different compared to the normal control group ($P < 0.05$). †Significant different compared to the MYL control group ($P < 0.05$). ‡Significant different compared to the MYL control group ($P < 0.05$). MYL = Myleran

Malondialdehyde

Levels of MDA revealed a significant growth in the MYL group compared to the control group ($P < 0.05$). Correspondingly, a significant reduction in MDA levels was shown in all THYM and *T. vulgaris* + MYL groups compared to the MYL group ($P < 0.05$), although had no significant effect on the levels of MDA in all *T. vulgaris* groups compared to the control group [$P > 0.05$, Figure 5].

Total antioxidant capacity

The consequences of measured TAC levels in the study groups displayed a significant reduction in the MYL group compared to the control group ($P < 0.05$). Furthermore, a significant rise in TAC levels was displayed in the completely treated *T. vulgaris* and *T. vulgaris* + MYL groups equated to the MYL group ($P < 0.05$), although had no significant effect on the levels of TAC in all *T. vulgaris* groups compared to the control group [$P > 0.05$, Figure 6].

Discussion

Chemotherapy as an agent of oxidative stress production in the body is able to disrupt the spermatogenesis

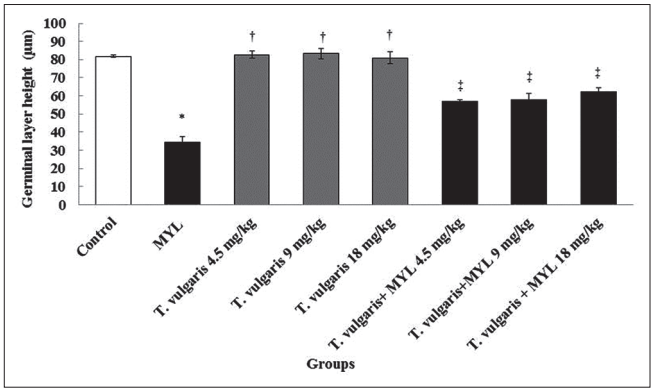

Figure 2: Comparison of germinal layer seminiferous tubule height in the treatment groups. *Significant different compared to the control group ($P < 0.05$). †Significant different compared to the MYL group ($P < 0.05$). ‡Significant different compared to the MYL group ($P < 0.01$). MYL = Myleran

Table 1: Effect of MYL and *T. vulgaris* on sperm parameters in male rats (n=8 for each group)

Groups	Mean of sperm count (106)	Sperm progressive motility (%)	Sperm viability (%)
Control	83.17±2.16	16.9±1.02	73.35±1.24
MYL	32.11±1.05*	2.23±0.41*	37.03±1.55*
THYM 4.5 mg/kg	82.75±2.63†	20.12±1.01†	77.61±4.09†
THYM 9 mg/kg	85.12±4.17†	22.07±1.54†	75.05±3.74†
THYM 18 mg/kg	86.25±4.09†	23.11±0.61†	78.22±1.47†
THYM+MYL 4.5mg/kg	48.50±2.51¶	8.14±1.63¶	52.05±4.18¶
THYM+MYL 9 mg/kg	52.36±3.17¶	7.27±0.91¶	53.32±2.13¶
THYM+MYL 18mg/kg	57.25±4.23¶	9.15±1.10¶	58.51±3.34¶

Data are presented as mean±SEM. * P<0.05 compared to the control group. † P<0.05 compared to MYL group. ¶ P<0.05 compared to the MYL group. THYM: Thymus vulgaris, MYL: Myleran

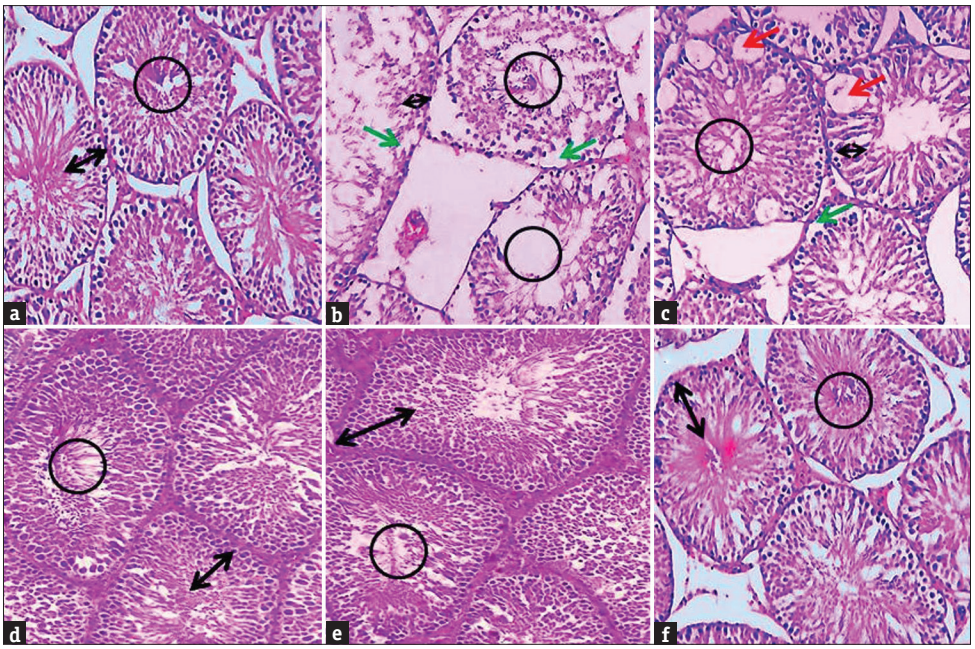

Figure 3: Normal seminiferous tubule structure was observed in control (a), *T. vulgaris* (18 mg/kg) (f), *T. vulgaris* + MYL (9 mg/kg) (d), and *T. vulgaris* + MYL (18 mg/kg) (e). Decrease in height of germinal layer, destruction of cell sequence, vacuolization and reduced sperm cell density were observed in MYL group (b and c). Black arrow: germinal layer height, circle mark: sperms density, green: irregularities in the margin of tubules (destruction of the membrane seminiferous tubules structure), and red identifies vacuolization. *T. vulgaris* = *Thymus vulgaris*, MYL = Myleran

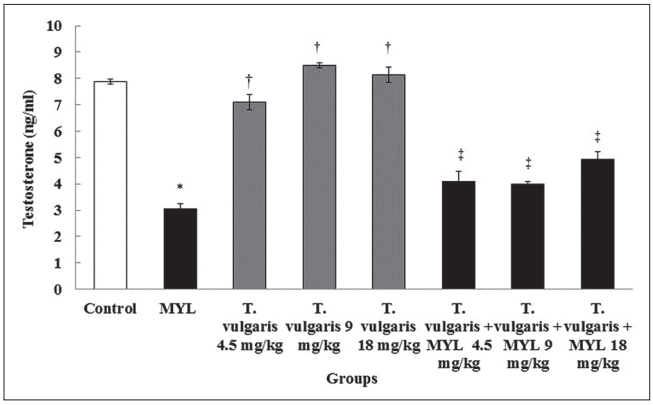

Figure 4: Comparison of testosterone hormone level in the treatment groups. *Significant different compared to the control group ($P < 0.05$). †Significant different compared to the MYL group ($P < 0.05$). ‡Significant different compared to the MYL group ($P < 0.05$). MYL = Myleran

process, reduce the production of spermatozoids, increase the number of abnormal spermatozoids, and decrease fertility rate.[19] Thus, the simultaneous use of potential antioxidant compounds and chemotherapy drugs has dramatically increase the protective effects for cells against the destructive side effects of free radicals.[20] The findings of this study proposed that the MYL had destructive testicular effects and sperm parameters, oxidant–antioxidant disorganization, and enhancement of testosterone hormone level. On the other hand, *T. vulgaris* as a phytoestrogen relief destructive effects of MYL administration. It also restores the cell damage caused by decreased level of MDA. The results of the current study also showed that *T. vulgaris* is able to reduce the lipid peroxidation (decreased MDA) and increase antioxidant capacity (increased TAC) of testis tissue; thus, it is reducing oxidative stress. Consistent with these findings, a large body of studies has shown antioxidant properties of *T. vulgaris*.[12-14] Seemingly, *T. vulgaris* inhibits tert-butyl-hydroperoxide-induced lipid peroxidation in sperms. *T. vulgaris* is also a lipophilic molecule that is able to prevent lipid peroxidation through Fenton reaction.[13] Thus, it appears that *T. vulgaris* with its antioxidant properties could reduce MDA and increase TAC in the treatment groups by inhibiting the production of ROS. The present study also indicated the recovery effects of *T. vulgaris* on some male reproductive parameters as well as decreasing the oxidative stress by showing declining of MDA level. Because sperms lose a large amount of their cytoplasm during spermatogenesis (lack of antioxidant systems), they apparently have a higher sensitivity to increased ROS than somatic cells.[11] The first outcome of the ROS attack to the membrane structures can be cellular peroxidation within the membrane of cells and organelles.[10] Antioxidants such as *T. vulgaris* eliminate toxins and free radicals from the cell surroundings, and inhibit lipid peroxidation, which results in the maintenance of the cellular biochemical structure.[11] The results of the present study showed that all sperm parameters in the MYL control group reduced significantly compared to the control group. In the *T. vulgaris* and MYL + *T. vulgaris* groups, a significant increase was

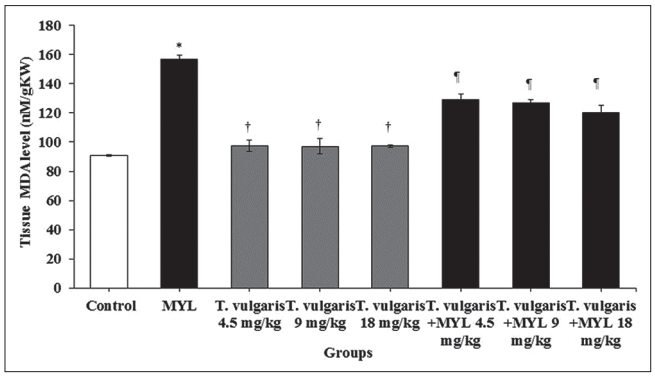

Figure 5: Comparison of testis MDA level between groups. **P* < 0.05 compared to the control group. †*P* < 0.05 compared to the MYL group. ¶*P* < 0.05 compared to the MYL group. MYL = Myleran, MDA = Malondialdehyde

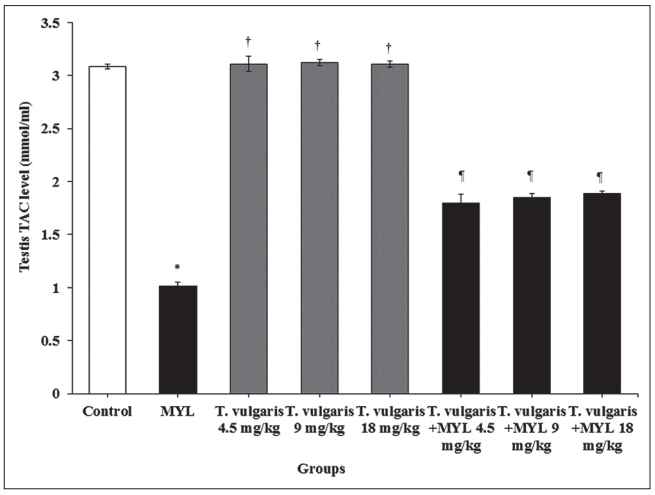

Figure 6: TAC level changes in the male rats. **P* < 0.05 compared to the control group. †*P* < 0.05 compared to the MYL group. ¶*P* < 0.05 compared to the MYL group. MYL = Myleran, TAC = Total antioxidant capacity

observed in all sperm parameters compared to the MYL group. Spermatogenesis is a highly complicated process that is influenced by numerous factors, which consequently leads to male infertility or reduced fertility status.[17] One of these factors is oxidative stress, which is induced as a result of the accumulation of ROS due to an imbalance between antioxidants and oxidants.[10] ROS can affect DNA and RNA synthesis and inhibit mitochondrial function.[14] It seems that oxidative stress induced by MYL administration causes dysfunctions in cell divisions and sperm differentiation.[21] The findings of Aitken and Baker confirmed the outcomes of this study in which that oxidative condition disturbed spermatogenesis leading to damaged gametes with remodeled chromatin. Thus, these sperms were disposed to be attacked by free radicals and reduced the number of spermatozoid cell lines.[22] Decrease of sperm parameters in the MYL group returns to the direct increase of oxidative stress-induced lipid peroxidation, which can alter the cell membrane structure, leading to loss of sperms transferring to epididymis as well as those within epididymis.[23] On the other hand, accumulated ROS induces mitochondrial damage and causes release of pro-apoptotic factors within intermembrane space.[10] MYL induces damage to DNA and destruction cell by increase in the expression of STRA8, MAK mRNA, and RAD51.[23] Bahmanpour *et al*. administrated MYL for male Wistar rats. They found decreased number of spermatogonial cells, spermatids, Leydig and Sertoli cells compared to the control group, confirming the results of the current research.[24] Increased free radicals due to the MYL administration can impair the Sertoli cells and destroy the cytoplasmic bridges through the loss of epithelial cells, thereby reducing the sperm count and elevating sperm deformity status.[16] It seems that MYL increases the number of immature Sertoli cells and cytokeratin 18 and consequently induces impaired spermatogenesis and infertility.[25] Reduced MYL-induced sperm motility may be due to the effect of drugs on DNA or impairment of mitochondria, an important organelle involved in sperm motility and ATP supply.[8] *T. vulgaris* seems to have inhibitory effects on free radicals, to possess antioxidant properties, and to increase the number of antioxidative enzymes.[14] The antioxidant actions of *T. vulgaris* are related to the sugar moiety in the *T. vulgaris* molecule which has a vital role in its chemical reactivity.[26] Maia *et al*. showed that *T. vulgaris* improved sperm parameters, such as count, motility, and viability in diabetic rats, which is in line with the results of the present study.[13] In fact, *T. vulgaris* prevents the formation of free radicals and lipid peroxidation.[13] *T. vulgaris* can inhibit the expression of MMP and proinflammatory factors such as tumor necrosis factor (TNF)-α, NF-Kβ, interleukin-1 β, and interleukin-6. It may also exert its antiapoptotic and cytoprotective effects by suppressing the expression of caspase-3 and TNF-α.[27] *T. vulgaris* seems to play a role in elevating sperm motility and viability by promoting the sperm antioxidant defense system, including superoxide dismutase, glutathione peroxidase, and catalase.[28] This study concluded that serum testosterone level and germinal layer thickness of seminiferous tubules was significantly attenuated in the MYL group than the normal control group. There was also a significant increase in the level of testosterone hormone and germinal layer height of seminiferous tubules in all *T. vulgaris* and *T. vulgaris* + MYL groups than MYL. Further, histological assessments showed loss of natural form, order, and consistency of cells of seminiferous tubule walls, creating vacuoles in them after all. Development of vacuole in the testis can be indicative of the effect of the oxidative stress

mechanism. It seems that MYL as an alkylating factor causes cell and DNA destruction, thus decreasing the thickness of the germinal layer in the testis.[29] The results of Vahdati *et al*. were in agreement with the findings of this research, indicating that MYL reduced the diameter of seminiferous tubules and sperm count, motility, and viability, increased the abnormal sperms, and decreased the epithelial thickness of seminiferous tubules.[30] As an antioxidant, *T. vulgaris* not only inhibits peroxidation of lipid and testicular oxidative stress but also plays a key role in the production of steroids in testis.[11] In addition, *T. vulgaris* reduces the effects of the oxidative stress induced under various conditions and empower of cells to cope with these conditions by preventing the reduction of glutathione and increasing antioxidant capacity.[31] The findings of Waly et al. were in line with our study, indicating that the phytoestrogens, including *T. vulgaris* attaches to the estrogen receptors in testis and stimulates spermatogenesis.[32] The present study showed that MYL-induced male reproductive damage in rats could be reduced by plant antioxidants such as *T. vulgaris*. Therefore, according to the previous data, THYM can improve some male reproductive dysfunctions, which has been caused by MYL-induced toxicity considering its antioxidant properties.

Conclusion

The results showed that MYL can produce damage to various reproduction values of male. Moreover, it was shown that a *T. vulgaris* scavenges the oxidation agents. It was concluded that *T. vulgaris* increases spermatozoa quality, normal morphology, viability, height of germinal layer seminiferous tubules, TAC, motility, and count. This also reduces MDA level in the testes. *T. vulgaris* can be used for subinfertile men. Antioxidant feature of *T. vulgaris* is known as the crucial cause of reproductive parameter enhancement. More investigations are needed to explore the exact mechanism of molecular action.

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Conflicts of interest

There are no conflicts of interest.

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