International Immunopharmacology 70 (2019) 37–46

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Diosgenin ameliorates testicular damage in streptozotocin-diabetic rats through attenuation of apoptosis, oxidative stress, and inflammation

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ARTICLE INFO

Keywords:
Diosgenin
Diabetes mellitus
Testis
Apoptosis
Oxidative stress
Inflammation

ABSTRACT

Diabetes mellitus (DM) is a prevalent metabolic disorder that is associated with development of some complications in male reproductive system including testicular damage, sexual dysfunction, abnormal spermatogenesis, and infertility. Diosgenin is a natural steroidal saponin with anti-diabetic, anti-oxidative, and anti-inflammatory effects. This research study was undertaken to explore the protective effect of diosgenin against diabetes-induced testicular damage in the rat. Ten days following streptozotocin (STZ; *i.p.*), diosgenin was daily administered for 6 weeks (*p.o.*). Diosgenin administration to diabetic rats significantly improved body weight and lowered serum glucose. In addition, diosgenin-treated diabetic group had a significantly lower level of malondialdehyde (MDA), protein carbonyl, greater level of glutathione (GSH), and higher activity of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) in addition to testicular improvement of ferric reducing antioxidant power (FRAP). Furthermore, diosgenin significantly improved serum insulin and testosterone level and alleviated testicular markers of inflammation including tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) in diabetic rats. Moreover, apoptotic markers including caspase 3 activity, Annexin V, and DNA fragmentation decreased, mitochondrial membrane potential (MMP) accentuated, and myeloperoxidase (MPO) activity as a biomarker of neutrophil infiltration decreased in diosgenin-treated diabetic group. Additionally, diosgenin was capable to improve sperm count, motility, and viability in addition to prevention of damage to seminiferous tubules in diabetic animals. Collectively, diosgenin ameliorates testicular damage in DM, at least via partial suppression of apoptosis, oxidative stress, inflammation, and neutrophil infiltration and also via partial restoration of mitochondrial integrity.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder with a global incidence of about 347 million people in 2008 and its incidence is estimated to double by 2050 [1]. DM is typified by hyperglycemia phenotype as a result of disturbed insulin production and/or insulin action [2]. Chronic hyperglycemia in DM is associated with oxidative stress [3,4] and inflammation [5,6]. DM adversely affects male genital system with complications including testicular damage, sexual dysfunction, abnormal spermatogenesis, and infertility [7–10]. In this regard, it has shown that diabetes induces several malfunctions in male germ cells [11]. Apoptotic cell death plays an important role in testicular damage in DM [12]. Furthermore, diabetes is associated with a higher tissue rate of neutrophil infiltration, as shown by elevated activity of myeloperoxidase (MPO) [13] in addition to testicular mitochondria dysfunction, as demonstrated by a lower mitochondrial membrane potential (MMP) [14].

Currently, natural products have increasingly suggested as a pivotal treatment strategy for DM [15,16]. Since some phytochemicals have lower complications and are capable to exert multiple beneficial effects including stimulation of insulin secretion and regeneration of pancreatic islets, they have obtained much attention [17]. Diosgenin is a plant-derived steroidal saponin that is found out in medicinal plants like fenugreek. It is structurally related to cholesterol and used as a precursor for steroidal hormones synthesis [18,19]. Diosgenin has shown cardiovascular protective [20] and anti-diabetic effects [20,21] and is capable to ameliorate oxidative stress [22–24] and inflammation [22,25]. In addition, anti-apoptotic [26] and protective effects of diosgenin in different tissues [22,24,27,28] have been confirmed. Moreover, diosgenin could protect rats against myocardial inflammatory

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https://doi.org/10.1016/j.intimp.2019.01.047
Received 17 March 2018; Received in revised form 13 December 2018; Accepted 17 January 2019
Available online 19 February 2019

injury due to ischemia-reperfusion through suppression of inflammation and myeloperoxidase (MPO) [25]. This study was conducted to evaluate possible effect of diosgenin on alleviation of testicular damage in streptozotocin-diabetic rats and to assess involvement of apoptosis, oxidative stress, and inflammation in addition to its modulation of mitochondrial dysfunction and neutrophil infiltration.

2. Materials and methods

2.1. Animals

In this research study, 32 male Wistar rats (10–12 weeks old, a weight range of 200–250 g) were kept in an animal house with a temperature of 21–23 °C and 12/12 h light/dark cycle. Food and water were freely provided in the home cages. The rats were adapted to the environmental conditions for at least one week before conducting the experiments. Animals were handled in compliance with the standards stipulated in the NIH Guide for the Care and Use of Laboratory Animals and our project was also approved by Ethics Committee of Shahed University (Tehran, Iran) in 2016.

2.2. Experimental design

After adaptation, the animals were randomly assigned to four equal groups (n = 8 for each group), i.e., control, diosgenin-treated control, diabetic, and diosgenin-treated diabetic. In the control group, the rats daily received the vehicle by oral gavage during the whole course of the study. Diosgenin-treated control received diosgenin (SigmaAldrich, USA; purity ≥ 93%) *p.o.* at a dose of 40 mg/kg/day dissolved in Kolliphor (SigmaAldrich, USA). Rats were made diabetic by *i.p.* injection of streptozotocin (STZ; 60 mg/kg; Santa Cruz Biotechnology, USA) dissolved in cold saline with pH adjusted to 4.5 immediately before use [29,30]. The control and diabetic groups received only the vehicles. Ten days following STZ, overnight fasting blood samples were obtained from anesthetized rats (under diethyl ether) and serum glucose was assessed (glucose assay kit, ParsAzmun, Tehran). Animals with a serum glucose > 250 mg/dl were chosen for further experiments. Diosgenin-treated diabetic group received diosgenin similar to treated control group. Diosgenin treatment started 10 days following STZ injection and continued for 6 weeks. Dose of diosgenin was according to an earlier study on its anti-diabetic effect [20]. In this regard, the effective dose range for diosgenin to preserve endothelium-dependent arterial relaxation in a rat model of early-stage metabolic syndrome has been 10–50 mg/kg and according to a dose-response pattern [31]. Body weight was weekly determined and serum glucose level was determined 1 week before and on weeks 4 and 8 after STZ injection. Fig. 1 shows schematic experimental design of the study.

2.3. Determination of serum insulin and testosterone

After collecting blood via cardiac puncture and serum preparation, serum levels of testosterone and insulin were determined by testosterone (Cayman Chemical, USA) and insulin (Mercodia, Sweden) ELISA kits, respectively, according to their instructions.

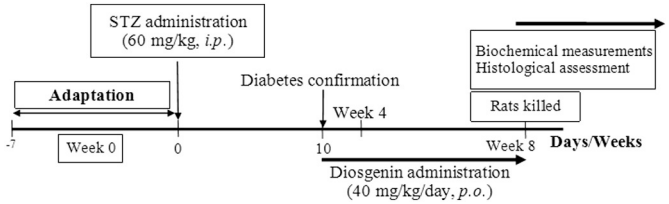

Fig. 1. Schematic experimental design of the study.

2.4. Biochemical studies

Rats were sacrificed by an overdose of diethyl ether and right testes were isolated, cleaned of excess tissues, weighed, washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4), and homogenized in cold lysis buffer containing protease inhibitor cocktail. The obtained homogenates were centrifuged at 2516 ×*g* for 10 min at 4 °C. All parameters were measured in duplicate. The obtained supernatant was used for determination of the following parameters:

Tissue level of MDA was determined using thiobarbituric acid method and was reported as nmole MDA/mg of protein with tetraethoxypropane as its standard [32,33].

Activity of the antioxidant enzyme superoxide dismutase (SOD) was assayed according to a previous report [34,35]. In short, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer for 40 min, and nitroblue tetrazolium (NBT) was added. Blue formazan formation was monitored at 550 nm.

Activity of catalase was measured according to Claiborne's method [36]. Concisely, hydrogen peroxide was added to a combination of 50 mM potassium phosphate buffer and supernatant and its rate of decomposition was measured at 240 nm.

Glutathione (GSH) level was determined in accordance to earlier reports [37,38]. Briefly, homogenate was centrifuged with trichloroacetic acid and obtained supernatant was mixed with phosphate buffer and 5′5 dithiobis (2-nitrobenzoic acid) and absorbance was read at 412 nm.

Measurement of glutathione peroxidase (GPX) activity was according to a method by Paglia & Valentine with some modifications [39]. For this purpose, changes of absorbance in the presence of H2O2, reduced glutathione, NADPH, sodium azide, and glutathione reductase were measured at 365 nm.

Bradford method was applied to determine protein content with bovine serum albumin as the standard [40].

The ferric reducing antioxidant power (FRAP) or total anti-oxidant capacity (TAC) was measured using the method of Benzi and Strain [41]. In this assay, freshly prepared FRAP reagent was mixed with tissue supernatant. After an incubation period of 15 min at 37 °C, changes of absorbance were obtained.

Protein carbonyl level as an alternate biomarker of oxidative stress and as an indicator of protein oxidation was determined as reported in literature [42,43]. Briefly, homogenate was re-centrifuged at 10,062 *g* for 20 min to isolate cytosolic portion and this was mixed with trichloroacetic acid at equal ratios. Then, dinitrophenyl hydrazine was added and it was kept for 60 min at room temperature. Pellet was washed 3 times with a mixture of ethanol-ethyl acetate and the pellet was dissolved using guanidine hydrochloride and absorbance was read at 366 nm.

The testicular level of TNFα and IL-6 was measured using sandwich enzyme-linked immunosorbent assay using primary rabbit anti-TNF alpha antibody (Abcam, USA) and anti-IL-6 antibody (Santa Cruz Biotechnology, USA) and secondary anti-rabbit IgG-peroxidase antibody raised in goat (SigmaAldrich, USA) and according to Abcam instructions (http://www.abcam.com/protocols/sandwich-elisa-protocol-1). The absorbance of samples was read at 450 nm by Synergy HT microplate reader (BioTek, USA) and their final concentrations were obtained from plotted and customized standard curves.

Mitochondrial membrane potential (MMP) as a consistent index of mitochondrial functional condition was determined in testicular supernatant. For this objective, obtained supernatant was re-centrifuged (10,062 ×*g* for 15 min). The formed precipitate includes mitochondrial fraction from testicular tissue. Assessment of MMP was done according to previous reports [44,45]. Mitochondrial fraction were incubated with 0.2 μmol/l of rhodamine 123 (Sigma-Aldrich, USA) at 37 °C for 5 min, and then the MMP was measured with excitation at 488 nm and emission at 525 nm using a fluorescent plate reader and fluorescence was shown as relative fluorescence unit (RFU).

For assessment of apoptosis, we measured testicular level of Annexin V, DNA fragmentation, and caspase 3 activity. Annexin V and DNA fragmentation were assessed using Elisa kit (MyBioSource, Inc., USA) and Cell Death Detection ELISA Plus kit (Sigma-Aldrich, USA), respectively and averaged ODs of samples were finally reported. Additionally, measurement of caspase 3 activity was measured as reported before [46,47].

Analysis of MPO activity as a marker of neutrophil infiltration was according to an earlier study [48]. For this purpose, a portion of testis was homogenized in CTAB buffer (50 mM cetyltrimethylammonium bromide in 50 mM potassium phosphate buffer at pH = 6) and centrifuged at 15,000 *g* for 20 min. For estimation of peroxidase activity, 10 µl of sample was combined with 80 µl of 0.75 mM H2O2 and 110 µl of 3,3′,5,5′-tetramethylbenzidine (2.9 mM in 14.5% DMSO and 150 mM sodium phosphate buffer at pH 5.4) and the plate was incubated at 37 °C for 5 min. The reaction was stopped by adding 50 µl of 2 M H2SO4 and absorption was measured at 450 nm to estimate enzyme activity as a percentage.

2.5. Evaluation of sperm parameters

The related procedure has been described before [49]. In this regard, laparotomy was done and after exposure of reproductive tract, the cauda epididymis was isolated, minced and incubated in a pre-warmed petri dish containing Ringer solution at 37 °C. Enough time was allocated for the spermatozoa to disperse into the medium. After 20 min, the cauda epididymis was removed and the suspension was gently shaken to homogenize. Then, approximately 15 µl of the sperm suspension was transferred onto the hemocytometer and allowed to stand for 5 min. The cells which settled during this time were counted under light microscope at a magnification of X400. The sperm heads were counted and expressed as million/ml of suspension. The motility assay was conducted by observing 20 µl of sperm suspension on a slide glass at 37 °C. The percentage of motile spermatozoa was determined by randomly counting > 100 spermatozoa in 10 selected fields under a light microscope and the mean number of motile sperms that showed progressive forward movement × 100/total number of sperms was calculated [49]. Sperm viability was assessed using eosin-nigrosin staining that is effective and simple. In this staining, only non-viable spermatozoa can absorb the stain. After counting viable and non-viable spermatozoa in a total of 100 cells, viability was reported as a percentage [50]. This experiment was done in duplicate.

2.6. Histological studies

For histological assessment, left testes were fixed in 10% phosphate buffered formalin solution for 72 h and processed for routine light microscopic analysis. The testes were sectioned at a thickness of 5 µm, stained with hematoxylin and eosin, dehydrated, cleared, and eventually mounted with Entellane (Merck Co., Germany) and coverslipped. The prepared slides were assessed with a light microscope (Optika Co., Italy) and photographed. In this respect, at least 3 slides from the upper, lower, and mid-portions of the testis were fully evaluated. Seminiferous tubule diameter was reported in micrometers. For assessment of testicular and spermatogenesis damage, Johnsen's mean testicular biopsy score (MTBS) was used [51]. In this respect, a score of 0–10 was assigned to each seminiferous tubule with regard to germinal epithelial maturation (Table 1). For quantitative analysis, Image J software (Version 1.49) was used. Histological analysis was done in duplicate.

2.7. Statistical analysis

All data are expressed as mean ± SEM. Analysis was performed using the SPSS statistical software (version 21.5; Chicago, IL, USA). Inter-group comparisons were done through one-way ANOVA followed by Tukey *post-hoc* multiple comparison test. Probability values < 0.05 were considered significant.

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

3. Results

3.1. The effect of diosgenin on body weight and serum glucose level

At baseline (1 week before STZ; week 0), there was no statistically significant differences between the experimental groups regarding the body weight and serum glucose level. In contrast, at weeks 4 and 8 post-STZ, body weight of diabetic rats was significantly lower ($p < 0.05$ and $p < 0.01$, respectively) relative to week 0 in the same group. Diosgenin-treated diabetic group did not show such a significant reduction of body weight. Even, body weight in this group was significantly greater at week 8 in comparison with vehicle-treated diabetic group ($p < 0.05$). In addition, both diabetic and diosgenin-treated diabetic groups had a significantly elevated level of serum glucose at weeks 4 and 8 as compared to weeks 0 ($p < 0.001$). However, serum glucose level was significantly lower in diosgenin-treated diabetic group versus vehicle-treated diabetic group ($p < 0.05$) (Fig. 2).

3.2. The effect of diosgenin on testicular oxidative stress and inflammation and serum levels of insulin and testosterone

With respect to testicular oxidative stress indices, STZ-diabetic group showed a significantly elevated level of MDA (Fig. 3A) ($p < 0.01$), protein carbonyl (Fig. 4) ($p < 0.01$), lower level of GSH (Fig. 3B) ($p < 0.01$) and lower activity of SOD (Fig. 3C) ($p < 0.01$), catalase (Fig. 3D) ($p < 0.01$), and GPx (Fig. 3E) ($p < 0.01$) in addition to lower levels of FRAP (Fig. 3F) ($p < 0.01$). Additionally, diosgenin treatment of diabetic group significantly lowered MDA ($p < 0.05$), protein carbonyl ($p < 0.05$), non-significantly increased GSH and significantly elevated FRAP ($p < 0.05$), SOD activity ($p < 0.05$), catalase activity ($p < 0.05$), and GPx ($p < 0.01$). Meanwhile, there was no significant alteration in diosgenin-treated control group when compared to control one with respect to these biomarkers of oxidative stress.

Diabetic group also showed a significantly lower serum concentration of insulin (Fig. 5A) ($p < 0.001$) and testosterone (Fig. 5B) ($p < 0.001$) as compared to the control one. In addition, diosgenin treatment of diabetic group significantly prevented decrease of insulin and testosterone level as a result of diabetes ($p < 0.05$). Furthermore, there was no significant difference between control and diosgenin-treated control groups with regard to serum levels of testosterone and insulin.

Regarding testicular inflammatory indices, diabetic rats showed significant elevation of TNFα ($p < 0.001$) (Fig. 5C) and IL-6 ($p < 0.01$) (Fig. 5D) in comparison with the control group. Upon chronic administration of diosgenin, diabetic group showed a significantly lower levels of TNFα ($p < 0.05$) and IL-6 ($p < 0.05$) as compared to diabetic group. Furthermore, there was no significant

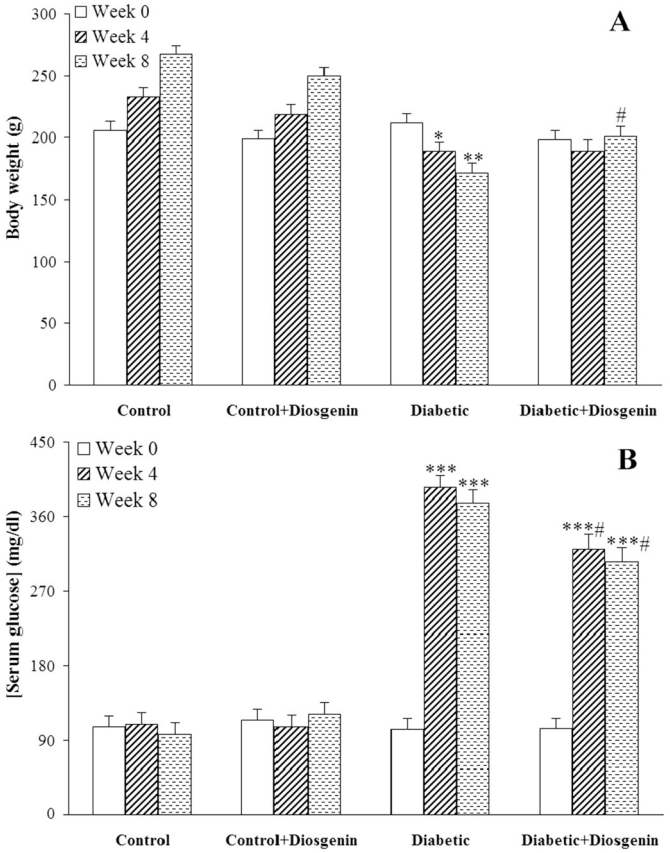

Fig. 2. Body weight (A) and serum glucose level (B) in different weeks in different groups (means ± S.E.M). Serum glucose levels were measured in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (relative to week 0 in the same group); # $p < 0.05$ (relative to diabetic group in the same week) (n = 7 for diabetic group and n = 8 for other groups).

alteration in diosgenin-treated control group relative to control group regarding these inflammatory indices.

3.3. The effect of diosgenin on testicular biomarkers of apoptosis

To assess the possible involvement of apoptotic processes in beneficial effect of diosgenin on testicular tissue of diabetic rats, we measured caspase 3 activity (Fig. 6A), DNA fragmentation (Fig. 6B), and Annexin V (Fig. 6C). Diabetic group displayed a significant elevation of caspase 3 activity ($p < 0.01$), DNA fragmentation ($p < 0.001$), and Annexin V ($p < 0.001$) as compared to the control group. Elevation of these apoptotic biomarkers was lower in diosgenin-treated diabetic group. In other words, the latter group had a significantly lower level of caspase 3 activity ($p < 0.05$), DNA fragmentation ($p < 0.01$), and Annexin V ($p < 0.01$) relative to the control group. Meanwhile, diosgenin treatment of control group did not significantly change level of these apoptotic indicators.

3.4. The effect of diosgenin on testicular mitochondrial membrane potential and MPO activity

In this study, MMP (Fig. 7A) was measured to evaluate mitochondrial integrity and its energy metabolism condition. In addition, MPO activity (Fig. 7B) was determined to assess testicular neutrophil infiltration. In this respect, MMP significantly reduced ($P < 0.01$) and MPO activity significantly increased ($p < 0.01$) in diabetic group when compared to the control group and diosgenin pretreatment of diabetic group successfully and significantly prevented MMP reduction ($p < 0.05$) and MPO elevation ($p < 0.05$) as compared to diabetic group.

3.5. The effect of diosgenin on sperm parameters and testis histology

The effect of diosgenin treatment on sperm parameters including epididymal sperm count, motility, and viability is shown in Fig. 8A–C. In this respect, diabetic group had a significantly lower level of sperm count ($p < 0.01$), sperm motility ($p < 0.01$), and sperm viability ($p < 0.01$) when compared to control group. Additionally, administration of diosgenin to diabetic rats resulted in significant improvement of sperm count, motility, and viability as compared to diabetic animals ($p < 0.05$). Furthermore, sperm parameters did not have a significant change in diosgenin-treated control group relative to control one.

Histological assessment of the testis tissue in different groups showed that two-month diabetes damaged seminiferous tubules, as demonstrated by a significantly lower tubular diameter (Fig. 9A) ($p < 0.001$) and mean testicular biopsy score (Fig. 9B) ($p < 0.01$). In contrast, diosgenin-treated diabetic group showed significant improvement of mean seminiferous tubule diameter (MSTD) ($p < 0.05$) and MTBS ($p < 0.05$) when compared to diabetic group. In addition, diosgenin administration to control group did not cause a noticeable and significant histological change.

4. Discussion

Male reproductive dysfunction and related pathologies are expected to increase in prevalence in human population during the next years [52,53]. Life style modification and environmental exposure to various toxic agents are mentioned as the principal causative factors for pathogenesis of male reproductive disorders in the society [54,55]. Amongst metabolic disorders, DM has pronounced destructive impacts on male reproductive system including alterations in testicular physiology, sperm maturation disturbance, and changes of sex hormone [56–58]. Additionally, increased blood glucose in DM could disturb oxidant/antioxidant balance and anti-oxidant status that leads to oxidative stress [59]. The development of oxidative stress in DM may finally damage DNA of germ cells, leading to subfertility or even infertility [60,61]. In our study, sperm viability of about 65% was found out for control group. Although findings close to our data had also obtained for sperm viability under control conditions [62,63], however, higher [64] or even lower levels [64,65] of viability had been reported for sperm viability in the literature for control group. These discrepancies may be attributed to differences in used animal species (mice or rat), method of analysis, and assessment of whole epididymis or only its caudal part, and so forth.

In this study, significant decreases in serum glucose and insulin level were observed in diabetic group following diosgenin treatment. Anti-diabetic effect of diosgenin in STZ-induced model of DM has been reported before [20,21]. In this respect, it has been shown that diosgenin could appropriately modulate enzymes of carbohydrate metabolism in muscular and renal tissues and is capable to elevate blood level of insulin [21]. Additionally, diosgenin can accelerate regeneration and/or reconstruction of beta cells and in this way could partly restore their secretory function after STZ challenge [66]. Furthermore, diosgenin may exert its anti-diabetic effect via alleviation of inflammation in the adipose tissue and through promotion of adipocyte differentiation process [67]. Besides, diosgenin could stimulate translocation of GLUT4 from intracellular compartment into plasma membrane in the muscular tissues [68]. However, compared to known anti-diabetic agents like glibenclamide [69], anti-hyperglycemic effect of diosgenin was less. It was better to have a positive control group such as gibenclamide-treated diabetic group and this is one of the design limitations of our study. This issue is better to be taken into account in future studies to have a better comparison.

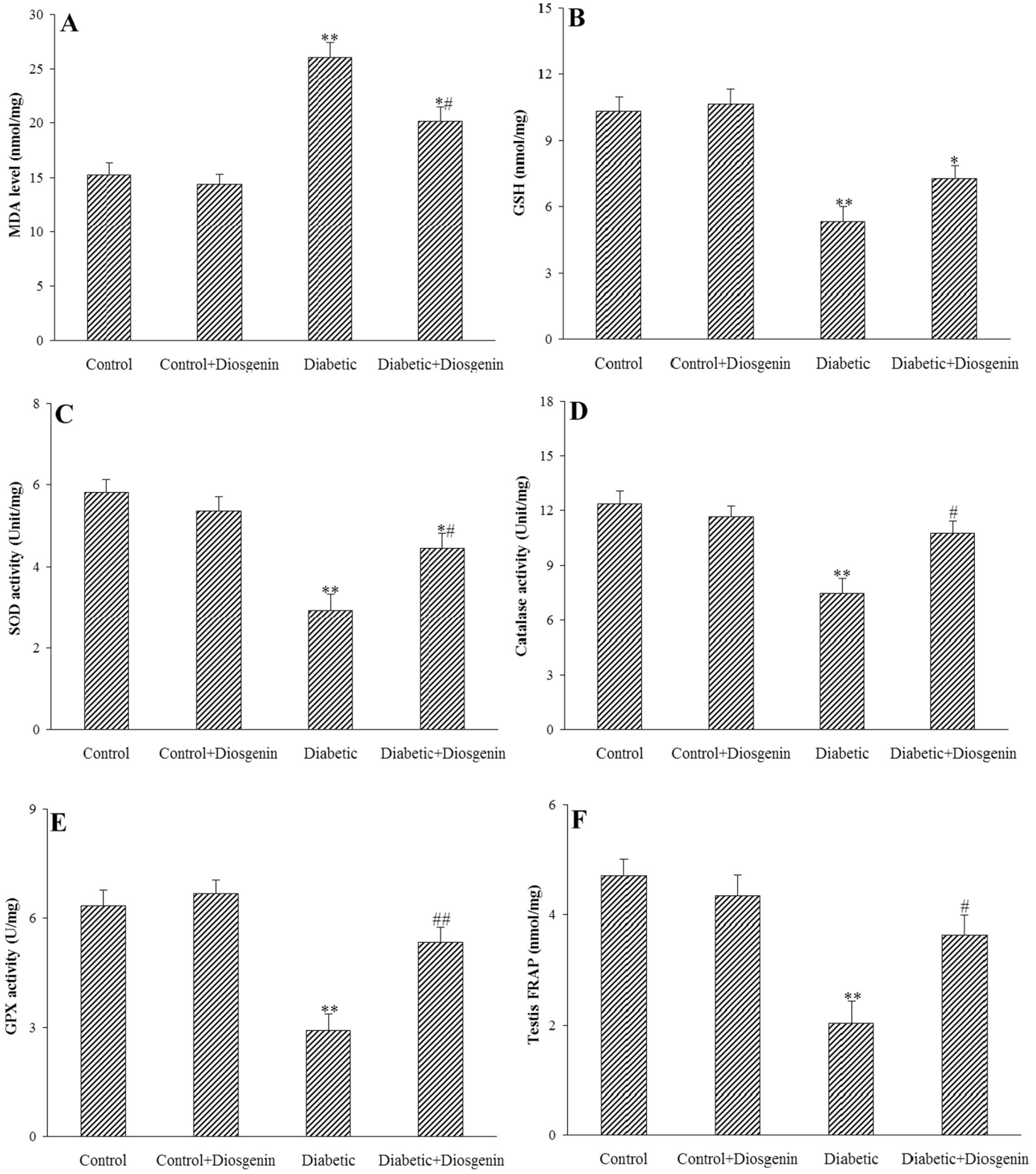

Fig. 3. Testicular tissue level of MDA (A), GSH (B) and activity of SOD (C), catalase (D), and GPx (E), and FRAP level (F) in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 6 for each group). * $p < 0.05$, ** $p < 0.01$ (relative to control); # $p < 0.05$, ## $p < 0.01$ (relative to diabetic).

Some complications of DM including its associated testicular problems are ascribed to development of oxidative stress phenomenon [9,70,71]. In our study, two-month diabetes caused testicular elevation of MDA as an index of lipid peroxidation and oxidative stress and concomitant reduction of GSH and the defensive enzymes SOD and catalase. Similar findings like those of us have been reported in the literature [72]. In addition, diosgenin successfully and significantly restored oxidative stress indices, as evidenced by a lower MDA and

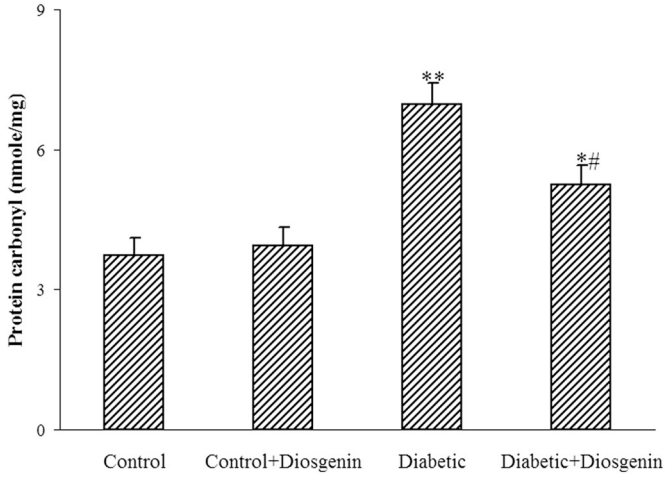

Fig. 4. Testicular tissue level of protein carbonyl in different groups. The parameter was measured in duplicate. All data are presented as mean ± S.E.M. (n = 6 for each group). * $p < 0.05$, ** $p < 0.01$ (relative to control); # $p < 0.05$ (relative to diabetic).

enhancement of antioxidants. Thus, part of beneficial effect of diosgenin in our study can be attributed to its inhibition of oxidative stress.

Tissue inflammation of varying degrees also develops in DM [73,74] with concurrent elevation of inflammatory biomarkers like TNFα and IL-6 [75]. Enhanced level of these biomarkers in testicular homogenate in our study clearly indicates an inflammatory event. Additionally, chronic diosgenin treatment was capable to alleviate inflammation in testis of STZ-diabetic rats. In agreement with our findings, it has shown that diosgenin could protect myocardial tissue under ischemia-reperfusion in rats through attenuation of severity of inflammation, as verified by lower levels of TNFα and interleukin-1 beta and inhibition of phosphorylation of transcription factor NF-κB and appropriate modulation of downstream inflammatory cytokines by regulating the activation of p38-MAPK and JNK pathways [25]. Furthermore, diosgenin is able to exert renoprotective effect in STZ-diabetic rats through suppression of inflammation in target tissues [28]. Interestingly, fenugreek that contains diosgenin could decrease gene expression of inflammatory-related molecules in adipose tissue [67].

Apoptosis is enhanced in in testicular tissue following diabetes induction by STZ. In this respect, STZ-induced diabetes causes severe histopathological damage of rat testes with concurrent enhancement of apoptotic tubule and apoptotic cell indices, caspase 8 and caspase 3 [76]. In addition, it has been demonstrated that diabetes augments testicular apoptotic cell death that mostly occurs in the spermatogonia and spermatocytes with strong involvement of mitochondrial cell death pathway [14]. In this study, we used caspase 3, Annexin V, and DNA fragmentation as apoptotic biomarkers and all of these parameters increased in STZ-diabetic group, indicating enhancement of apoptosis in

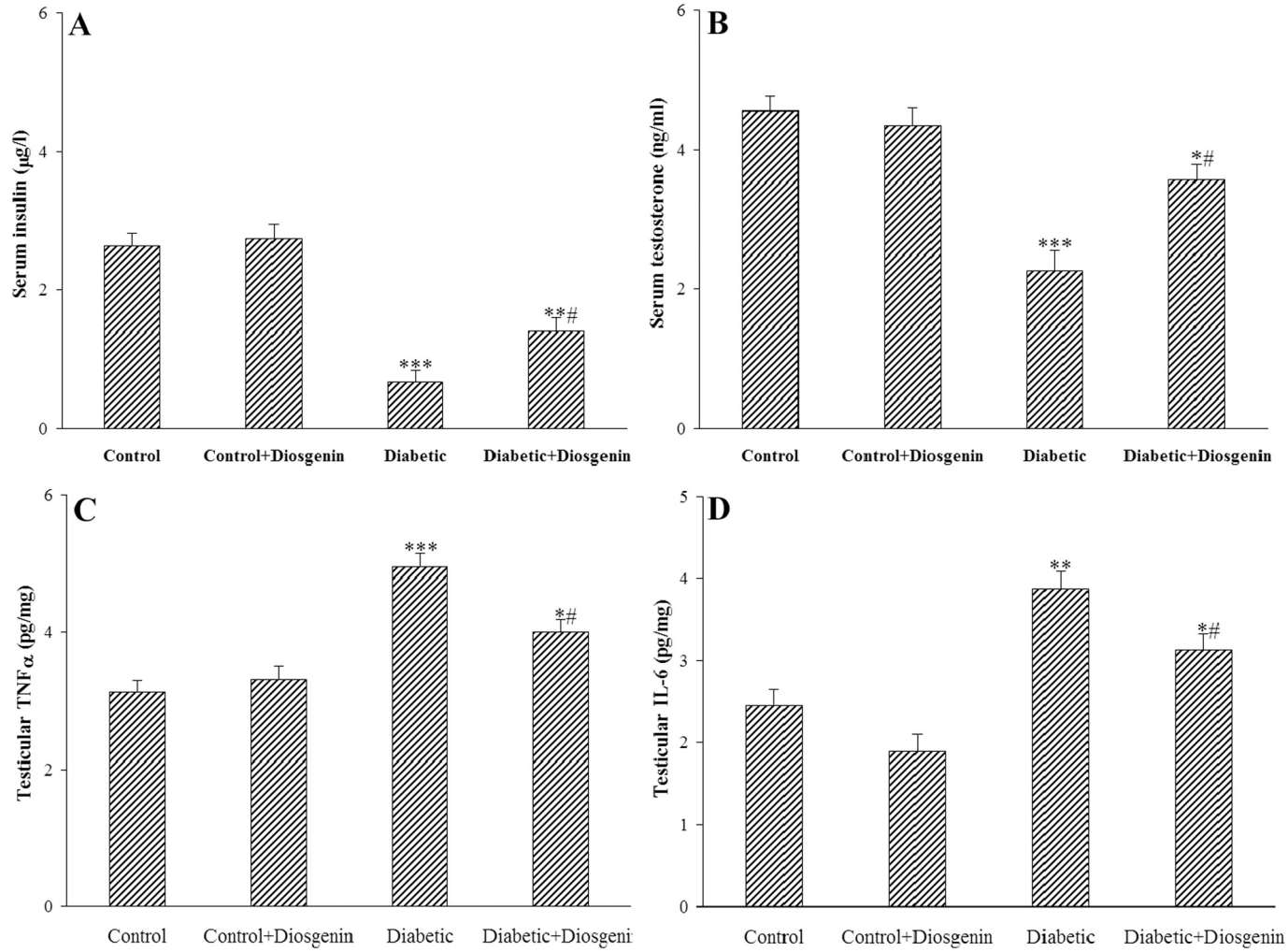

Fig. 5. Serum insulin (A) and testosterone (B) level and testicular tissue level of TNFα (C) and IL-6 (D) in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 6 for each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (relative to control); # $p < 0.05$ (relative to diabetic).

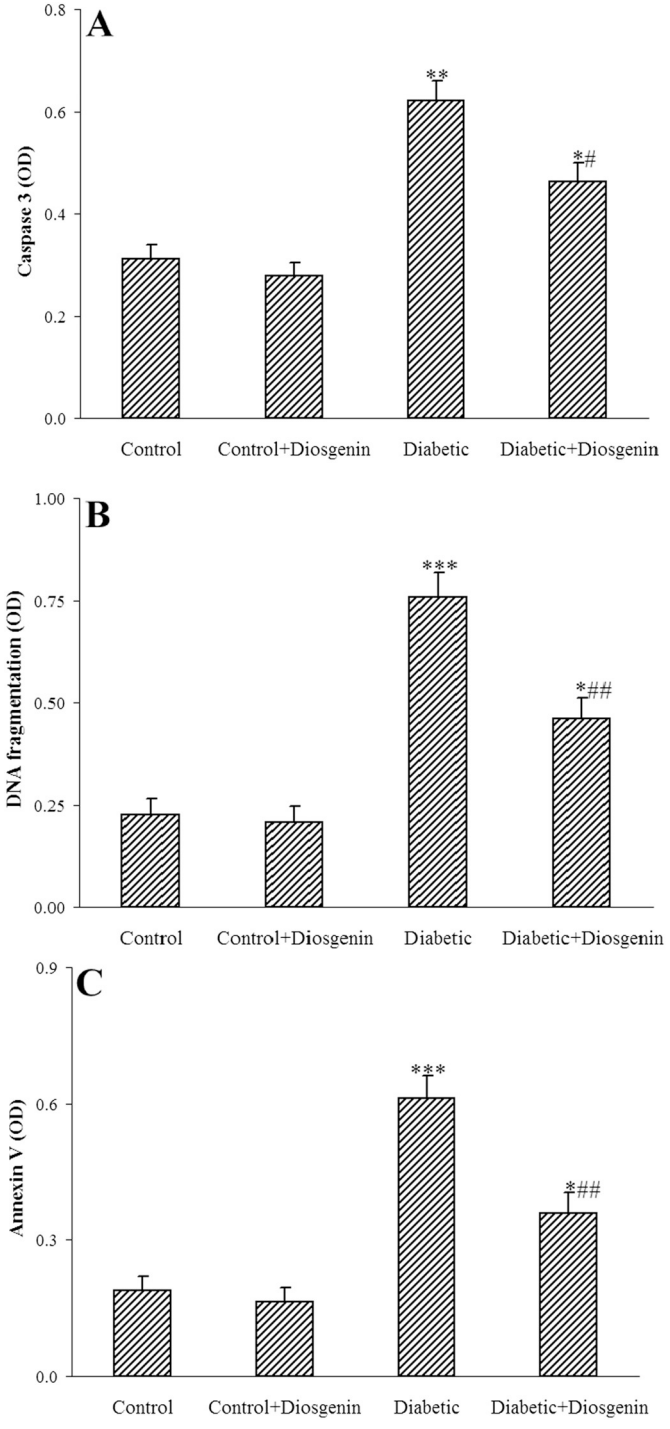

Fig. 6. Testicular tissue level of apoptotic biomarkers including caspase 3 activity (A), DNA fragmentation (B), and Annexin V (C) in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 6 for each group). * $p < 0.05$, *** $p < 0.001$ (relative to control); # $p < 0.05$, ## $p < 0.01$ (relative to diabetic).

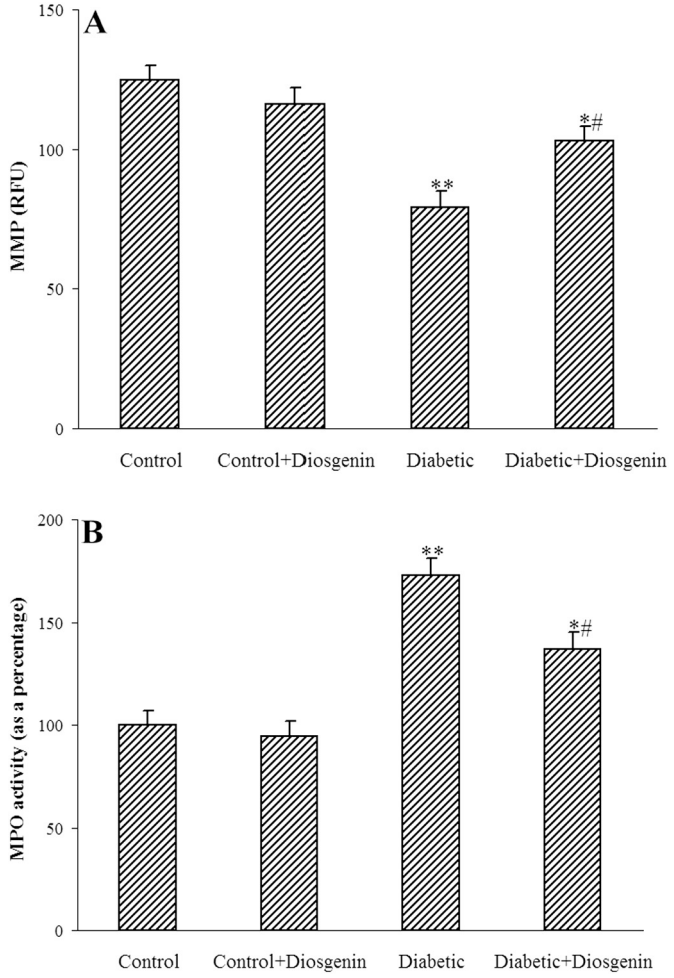

Fig. 7. Testicular tissue level of mitochondrial membrane potential (MMP) (A) and myeloperoxidase 3 activity (MPO) (B) in different groups. The parameters were measured in duplicate. All data are presented as mean ± S.E.M. (n = 6 for each group). * $p < 0.05$, ** $p < 0.01$ (relative to control); # $p < 0.05$ (relative to diabetic).

testicular tissue. Moreover, diosgenin treatment of diabetic group attenuated apoptosis severity. Consistent with our findings, earlier studies have shown that diosgenin could ameliorate diabetes-induced vascular dysfunction partly via inhibition of apoptosis [20] and diosgenin is capable to prevent high glucose-induced apoptosis of cardiomyocytes [26]. Mitochondrial disturbances are responsible for testicular dysfunction in DM, as shown by lower MMP following diabetes induction by STZ [62]. In this respect, maintenance of MMP is vital for cell survival and its reduction indicates a cascade of reactions, finally leading to cellular apoptosis [77]. MMP reduction induces the release of cytochrome *c* into the cytosolic compartment with triggering downstream apoptotic pathway [78]. Lower levels of MMP have been reported for liver tissue of diabetic rats [79]. Our findings showed that MMP reduces due to STZ-diabetes and diosgenin treatment successfully and partially improved MMP, indirectly suggestive of its anti-apoptotic and protective property.

Increased MPO as a biomarker and consistent indicator of neutrophil infiltration has been reported in cardiac tissue from diabetic rats, indirectly indicating the occurrence of inflammation [13]. In addition, diosgenin was successful to ameliorate MPO activity in testicular tissue of diabetic group in our study that was also associated with lower inflammation. Consistent with our finding regarding MPO, it has been shown that diosgenin could protect against myocardial inflammatory injury due to ischemia-reperfusion in the rat [25].

It is probable that part of beneficial effect of diosgenin on testicular tissue and testosterone level in our study may have exerted through modulation of estrogenic signaling. In this respect, the saponin diosgenin is a precursor for synthesis of oral contraceptives, sex hormones and other steroidal compounds [80,81]. Diosgenin regulates Sertoli cells proliferation through estrogen receptor signaling and in this way

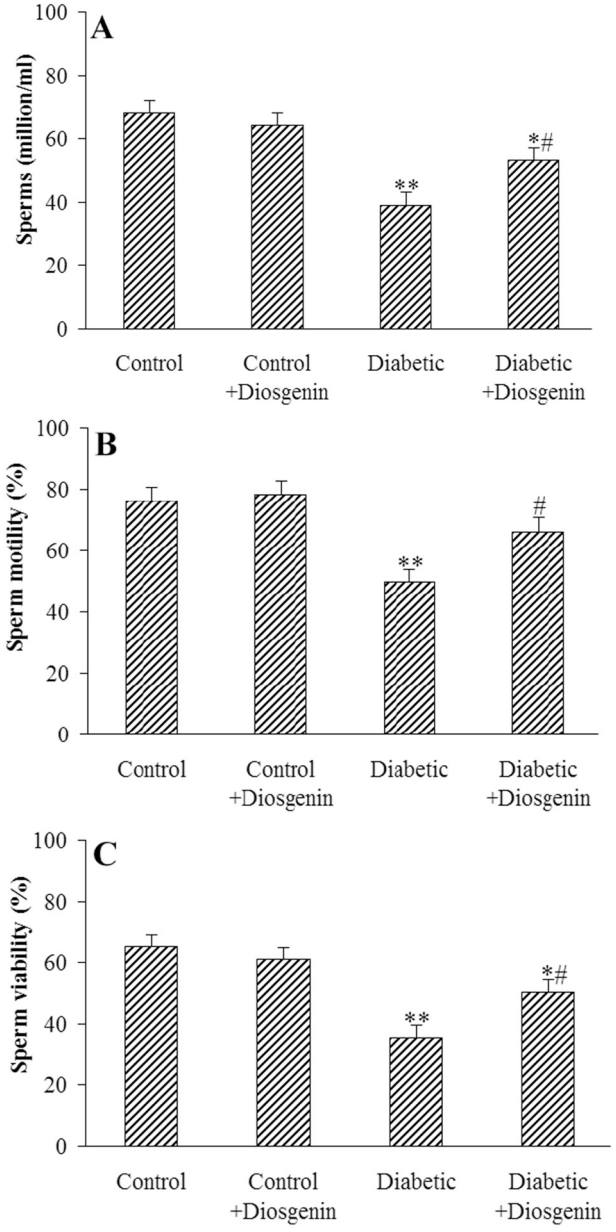

Fig. 8. Male fertility indices including sperm count (A), motility (B), and viability (C) in different groups. These parameters were assessed twice. All data are presented as mean ± S.E.M. (n = 4 for each group). * $p < 0.05$, ** $p < 0.01$ (relative to control); # $p < 0.05$ (relative to diabetic).

may increase cell viability and proliferation [82]. The biological effects of estrogen are exerted by the particular receptors ERα and ERβ, which are extensively distributed in the male reproductive tract [83,84]. ERα receptors are expressed in some cells including Sertoli and Leydig cells and ERβ receptors are expressed in various cells such as germ cells [85,86]. Diosgenin could induce an immediate and temporary translocation of ERα and ERβ from the nucleus to plasma membrane through SRC-dependent pathway [82] and in such way may affect reproductive system. However, further investigation is warranted to better clarify such interaction.

Collectively, results of this study showed that diosgenin ameliorates testicular damage in DM, at least via partial suppression of apoptosis, oxidative stress, inflammation, and neutrophil infiltration and also via partial restoration of mitochondrial integrity.

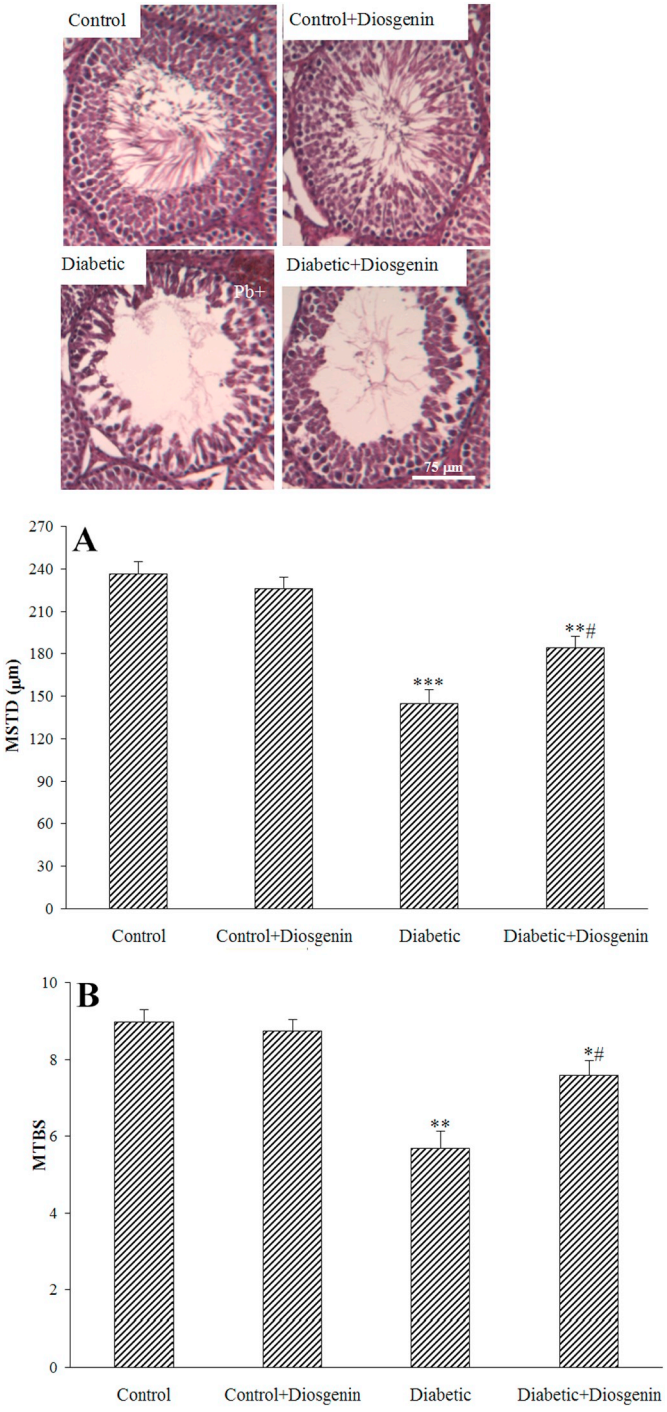

Fig. 9. Mean seminiferous tubule diameter (MSTD) (A) and Johnsen's mean testicular biopsy score (MTBS) (B) in different groups and photomicrographs showing sections of seminiferous tubules stained with Hematoxylin and Eosin. These parameters were assessed twice. All data are presented as mean ± S.E.M. (n = 4 for each group). * $p < 0.05$, ** $p < 0.01$ (relative to control); # $p < 0.05$ (relative to diabetic).

Acknowledgement

This research project was the result of MD thesis project (Mrs. Zeinab Khosravi), approved and financially supported in part by Shahed University in 2016.

Conflict of interest

The authors declare that they have no competing interests.

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