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Original article

Therapeutic potential of diosgenin against methotrexate-induced testicular damage in the rat

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ABSTRACT

This study evaluated diosgenin effects on methotrexate-induced testicular injury in the rats. A single dose of methotrexate (MTX) (20 mg/kg, i.p) was administered, followed by two weeks of diosgenin treatment via gavage starting one day before methotrexate injection. Testicular damage was evaluated through histological examination of seminiferous tubules, as well as analysis of serum testosterone level, oxidative stress and inflammation biomarkers, and antioxidant levels. The results of this study showed that in the MTX-exposed group, oxidative stress indices of malondialdehyde (MDA), reactive oxygen species (ROS), nitrite and indices of inflammation consisting of tumor necrosis factor α (TNFα), and interleukin 6 (IL-6) have a significant increase compared to the control group. Additionally, reductions were observed in antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). In addition, testosterone level decreased and signs of testicular damage were observed in the MTX group. Conversely, in the group treated with diosgenin alongside MTX at a dosage of 50 mg/kg, there was a significant decrease in oxidative stress markers (MDA, ROS, nitrite) and inflammatory markers (TNFα and IL-6). Moreover, there was a significant increase in the levels of antioxidant enzymes (SOD, CAT, and GSH). Diosgenin appears to have the potential to protect testicular tissue from damage caused by the toxic effects of MTX through the reduction of oxidative stress and inflammation.

1. Introduction

Testicular tissue is involved in the production of sperm germ cells and hormones. Germ cells transmit genetic information from one generation to the next [1]. Congenital and genetic factors such as testicular failure, disease, radiation, drugs and trauma cause testicular dysfunction and infertility by causing biochemical and hormonal changes [2]. Oxidative stress causes damage to the testicular by upsetting the balance between the production of free radicals and antioxidants within the cell [3]. Methotrexate is used in the treatment of various cancers and by increasing oxygen metabolites called reactive oxygen species (ROS) and enzyme inhibition, it increases oxidative stress and destroys the cell membrane structure [4]. Methotrexate leads to oxidative stress by increasing cellular lipid peroxidase [5,6]. Increases plasma SOD and MDA levels and decreases CAT levels and body weight [7,8]. Methotrexate increases inflammation and apoptotic factors and causes infertility by increasing the interstitial space of the testicular and causing severe edema and disruption of the seminal vesicle, reducing the diameter of the tubes and reducing the weight of the testicles and incomplete spermatogenesis [9,10].

Diosgenin is a steroidal sapogenin used in the synthesis of steroid derivatives and sex hormones [11,12]. According to studies, in diabetic mice, diosgenin significantly improved serum insulin and testosterone levels, reducing inflammatory factors IL-6 and TNFα. It prevented damage to the seminiferous tubules and increased the number and motility of sperm [13]. Diosgenin increased hepatic insulin levels and glycogen content inhibited oxidative stress in pregnant mice with diabetes, and increased glutathione levels and superoxide dismutase and catalase activities [14]. and by inhibiting apoptosis and reducing ROS levels, diosgenin prevents high-glucose-induced myocardial damage [15]. Diosgenin has a beneficial effect on oxidative stress caused by oxygen-free radicals and can be effective in treating male infertility

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[16]. Numerous animal studies and clinical trials have demonstrated that diosgenin can lower blood lipids by attenuation of plasma low-density lipoprotein and elevating high-density lipoprotein, as reviewed before [17]. Of relevance to this study, it has been shown that natural compounds such as diosgenin are capable to enhance ejaculation control in individuals suffering from premature and erectile dysfunction without any side effects [18]. Since no study has been conducted on diosgenin effect after MTX toxicity, this study was designed to evaluate the effects of diosgenin on methotrexate-induced testicular damage and to unravel the involvement of oxidative stress and inflammation.

2. Materials and methods

2.1. Animals

In this study, 40 male Wistar rats weighing (200–250 g) at (21–22°C) temperature, and 12-hour dark-light cycle and, 30–40 % humidity were kept in the Animal Studies Center of Shahed University (Tehran, Iran). Water and food were provided to them without any restrictions. The rats were adapted to the laboratory for at least one week before performing any experiments. All rats were tested according to the standards set by the NIH guidelines. The project was approved in 2020 by the Shahed University Ethics Committee.

2.2. Experimental strategy

The rats were randomly divided into five equal groups (n = 8 per group) consisting of a control group, diosgenin-treated control at 50 mg/kg/b.w, methotrexate, and two groups of diosgenin-treated-methotrexate at doses of 10 and 50 mg/kg/b.w were classified. After two weeks of adaptation, the rats were weighed and the dose was determined based on the weight of the rats. The control group did not receive any medication. The control group treated with diosgenin received 50 mg/kg/b.w diosgenin (Sigma-Aldrich, USA, purity>93 %) in the form of gavage for two weeks [19]. The methotrexate group received 20 mg/kg/b.w, methotrexate (Orion, Finland) single-dose i.p [20]. The two groups of methotrexate treated with diosgenin received 10 and 50 mg/kg/b.w diosgenin by gavage for two weeks, and one day after administration of diosgenin methotrexate at a dose of 20 mg/kg/b.w received a single dose intraperitoneally.

2.3. Biochemical studies

Following two weeks of gavage, rats were anesthetized using ketamine, and blood was collected via cardiac puncture into heparin tubes. The serum was then centrifuged at 106 g for 10 min and collected to analyze testosterone levels. Subsequently, the testes were immediately extracted. The right testicular was cleaned of excess tissues, weighed, washed with ice-cold phosphate-buffered saline (PBS) at pH 7.4, and homogenized in ice-cold Tris-HCl buffer (150 mM, pH 7.4). The resulting homogenates were then centrifuged at 5000 rpm for 10 min at 4 °C. The left testicular was fixed in a 10 % phosphate-buffered formalin solution and processed for routine light microscopic analysis.

2.3.1. Oxidative stress and antioxidant assessment

MDA content, indicating lipid peroxidation, was assessed using the thiobarbituric acid method and expressed as nmol MDA/mg of protein, with tetraethoxypropane serving as the standard [21]. The activity of the antioxidant enzyme superoxide dismutase (SOD) was determined following established procedures [21]. Briefly, the supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer for 40 min, followed by the addition of nitro blue tetrazolium (NBT), and the formation of blue formazan was monitored at 550 nm. The level of reduced glutathione (GSH) was measured spectrophotometrically using the Ellman method [22,23]. In summary, the homogenate was centrifuged with trichloroacetic acid, and the resulting supernatant was mixed with phosphate buffer and 5,5′-dithiobis (2-nitrobenzoic acid), with absorbance readings taken at 412 nm.

The activity of catalase was measured according to Claiborne's method [24]. Concisely, hydrogen peroxide was added to a mixture of 50 mM potassium phosphate buffer and supernatant, and the rate of its decomposition was measured at 240 nm. Protein content was determined using the Bradford method, with bovine serum albumin as the standard [25].

The nitrite content in the supernatant was assayed using the Griess method, as described in previous studies [26]. Nitric oxide (NO) has a short half-life and quickly converts to stable end products, nitrate (NO3-) and nitrite (NO2-). In this assay, $NO3^-$ is converted to $NO2^-$ by cadmium, followed by color development with Griess reagent (sulphanilamide and N-naphthyl ethylenediamine) in an acidic environment. The total nitrite concentration was measured through the Griess reaction, and absorbance was determined at 540 nm using a spectrophotometer.

The estimated level of ROS was evaluated using dichlorofluorescein diacetate, a non-fluorescent substance. This substance is cleaved by intracellular esterase enzymes in the presence of ROS, resulting in the formation of 2,7-dichlorofluorescein which fluoresces [27,28]. Fluorescence intensity was measured at 488 nm excitation and 525 nm emission wavelengths. A standard curve was constructed using dichlorofluorescein (DCF).

2.3.2. Assessment of inflammation

The testicular level of TNFα and IL-6 was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). For TNFα measurement, a primary rabbit anti-TNF alpha antibody from Abcam (USA) was utilized, while for IL-6 measurement, an anti-IL-6 antibody (Santa Cruz Biotechnology, USA) was employed. Subsequently, a secondary anti-rabbit IgG-peroxidase antibody raised in goat (Sigma-Aldrich, USA) was used.

The assay was conducted following the instructions provided by Abcam at the following link: http://www.abcam.com/protocols/sandwich-elisa-protocol-1. The absorbance of samples was read at 450 nm by a Synergy HT microplate reader (BioTek, USA) and their final concentrations were obtained from plotted and customized standard curves.

2.4. Histological studies

For histological assessment, the left testes were fixed in 10 % phosphate-buffered formalin solution for 3 days and processed for routine light microscopic analysis. Testicular tissue was sectioned at a thickness of 5 μm, stained with Hematoxylin and Eosin, and then subjected to dehydration, clearing, and mounting with Entellane (Merck Co., Germany), followed by coverslip. At least, three slides from the upper, lower, and mid-portions of the testicular were thoroughly examined. To assess testicular and spermatogenesis damage, Johnsen's mean testicular biopsy score (MTBS) was used [29,30]. This scoring system assigns 0–10 to each seminiferous tubule, indicating the degree

Table 1
Mean testicular biopsy score (MTBS) classification.

Score	Description
1	No cells
2	Sertoli cells without germ cells
3	Only spermatogonia
4	Only a few spermatocytes
5	Many spermatocytes
6	Only a few early spermatids
7	Many early spermatids without differentiation
8	Few late spermatids
9	Many late spermatids
10	Full spermatogenesis

of germinal epithelial maturation and damage (Table 1). In addition, mean seminiferous tubular diameter (MSTD) was determined. Quantitative analysis was performed using ImageJ software (Version 1.49).

2.5. Statistical analysis

All data are presented as means ± SEM. Statistical analysis was performed using SigmaPlot software (version 12). Inter-group comparisons were performed via parametric one-way ANOVA followed by Tukey post-hoc multiple comparison test. The significance level was set at $p < 0.05$.

3. Results

3.1. The effect of diosgenin on testicular indices of oxidative stress and antioxidants

Oxidative stress typically rises while enzymatic and non-enzymatic antioxidant defenses decrease after chemotherapy. In our study, various oxidative stress parameters in the testes (Table 1). The results

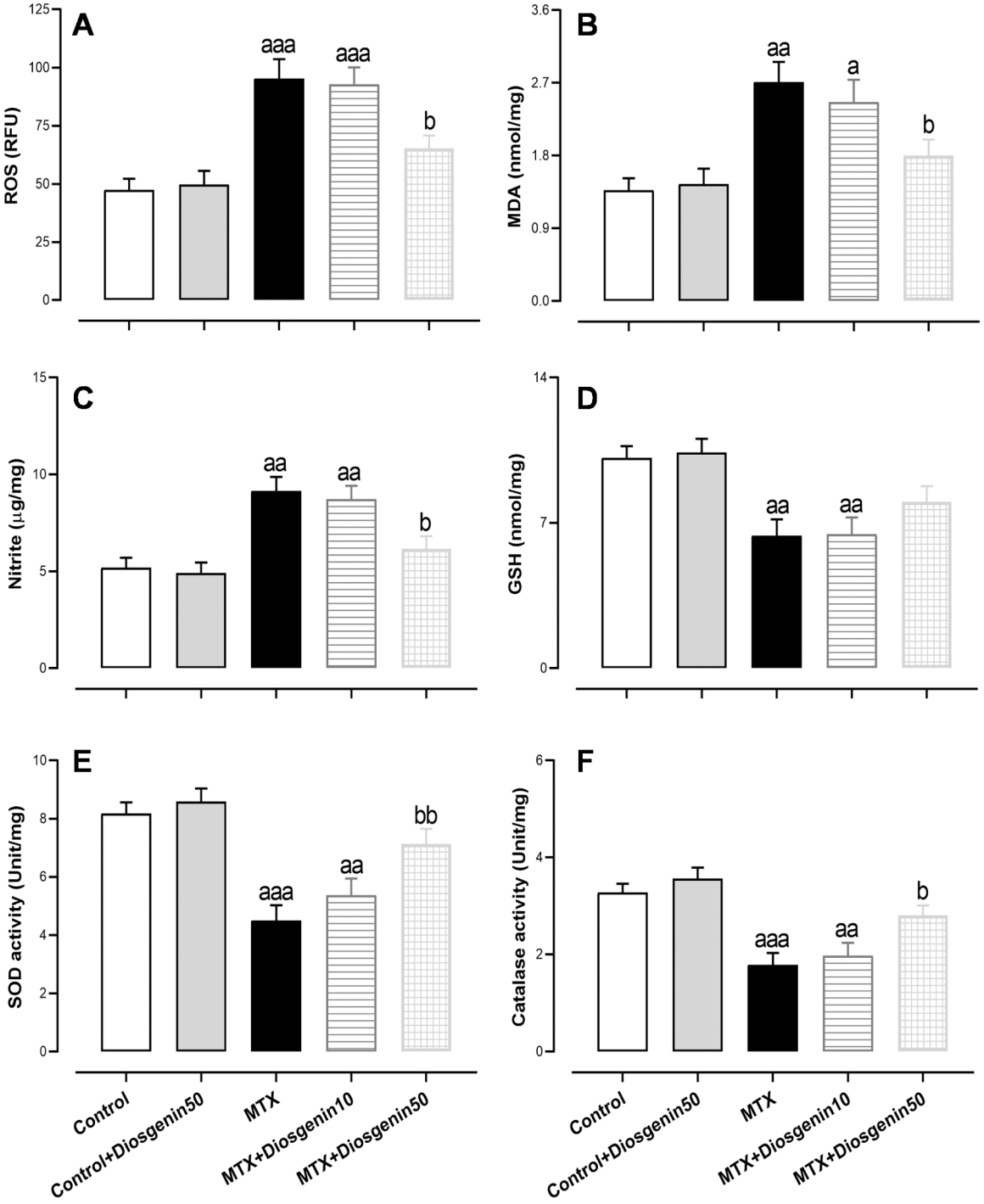

Fig. 1. Testicular level of ROS (A), MDA (B), nitrite (C) and activity of SOD (D), GSH (E), and CAT (F) in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 8 for each group). a $p < 0.05$, aa $p < 0.01$, aaa $p < 0.001$ (versus the control); b $p < 0.05$, bb $p < 0.01$ (versus the MTX).

demonstrated a significant increase in oxidative stress markers in the MTX group compared to the control group. These markers including levels of ROS (Fig. 1A) ($p < 0.001$), malondialdehyde (MDA) (Fig. 1B) ($p < 0.01$), nitrite (Fig. 1C) ($p < 0.01$), glutathione (GSH) (Fig. 1D) ($p < 0.01$), superoxide dismutase (SOD) (Fig. 1E) ($p < 0.001$), and catalase (CAT) (Fig. 1F) ($p < 0.001$).

In contrast, in the MTX-treated group receiving diosgenin, oxidative stress markers decreased and levels of antioxidant agents increased compared to the MTX group alone. Specifically, in the MTX-treated groups supplemented with diosgenin at a dose of 10 mg/kg, no significant changes were observed in ROS, MDA, nitrite, GSH, SOD, or CAT levels compared to the MTX group (Fig. 1A-F) ($p > 0.05$). However, in the MTX-treated groups supplemented with diosgenin at a dose of 50 mg/kg, significant reductions were noted in ROS, MDA, and nitrite levels, along with increased SOD and CAT levels compared to the MTX group (Fig. 1A-F) ($p < 0.05$ for ROS, MDA, nitrite; $p < 0.01$ for SOD; $p < 0.05$ for CAT).

Furthermore, no significant alterations were observed in the oxidative stress and antioxidant indices in the control group treated with diosgenin at a dose of 50 mg/kg compared to the untreated control group. This suggests that diosgenin treatment did not impact normal oxidative stress and antioxidant levels in the absence of methotrexate-induced stress.

3.2. The effect of diosgenin on testicular markers of inflammation

Methotrexate is known for its ability to increase inflammatory factors, and in our study, we measured testicular indicators of inflammation, including IL-6 and TNFα. Our findings showed a significant increase in inflammation markers in the MTX group compared to the control group, with IL-6 (Fig. 2A) ($p < 0.001$) and TNFα (Fig. 2B) ($p < 0.001$) levels being notably elevated.

In the MTX group treated with diosgenin at a dose of 10 mg/kg, no significant change was observed in IL-6 levels compared to the MTX group (Fig. 2A) ($p > 0.05$). However, in the MTX group treated with diosgenin at a dose of 50 mg/kg, IL-6 levels were significantly reduced compared to the MTX group (Fig. 2A) ($p < 0.05$). Additionally, TNFα levels were significantly lower in the MTX treated with diosgenin at 50 mg/kg compared to the MTX group (Fig. 2B) ($p < 0.01$). Furthermore, no significant changes were observed in these inflammation

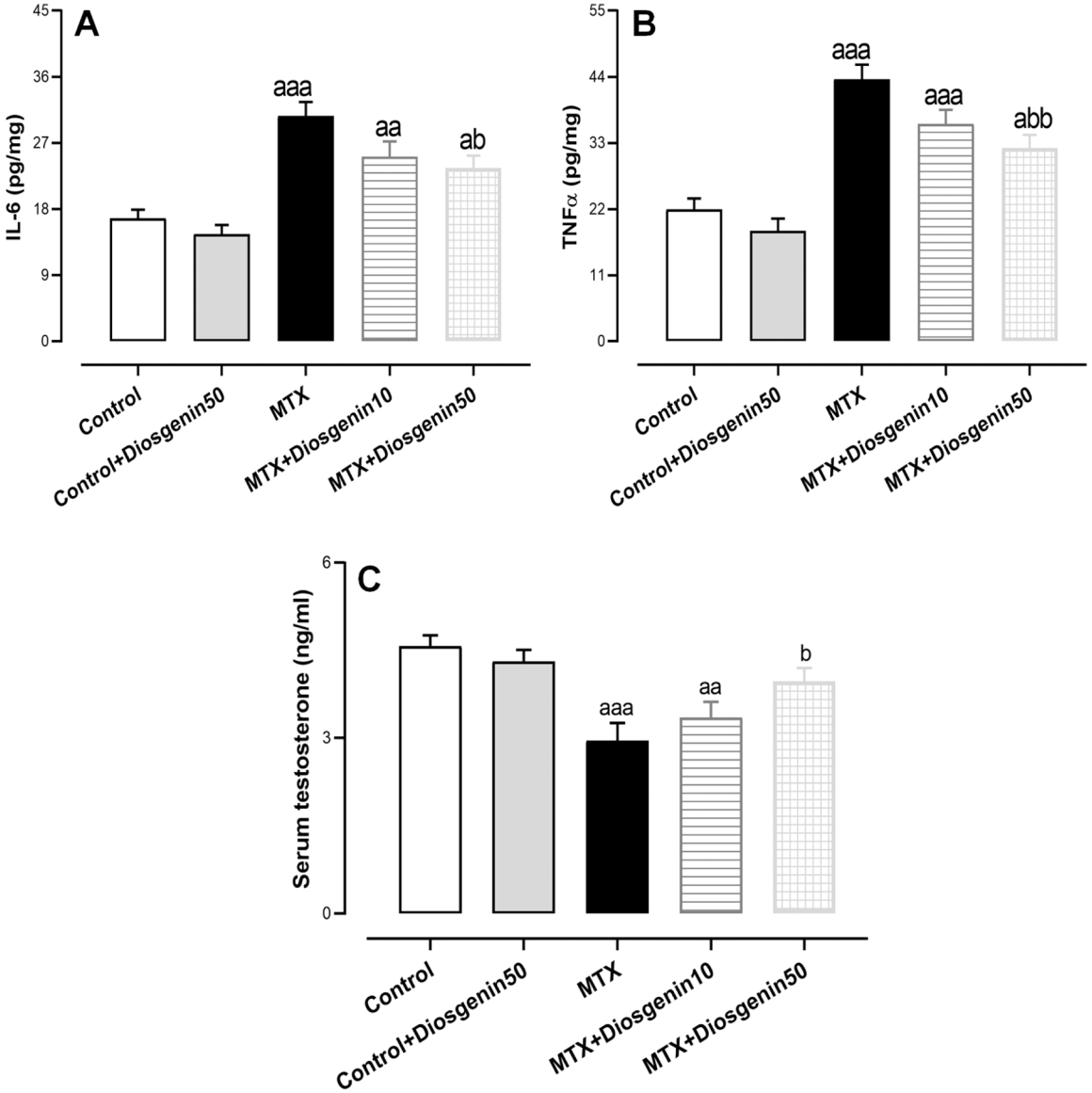

Fig. 2. Testicular level of TNFα (A) and IL-6 (B) and serum testosterone (C) level in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 8 for each group). a $p < 0.05$, aa $p < 0.01$, aaa $p < 0.001$ (versus the control); b $p < 0.05$, bb $p < 0.01$ (versus the MTX).

indices in the control group treated with diosgenin at 50 mg/kg compared to the untreated control group. This suggests that diosgenin treatment did not impact normal inflammation levels in the absence of methotrexate-induced inflammation.

3.3. The effect of diosgenin on serum levels of testosterone

Methotrexate significantly reduced serum testosterone levels compared to the control group (Fig. 2C) ($p < 0.001$). However, in the methotrexate-treated groups supplemented with diosgenin at doses of 10 mg/kg and 50 mg/kg, there was a notable increase in serum testosterone levels compared to the methotrexate group (see Fig. 2C) ($p < 0.01$ for 10 mg/kg diosgenin; $p < 0.05$ for 50 mg/kg diosgenin). Additionally, no significant alterations were observed in serum testosterone levels in the control group treated with diosgenin at 50 mg/kg compared to the untreated control group. This suggests that diosgenin treatment did not impact normal serum testosterone levels in the absence of methotrexate-induced testosterone reduction.

3.4. The effect of diosgenin on testicular histology

The histological analysis of the testicular showed that the MTX group exhibited damage to seminiferous tubules, as indicated by significantly lower mean testicular biopsy score (MTBS) (Fig. 3A) ($p < 0.001$) compared to the control group. In the MTX-treated groups supplemented with diosgenin at a dose of 10 mg/kg, there was also significant reduction of MTBS ($p < 0.001$) compared to the MTX group. However, in the MTX-treated group supplemented with diosgenin at a dose of 50 mg/kg, there was no significant reduction of MTBS ($p > 0.05$). In addition, MTBS score was significantly higher in the MTX group supplemented with diosgenin at a dose of 50 mg/kg when compared to the MTX group ($p < 0.01$). Regarding mean seminiferous tubular diameter (MSTD) (Fig. 3B), there was no significant differences amongst the groups ($p > 0.05$). Fig. 4 shows comparable photomicrographs of the seminiferous tubules in different groups.

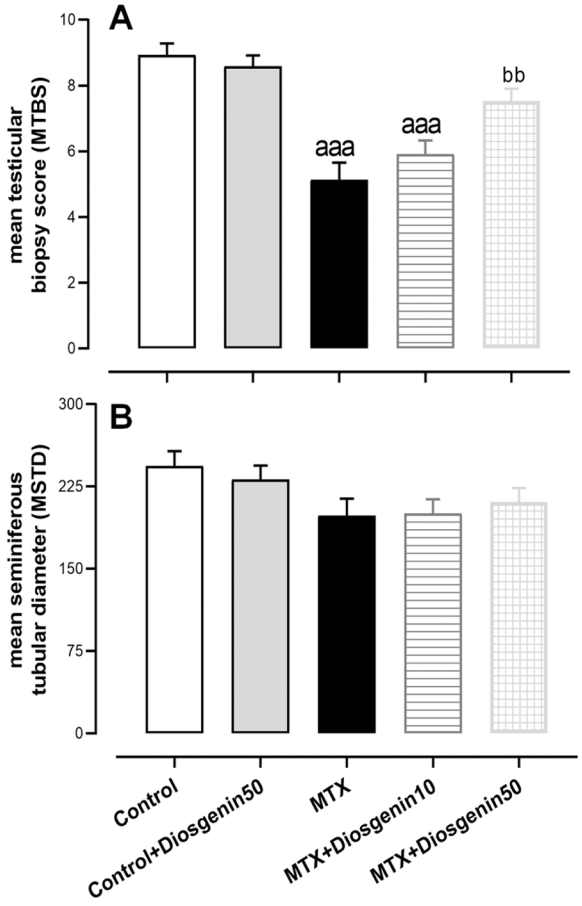

Fig. 3. Johnsen's mean testicular biopsy score (MTBS) (A) and mean seminiferous tubule diameter (MSTD) (B) in different groups. All data are presented as mean ± S.E.M. ($n = 6$ for each group). aaa $p < 0.001$ (versus the control); bb $p < 0.01$ (versus the MTX).

4. Discussion

Infertility disorders are widespread among men, and oxidative stress is identified as a significant contributing factor [31]. Oxidative stress occurs when there's an imbalance between oxidants, such as reactive oxygen species (ROS), and antioxidants in the body, resulting in tissue damage and destruction [32,33]. Methotrexate, a frequently prescribed medication for a range of medical conditions, including cancer and autoimmune diseases, has been implicated in disrupting this delicate balance by increasing oxidative stress levels. Consequently, this imbalance can lead to damage and disruption of the testis, ultimately contributing to infertility [33,34].

In this study, we investigated the potential of diosgenin in alleviating the determined impacts of methotrexate on testicular function. Diosgenin, known for its potent antioxidant properties, emerges as a promising contender for counteracting this oxidative stress cascade. By effectively scavenging ROS and enhancing the body's natural antioxidant mechanisms, diosgenin works to restore the redox balance within the testes. Consequently, is attenuates oxidative damage and helps preserve testicular function.

Our result demonstrate that oral administration of diosgenin to the MTX-exposed groups led to a significant decrease in complications associated with methotrexate, particularly testicular dysfunction stemming from oxidative stress in germ cells [35]. Methotrexate triggers the generation of ROS through several mechanisms, including inhibition of tetrahydrofolate production, which disrupts normal cellular functions. These ROS have the potential to cause significant damage to cell membranes, inhibit enzymatic activities, and increase oxidative stress by inducing lipid peroxidation. [6,7]. Diosgenin, by virtue of its antioxidant properties, scavenges these ROS and helps restore the redox balance within the testicular environment. This protective effect of diosgenin against oxidative stress-induced damage is further supported by previous research demonstrating its ability to increase glutathione levels, reduce malondialdehyde levels, and alleviate tissue damage in various pathological conditions, such as Parkinson's disease and diabetes [36].

This improvement was associated with increased glutathione levels and reduced malondialdehyde levels in striatal tissue. Additionally, diosgenin reduced glial fibrillary acidic protein levels and the number of bipolar neurons in the corpus striatum compared to the Parkinson's surgical group.

Similarly, Khosravi et al. reported that diosgenin has a protective effect against testicular damage induced by diabetes. Diosgenin treatment significantly improved serum insulin and testosterone levels in diabetic animals, indicating its potential therapeutic benefit in preserving testicular function under diabetic conditions. These studies collectively suggest that diosgenin's antioxidant properties contribute to its protective effects against oxidative stress-related tissue damage in various pathological conditions [13].

Increased inflammation leads to tissue damage characterized by elevated levels of tissue markers such as TNFα and IL-6. Moreover, diosgenin has shown promising anti-inflammatory effects in mitigating tissue damage associated with methotrexate administration. Our findings reveal that methotrexate exposure led to increased levels of inflammatory markers such as TNFα and IL-6, indicative of heightened inflammation within the testis. Treatment with diosgenin resulted in a significant reduction in these inflammatory factors, particularly at a dose of 50 mg/kg. This anti-inflammatory effect of diosgenin is

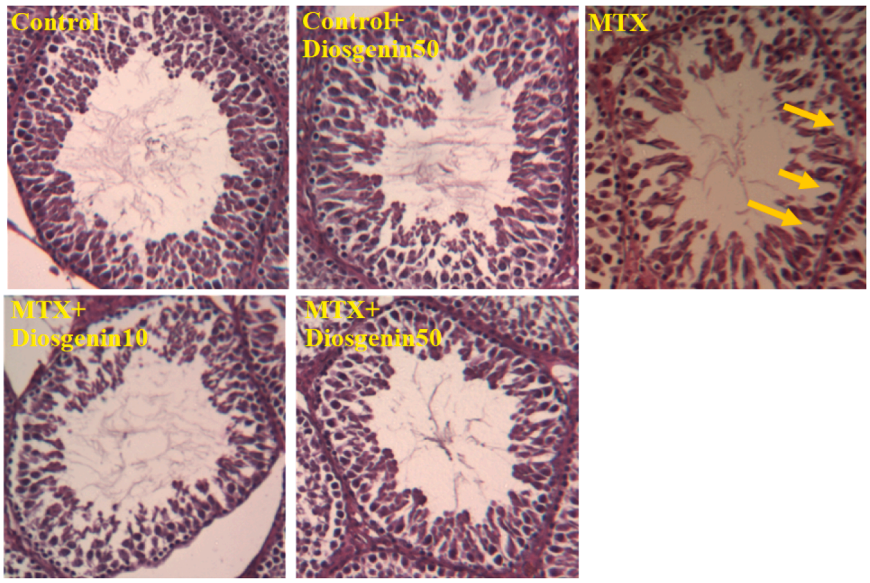

Fig. 4. Photomicrographs showing sections of seminiferous tubules in different groups stained with Hematoxylin and Eosin. Solid yellow arrows indicate some injured areas of the germinal epithelium.

consistent with previous research demonstrating its ability to attenuate inflammation in conditions such as acute liver damage and improve glucose tolerance while reducing levels of inflammatory cytokines and serum cardiac injury markers[14].

Additionally, Wu et al. demonstrated that long-term diosgenin treatment improved glucose tolerance, reduced levels of inflammatory cytokines (IL-1β, IL-6, TNF-α), and decreased serum levels of cardiac injury markers.

In this study, rats injected with MTX showed signs of severe injury to the seminiferous tubules, as demonstrated by significantly lower MTBS, which was in line with previous studies [37,38]. Conversely, diosgenin treatment was associated with protection of the testicular tissue against MTX, as was shown by higher rating for MTBS. In agreement with this finding, it has been shown that diosgenin with protective potential could ameliorate testicular injury in the streptozotocin-diabetic rats via attenuation of apoptosis, oxidative stress, and inflammation [39] and diosgenin is capable to ameliorate carbon tetrachloride-induced liver injury [40]. A significantly lower serum level of testosterone in the MTX-injured group indicated an injury to Leydig cells. This reduction was ameliorated following diosgenin treatment at 50 mg/kg. This clearly shows protective effect of diosgenin on the Leydig cells against MTX toxicity. Such beneficial effect of diosgenin has been shown before in diabetic rats [39].

Overall, the findings from this study underscore the potential therapeutic utility of diosgenin in protecting against the adverse effects of methotrexate on the testicular function. By targeting oxidative stress and inflammation, diosgenin offers a dual protective mechanism that helps maintain the structural and functional integrity of the testis, thus preserving male fertility. Further research is warranted to elucidate the precise molecular mechanisms underlying the protective effects of diosgenin and to explore its therapeutic potential in clinical settings.

Lack of assessment of testicular interstitial tissue and morphometric analysis of the sperms is one of the limitations of this study which should be considered in relevant studies. In addition, lack of determination of apoptotic factors besides not performing Western blotting experiments are other limitations of the present work.

5. Conclusion

According to the results of this study, it is evident that methotrexate administration leads to testicular damage and dysfunction by inducing oxidative stress and increasing inflammatory factors, potentially resulting in male infertility. However, diosgenin, known for its antioxidant and anti-inflammatory properties, acts by enhancing antioxidant activity and reducing inflammatory responses. As a result, diosgenin effectively mitigates the adverse effects of methotrexate on the testis. These results suggest that diosgenin exhibits significant therapeutic promise for addressing male infertility and may offer valuable treatment option for individuals experiencing testicular damage and disorder due to methotrexate toxicity.

Ethical approval statement

This study was approved by ethics committee of Shahed university (IR.SHAHED.REC.1398.120) at 2020.

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In this study, we do not have any funding statements.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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