Original Article

Modulatory Effect of Pioglitazone on Sperm Parameters and Oxidative Stress, Apoptotic and Inflammatory Biomarkers in Testes of Streptozotocin-Induced Diabetic Rats

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A B S T R A C T

Background and Aims: Diabetes mellitus causes testicular damage by increasing oxidative stress and inflammation. In the present study, modulation of oxidative stress by pioglitazone, a synthetic ligand of peroxisome proliferator-activated receptor-γ, was examined in testis of streptozotocin-induced diabetic rats.

Materials and Methods: Diabetes was induced by a single dose of streptozotocin (65 mg/kg, i.p.) injection in male Wistar rats. 32 adult male rats were divided into four groups (n=8): control, diabetic, diabetic pioglitazone (1 mg/kg/day) and diabetic pioglitazone (10 mg/kg/day). Rats were treated with pioglitazone for 5 weeks. Serum testosterone levels were estimated. Reproductive damage was evaluated by sperm parameters (viability, motility, morphology and count). Oxidative stress markers were evaluated in testicular homogenate. Pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1β) levels, expressions of inflammatory (inducible nitric oxide synthase and nuclear factor-κ B) and apoptotic markers (caspase-3) in testicular tissue were performed by enzyme-linked immunosorbent assay and western blot.

Results: Pioglitazone treatment significantly increased sperm parameters ($p<0.05$). No significant decrease in serum level of testosterone was observed in the pioglitazone treated mice ($p>0.05$). Aside from reducing the elevated tissue nitric oxide and malondialdehyde levels, pioglitazone increased the reduced superoxide dismutase, catalas and total antioxidant capacity in testes compared to diabetic rats ($p<0.05$). Pioglitazone reduced testicular inflammation by decreasing the expressions of inducible nitric oxide synthase, nuclear factor-κB and pro-inflammatory cytokines levels and inhibited cell death by decreasing the expressions of caspase-3 ($p<0.05$).

Conclusions: Pioglitazone attenuated testicular damage in diabetic rats by decreasing oxidative stress, testicular inflammation and testicular damage.

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Introduction

Diabetes is considered as one of the greatest health problems globally [1]. It has been shown that diabetes mellitus has adverse effects on the male sexual and reproductive functions [2]. Impaired homeostasis under diabetic conditions is connected with the increased production of free radicals and defiant capacity of antioxidant systems [3]. Previous studies have shown that diabetes induces changes in reproductive functions especially on steroidogenesis, histology of testes, spermatogenesis, sperm quality and fertility both in diabetic men and experimental diabetic animals [4, 5]. Oxidative stress is one of the major pathophysiological routes during diabetes [6]. Enhanced oxidative stress and changes in antioxidant capacity have important roles in the pathogenesis of chronic diabetes mellitus [7]. Oxidative stress causes the activation of various transcription factors including the nuclear factor-κB (NF-κB). NF-κB plays a crucial role in inflammation and activation of inducible nitric oxide synthase (iNOS) [8, 9]. Pioglitazone is a synthetic ligand of the peroxisome proliferator-activated receptor-γ (PPAR-γ) that is an antidiabetic agent, which improves insulin production in the patients with Type 2 diabetes [10]. Several investigators have demonstrated that pioglitazone is able to reduce oxidative stress [11-14]. The activation of PPAR-γ protects pancreatic β-cells from cytotoxicity by preventing NF-κB activation [15, 16]. Furthermore, pioglitazone increases insulin sensitivity thereby elevating glucose uptake and inhibits hepatic glucose output [10]. Previous studies have demonstrated that pioglitazone is potent inhibitor of inflammatory [14, 17, 18] and potent antioxidants [12, 13, 19]. However, the effect of pioglitazone on insulin sensitivity and reproductive damage in type 2 diabetes has been examined [10] No study to date has explored the effect of pioglitazone on reproductive damage and its roles in pro-inflammatory cytokines response such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), expressions of inflammatory (iNOS and NF-κB) and apoptotic markers (caspase-3) in testicular tissue of type 1 diabetic rats. The present study was thus undertaken to examine the protective effect of pioglitazone on anti-oxidative and anti-inflammatory system in the testis of streptozotocin (STZ)‑induced diabetic rats.

Materials and Methods

Animals

Adult male Wistar rats, weighing 180-210 g, were obtained from animal house of the faculty of veterinary medicine in Iranian Urmia University. Animals were housed in a room with a 12-h light/dark cycle and 25±2℃ temperature for one week and had free access to tap water and ad standard rodent chow (supplemented with 18% proteins, 3% lipids and 10% fibers). This experimental study was conducted with the approval of the local Ethics Committee of the faculty of veterinary medicine in Urmia University.

Experimental design

Diabetes was induced via a single intra-peritoneal injection dose of 65 mg/kg STZ (Sigma, Germany). STZ was dissolved in 0.01 mM citrate buffer (pH 4.5) and used within 10 min. after preparation [20]. Control animals received intraperitoneal injection of citrate buffer only as a vehicle. The blood samples were obtained from the tail vein of non-fasted rats and glucose was measured using a glucometer (Accu-Chech Active). Rats were considered diabetic when their non-fasting blood glucose levels were >250 mg/dl. All the 32 adult male rats were divided into four groups. They included the control group, STZ-induced diabetic group and STZ-induced diabetic group treated with low or high dose of pioglitazone (Sigma, Germany) of 1 or 10 mg/kg/day oral gavage for 5 weeks after the induction of diabetes [21, 22].

Sampling

At the end of the 5th week, Blood samples were collected from the orbital plexus of veins by capillary tube and left to clot formation for separating the serum after centrifugation at 3000 rpm for 15 minutes. Animals were then sacrificed and their testicular tissues were dissected and stored at −80 °C till analysis.

Blood glucose estimation

Blood glucose level was measured by using a glucometer (Accu-Chech Active, Roche Diagnostics, Germany). The blood samples were obtained from the tail vein of non-fasted rats. The blood glucose level was recorded immediately as mg/dl.

Sperm analysis

Semen collection

Epididymal sperms were collected by slicing the caudal region of the epididymis in 5 ml of human tubal fluid and incubated for 5 min. at 37˚C in an atmosphere of 5% CO_2 to allow sperm to swim out of the epididymal tubules.

Assessment of sperm count

In order to count sperms, a 1:20 dilution was prepared in a 1 ml micro tube 190 µl of distilled water was poured and 10 µl of sperm mixture was added to it. Then, 1 µl of the mixture was dropped on a Neobar slide and the sperms were counted [23].

Evaluating sperm motility

In order to evaluate sperm motility, 10 µl sperm suspension was placed on a pre-heated slide covered with a slip and then the motility was observed under a light microscope (Nikon, Tokyo, Japan) with 400×magnification [24].

Sperm viability

Sperm viability was evaluated as follows: Volume of 20 µl of 0.05% Eosin Y-nigrosin was added into an equal volume of sperm suspension. After 2 min. incubation at room temperature, slides were observed by a light microscope with magnification of 400×. Dead sperms were stained pink but the live ones took no color. Sperms (n=400) were counted in each sample and the viability percentage was computed [25].

Sperm morphology

To evaluate sperm morphology, aniline blue staining method was implemented and abnormal morphologies percentage was determined. The cytoplasmic residual of

sperms was especially considered as abnormal morphology [26].

Determining damage to DNA

Fragmentation of sperm DNA was applied as a biomarker for male infertility. Acridine orange staining was used, after challenging at low PH, to distinguish between denatured, native, and double-stranded, DNA regions in sperm chromatin [27]. Results indicated high level of fluorescent in denatured DNA. Thick smears were placed in carnoy's fixative (methanol/ acetic acid 1:3) for 2 hr. for fixation [28]. The slides were removed from the fixative and they were left on the outside to be dried for 5 min. at laboratory temperature. Then, slides were placed in a stock solution of 1 mg of Acridine orange in 1000 ml distilled water and stored in a dark place at 4˚C. The stained solution was produced and 10 ml of the stock was added to 40 ml 0.3 M NaHPO4.7H2O solution [29]. After 5 min. staining, sperms were examined using fluorescent microscope (Olympus BX51) at 490 nm. Green colored sperms were observable among normal sperms and yellow-red sperms were categorized in abnormal or damaged DNA [30].

Total antioxidant capacity assay

The total antioxidant capacity of the semen was done by ferric reduction antioxidant power (FRAP) assay [31]. 100 µl of cellular supernatant was added to 1 ml of fresh Ferric Reducing Antioxidant Power reagent (FRAP; Tripiridyl triazine; Merck) and was incubated in 37°C for 10 min. at dark condition. Reading of the blue-colored reagent was then taken at 595 nm every 20 second for 10 min. Aqueous solution of Fe^{II} (FeSO4.7H2O) and appropriate

concentration of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.

Estimations of testosterone

Serum levels of testosterone were determined using the enzyme-linked immunosorbent assay kit (Cayman Ltd., USA) according to the manufacturer's instructions.

Analysis of antioxidant status markers in testicular tissues

Testes were homogenized in ice-cold phosphate buffer (0.01 M, pH 7.4; 20% w/v). Assessment of testicular antioxidant defense mechanisms was carried out in tissue homogenate. Determination of Malondialdehyde, marker of lipid peroxidation, measurement was determined as measurement of TBARS according to the method of Uchiyama and Mihara [32]. The total NO content of the testicular tissue was measured according to the Griess reaction [33]. Reduced glutathione was estimated by spectrophotometric kit (Biodiagnostic, Egypt). In brief, the method was based on the sulfhydryl component of glutathione reacts with 5,5-dithio-bis-2-nitro benzoic acid (Ellman's reagent) producing 5-thio-2-nitrobenzoic acid having a yellow color, that was measured colorimetrically at 405 nm.

Testicular superoxide dismutase activity was estimated following the method described by Kono [34]. One unit of superoxide dismutase is described as the amount of enzyme required to cause 50% inhibition of pyrogalol auto-oxidation at alkaline PH. Catalase activity of testicular tissue was estimated following the method described by Aebi [35].

Analysis of myeloperoxidase activity in testicular tissues

Myeloperoxidase activity in testicular tissues was determined as described by Hillefass et al. [36]. One unit of meloperoxidase was defined as the amount of myeloperoxidase that degrades one µM peroxide per minute.

Estimations of TNF-α and IL-1β levels in testicular cells

Proinflammatory cytokines including TNF-α and IL-1β levels in testicular cells were assessed and quantified pg/mg protein) by using enzyme-linked immunosorbent assay technique (R & D systems, USA).

Western blot analysis

Testicular tissue samples (50 mg) were homogenized in lysis buffer containing 20 mM TrisHCl PH 7.5, 2 mM EDTA, 50 mM 2-mercaptoethanol, 5 mM EGTA, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 25 mg/ml leupeptin, and 2 mg/ml aprotinin. The protein concentration was determined [37] and 25 µg was applied in each lane on 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis and transfer to the polyvinyl difluoride membrane, the membrane was incubated in blocking buffer containing 0.1% Tween-20 in Tris-buffered saline and 5% non-fat milk powder and dried for 1 hr at the room temperature. Immunoblotting was carried out with primary antibodies (diluted 1:1000 for iNOS, NF-κB, caspase-3) (Santa Cruz, USA, cat. no. sc-7271, sc-372, sc-7148) at 4°C overnight. β-actin antibody was used for internal controls (Sigma). After four washes in phosphate-buffered saline containing Tween-20, the membrane was incubated with anti-rabbit IgG (1:5000; Cell Signaling Technology Inc., MA, USA cat .no. #7074BC) conjugated to horseradish peroxidase for 1 h at the room temperature. Protein bands were detected by a standard enhanced chemiluminescence method. Densitometric analysis was made using ImageJ software (freeware; rsbweb.nih.gov/ij). The densities of target protein bands were normalized to the corresponding density of β-actin band. All experiments were repeated for triplicate.

Statistical analysis

One-way analysis of variance followed by Tukey's test were used for multiple comparisons between groups. Data are expressed as means±SEM. $P<0.05$ was considered as significant.

Results

Effect of pioglitazone on serum glucose and testosterone levels

Non-fasting blood glucose levels in STZ-induced diabetic rats significantly increased compared to the control group ($p<0.05$) (Table 1). Administration of pioglitazone for 5 weeks did not affect blood glucose. All STZ-induced diabetic rats with pioglitazone treatment remained hyperglycemic on day 35, and no significant difference in blood glucose levels was observedbetween pioglitazone and diabetic control groups.

To evaluate testes function in terms of testosterone release, we measured the plasma testosterone levels. Pioglitazone prevented the STZ-induced reduction in plasma testosterone ($p<0.05$) indicating a possible protective effect

of pioglitazone against testes damage compared to the control group (Table 1).

Effect of pioglitazone on semen parameters

The results revealed that the number of sperms, sperm motility and viability, sperm with normal morphology and damaged DNA were decreased significantly in the diabetic group (p<0.05). Pioglitazone treatments significantly increased these parameters when compared to the diabetic rats (p<0.05) (Table 2).

Effect of pioglitazone on testicular oxidative stress markers in STZ‑induced diabetic rats

STZ caused significant reduction (p<0.05) in three powerful endogenous anti‑oxidants (glutathione level, superoxide dismutase and catalase activity), total antioxidant capacity

levels, and increase testicular malondialdehyde and NO levels compared with control group. Treatment of rats with pioglitazone, in low and high dosage, significantly increased glutathione, superoxide dismutase, catalase in testicular tissue of diabetic rats and ameliorated abnormalities in testicular NO and malondialdehyde levels (p<0.05) (Table 3). The total antioxidant capacity levels in treatment groups with pioglitazone were significantly higher (p<0.05) than those in the diabetic group. It also appeared that treatment with high dose of pioglitazone caused the highest protective effects in these parameters and nearly normalized them to control values in diabetic rats (Table 3).

Table 1. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on serum glucose and testosterone levels in streptozotocin-induced diabetic rats

Groups	Non-fasting blood glucose level (mg/dl)	Testosterone level (mg/dl)
Control	99±0.1	4.09±0.32
Diabetic	345±0.54*	1.92±0.22*
Diabetic+ pioglitazone (1 mg/kg)	338±0.43*	2.03±0.42*
Diabetic+ pioglitazone (10 mg/kg)	330±0.48*	2.07±0.31*

Data are represented as mean±SEM of observations from 8 rats. *p<0.05 vs. control

Table 2. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on semen parameters in streptozotocin-induced diabetic rats

Groups	Count (10⁶)	Motility (%)	Viability (%)	Morphology (%)	DNA integrity (%)
Control	20.63±0.04	74.08±0.07	92.34±0.45	85.46±0.03	1.74±0.21
Diabetic	13.43±0.12*	51.23±0.41*	67.44±0.02*	54.08±0.09*	13.03±0.44*
Diabetic+pioglitazone (1 mg/kg)	16.22±0.24*#	69.81±0.37*#	76.63±0.18*#	74.95±0.38*#	7.72±0.54*#
Diabetic+pioglitazone (10 mg/kg)	19.16±0.31#	70.18±0.28#	88.95±0.07#	75.04±0.66#	6.69±0.09#

Data are represented as mean±SEM of observations from 8 rats. *p<0.05 vs. control ;#p<0.05 vs. diabetic; •p<0.05 vs. diabetic+pioglitazone (1 mg/kg) group.

Table 3. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on testicular oxidative stress markers in streptozotocin-induced diabetic rats.

Groups	Glutathione (µmol/g tissue)	Malondialdehyde (nmol/g tissue)	Nitric oxide (nmol/g tissue)	Superoxide dismutase (U/mg protein)	Catalase (U/mgprotein)	Total antioxidant capacity (µmol/L)
Control	4.95±0.56	32.45±0.11	50.04±0.03	3.32±0.28	6.12±0.31	1.51±0.07
Diabetic	2.16±0.38*	58.92±0.32*	101.75±0.24*	1.74±0.32*	4.55±0.72*	0.21±0.12*
Diabetic+ pioglitazone (1 mg/kg)	3.22±0.65*#	41.87±0.28*#	83.41±0.12*#	2.22±0.42*#	4.31±0.21*#	1.09±0.33*#
Diabetic+ pioglitazone (10 mg/kg)	3.99±0.44#	34.44±0.15#	52.09±0.20#	3.15±0.48#	5.73±0.53#	1.49±0.85#

Data are represented as mean±SEM of observations from 8 rats. *$p<0.05$ vs. control ;#$p<0.05$ vs. diabetic; •$p<0.05$ vs. diabetic+pioglitazone (1 mg/kg) group.

Effect of pioglitazone on diabetes-induced apoptosis in testicular tissues

Levels of caspase 3 expression in the treatment groups (low and high dosage) with pioglitazone showed a significant decrease as compared with that in the diabetic control group ($p<0.05$). High doses of pioglitazone succeeded in reverting caspase 3 expression to levels not significant from normal control ($p< 0.05$) (Fig. 1).

Effect of pioglitazone on testicular pro-inflammatory cytokines and inflammatory markers in diabetic rats

Testicular levels of pro-inflammatory cytokines including TNF-α and IL-1β were increased in diabetic rats compared to the control animals ($p<0.05$). IL-1β and TNF-α levels significantly decreased only in high (10 mg/kg) dose of

pioglitazone group when compared to the diabetic rats ($p<0.05$) (Fig. 2).

Diabetic rats exhibited significant high level in the expressions of both iNOS and NF-κB-p65 in testicular tissues ($p<0.05$). Administration of pioglitazone effectively inhibited these increases in both iNOS and NF-κB-p65 expressions ($p<0.05$) (Fig. 3).

Effect of pioglitazone on testicular myelo-peroxidase activity in diabetic rats

In testicular tissues, we measured myelo-peroxidase activity to evaluate anti-inflammatory effect of pioglitazone. Treatment of diabetic rats with pioglitazone significantly decreased myeloperoxidase activity when compared to the diabetic rats ($p<0.05$) (Fig. 4).

A

35 kDa   caspase 3

42 kDa   β-actin
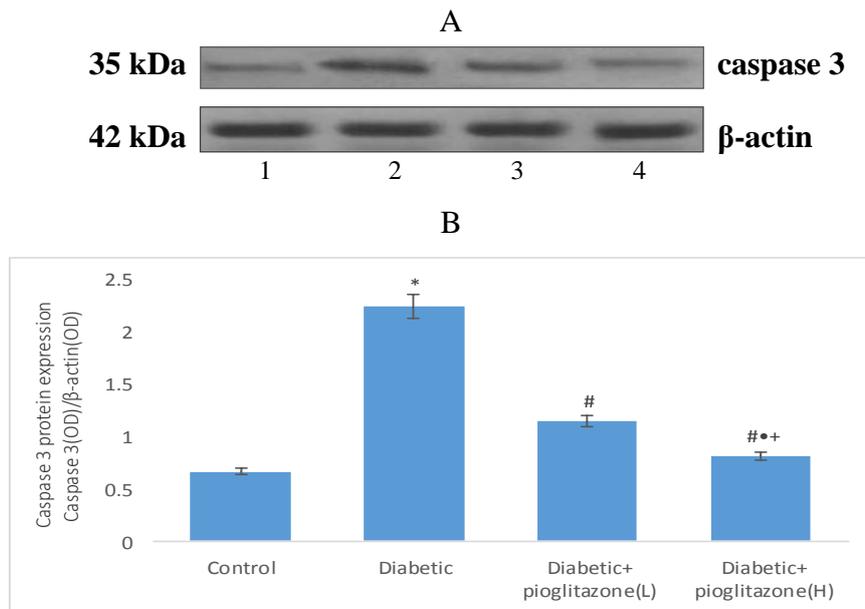
       1   2   3   4

B

Fig. 1. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on testicular caspase 3 protein expression in streptozotocin-induced diabetic rats. A: Electrophoretic analysis of western blotting products B: Graphs present the ratio of densitometric measurements (OD) of caspase 3 to the corresponding reporter gene (β-actin). Data are represented as mean±SEM Lane 1, Control group; Lane 2, Diabetic group; Lane3, Diabetic+ pioglitazone (L) group; Lane 4, Diabetic+pioglitazone(H) group. *$p<0.05$ vs. control; #$p<0.05$ vs. diabetic ; •$p<0.05$ vs. diabeti + pioglitazone (L) group;+no significant difference compared with control. pioglitazone (L)= low dose of pioglitazone; pioglitazone (H)= high dose of pioglitazone
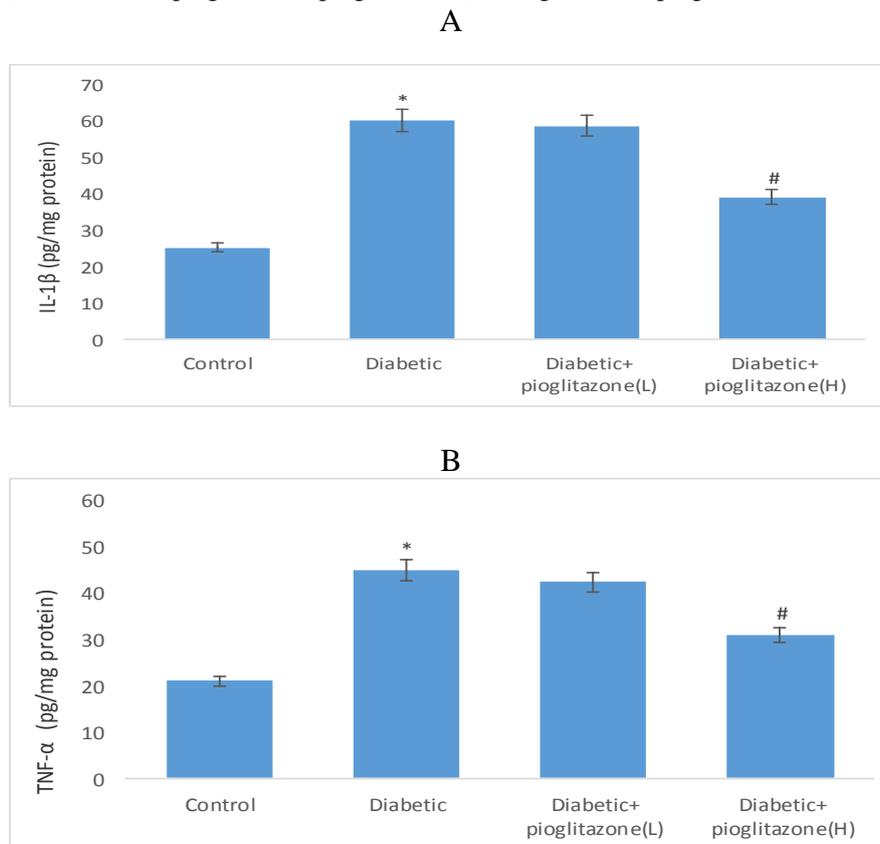
A

B

Fig. 2. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on IL-1β (A) and TNF-α (B) levels in testicular tissue of diabetic rats. Data are represented as mean±SEM *$p<0.05$ vs. control; #$p<0.05$ vs. diabetic. pioglitazone (L)= low dose of pioglitazone; pioglitazone (H)= high dose of pioglitazone
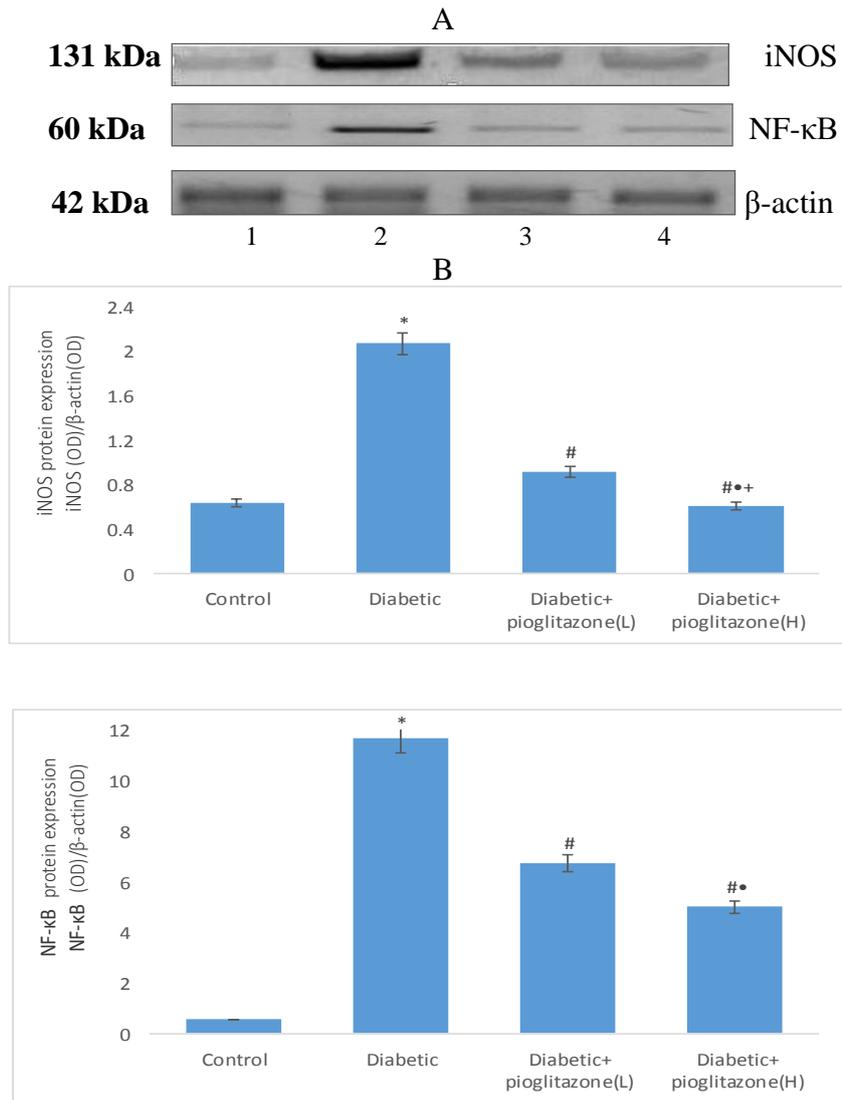
Fig. 3. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on testicular iNOS and NF-κB protein expressions in streptozotocin-induced diabetic rats. A: electrophoretic analysis of western blotting products B: graphs present the ratio of densitometric measurements (OD) of iNOS and NF-κB to the corresponding reporter gene (β-actin). Data are represented as mean±SEM Lane 1, Control group; Lane 2, Diabetic group; Lane3, Diabetic+ pioglitazone(L) group; Lane 4, Diabetic+ pioglitazone(H) group .*p<0.05 vs. control; #p<0.05 vs. diabetic; •p<0.05 vs. diabeti+pioglitazone (L) group; +no significant difference compared with control. pioglitazone (L)= low dose of pioglitazone; pioglitazone (H)= high dose of pioglitazone
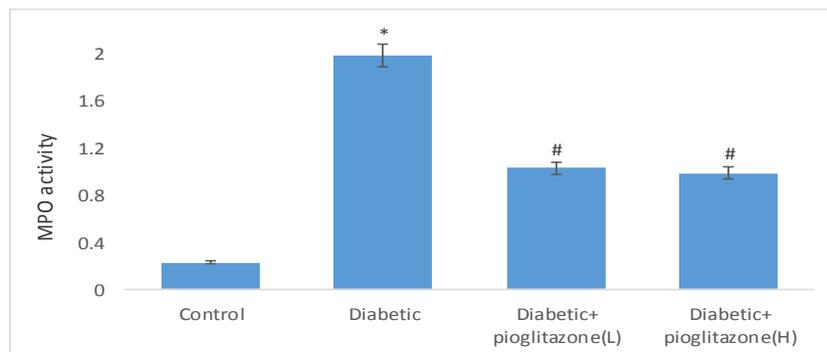

Fig. 4. Effect of low (1 mg/kg) and high (10 mg/kg) doses of pioglitazone on testicular myeloperoxidase activity (MPO) in streptozotocin-induced diabetic rats. Data are represented as mean±SEM *p<0.05 vs. control; #p<0.05 vs. diabetic. pioglitazone (L)= low dose of pioglitazone; pioglitazone (H)= high dose of pioglitazone

Discussion

The present investigation outlines the protective effects of pioglitazone against experimentally-induced diabetic sexual oxidative dysfunction in Wistar rats. Administration of pioglitazone for five consecutive weeks markedly reduced signs of testicular inflammation, lipid peroxidation, oxidative stress. The preventative properties of pioglitazone were confirmed by semen parameters evaluation. It was also shown that treatment with high dose of pioglitazone caused the highest protective effects in some of these parameters and nearly normalized them to control values in diabetic rats

The most suitable animal model of diabetes is STZ-induced diabetes in rodents because it reflects the symptoms of diabetes in human [38]. In diabetes, impairment of glucose transport from the blood to testicular germ cells is followed by an increase in free radical formation and oxidative stress [39] the result of which is sperm nuclear damage [40]. Thus an apoptotic response arises in sperm cells causing higher percentage of spermatic cellular death [41]. Pioglitazone is high-affinity PPAR-γ agonists with potent anti-diabetic and antioxidant properties and potential anti-inflammatory effects. PPAR-γ is expressed in a wide range of human tissues. It has been suggested that PPAR-γ is an important immunomodulator and suppresses the production of inflammatory cytokines [17, 18, 42, 43]. PPAR-γ agonists, main tissue expressing isoform, have potential to influence inflammation processes, reduce oxidative stress, improve endothelial function and play an important role in lipid metabolism [44]. The potential anti-inflammatory properties of PPAR-γ ligands have been investigated in experimental arthritis models [45].

Previous studies have shown that diabetes can perturb spermatogenesis by significantly reducing sperm density, sperm motility and progressivity, and volume of the ejaculate while increasing the incidence of deformed sperm cells both in diabetic men and experimental diabetic animals [46, 47]. Based on our results, in semen, pioglitazone addition had a positive impact on all semen parameters. Results of the present study were consistent with the previous observation where pio-glitazone significantly reduced sperm shape abnormalities and increased sperm count in type-2 diabetic Wistar rats due to its ability to enhance the antioxidant enzyme levels [48]. Another study also showed that pioglitazone restores testicular histomorphometry and improves semen quality in alloxan-induced diabetic rats [49]. Men with diabetes were found to have a significantly higher percentage of spermatozoa with nuclear DNA damage [50]. The present data demonstrated that pioglitazone candecrease the percentage of sperm with damaged DNA in diabetic rats.

A significant reduction in the serum levels of the main androgenic hormone, testosterone, was observed in diabetic rats [5]. No significant difference was reported in intratesticular testosterone levels between the diabetic and non-diabetic rats with and without

pioglitazone treatment by Akinola et al. [49]. In the present study, STZ caused a significant decrease in the testosterone level and this level was not changed by administration of pioglitazone compared to diabetic group. It has been reported that hyperglycemia causes dysfunction of Leydig cells thus these cells can not secrete testosterone [51]. In this study, it was shown that dysfunction of leydig cells under hyperglycemic effect can cause reduction of testosterone level in diabetic rats. Moreover, diabetic animals treated with pioglitazone demonstrated the activity of some anti-oxidative enzymes. Pioglitazone had enhanced the level of glutathione, superoxide dismutase, catalase and total antioxidant capacity. The improvement in the level of glutathione, superoxide dismutase, catalase and total antioxidant capacity indicated that pioglitazone can either increase the proliferation of the antioxidant enzymes or reduce the formation of reactive oxygen species. This means that administration of pioglitazone can reduce excessive production of reactive oxygen species with a resulting decrease in the anti-oxidative defense. Modulation of superoxide dismutase and catalase activity by pioglitazone was demonstrated in the heart and liver of the alloxan-induced diabetic rabbits [21, 52]. Furthermore, it was shown that treatment of alloxan-induced diabetic rabbits with pioglitazone produces increased activity of antioxidant enzymes including glutathione peroxidase, superoxide dismutase, catalase and glutathione reductase, as well as elevated glutathione levels in testicular tissue [12].

Mehta et al. [53] identified that pioglitazone treatment can reduce superoxide radical generation in human coronary artery endothelial tissue. These findings are indicative of the potential of pioglitazone at improving antioxidant capacity in different tissue types.

In our research, pioglitazone did not affect blood glucose levels. This is in line with findings in which pioglitazone did not produce any significant changes in blood glucose levels in the treated animals [11, 49]. It seems that the effect of pioglitazone is to increase the sensitivity of the cells to insulin. Therefore, this drug is used in the treatment of type 2 diabetes. However, in type 1 diabetes, due to the degeneration of beta-cells and the absence of insulin secretion, the drug does not reduce blood glucose levels. Thus, the observed anti-oxidative effects can not be attributed to the drugs anti-hyperglycemic action.

Previous studies have suggested that the anti-inflammatory effect of pioglitazone is mediated by the inhibition of NF-κB activation [17]. It has been shown that native or synthetic PPAR-γ ligands inhibit the production of several inflammatory mediators, nitric oxide synthase and NF-κB transcription activity [54]. NF-κB plays a pivotal role in regulating programmed cell death by having the ability to activate both pro-apoptotic and anti-apoptotic genes and regulating a variety of additional genes, immune function, expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [55]. Biological markers of inflammatory responses are elevated level of NF-κB and the associated inflammatory mediators such as iNOS. This substance

catalyses the production of large amount of NO from L-arginine, and importantly, NO inhibited insulin secretion [9]. IL-1β, alone or in combination with TNF-α and IFNγ induces transcription of the iNOS gene [56]. In diabetes, a reduction of plasma antioxidant power and an increased production of NO have clearly been shown [57]. This over-production of NO induces testicular damage and chronic testicular inflammation, leading to the destruction of germ cells, testicular atrophy and apoptosis in diabetes [9, 58].

Previous reports have also shown that diabetes is associated with up-regulations of iNOS and NF-κB-p65 expressions with a concomitant increase in the level of NO in testicular tissues of diabetic rats [59, 51]. Takagi et al. [17] reported that pioglitazone can reduce expression of iNOS mRNA in mice model of colitis. The present study showed that pioglitazone can not only inhibit the expression of iNOS and NF-κB-p65 but can also decrease the level of NO in testicular tissues of diabetic rats.

Moreover, a significant increase in caspase-3 level was observed in diabetic testicular tissue which was further decreased by the administration of pioglitazone. This can indicate the anti-apoptotic effect of pioglitazone on diabetes induced germ cell death. Several compounds, such as parkia biglobosaprotein isolate [60], carvedilol [61], lycium barbarum polysaccharide [62] curcumin [63] and quercetin [64] possessing antioxidant properties might confer testicular protection against diabetes-induced oxidative stress and apoptotic markers. The present data

demonstrated the significant elevation of myeloperoxidase activity, a marker of neutrophil infiltration, inflammation and oxidative stress, in testicular tissues of diabetic animals. In pioglitazone treated diabetic group, significant reduction in myeloperoxidase activity was observed in testicular tissue. Our findings are in agreement with the previous studies [59, 54, 65] indicating the anti-inflammatory action of pioglitazone.

Results indicated significant elevation in the levels of TNF-α and IL-1β in the diabetic group of animals. These elevated proinflammatory mediators antagonize insulin action because of their ability to augment insulin receptor substrate phosphorylation leading to insulin resistance. Therefore, attenuation of free radical induced NF-κB translocation and ameliorating oxidative stress in diabetic rats explains an associative relationship between the inflammatory cytokines and diabetes. Rosiglitazone, another PPAR-γ ligand, inhibited NF-κB and expression of TNF-α and IL-1β in a mouse 2,4,6-trinitrobenzene sulfonic acid-induced colitis [66]. Our results are consistent with the previous studies showing that pioglitazone treatment decreases the levels of TNF-α and IL-1β in the diabetic group of animals; this indicates the anti-inflammatory effect of pioglitazone.

Conclusions

In summary, the results of the present study indicate that pioglitazone treatment protects against diabetes−induced testicular damage. The protective effect of pioglitazone possibly involves the reduction of oxidative stress by

decreasing nitric oxide and malondialdehyde levels and increasing the level of superoxide dismutase, catalas and total antioxidant capacity, and inflammatory status by decreasing the expressions of iNOS, NF-κB and pro-inflammatory cytokines (TNF-α and IL-1β) levels, and cell death via down-regulation of caspase-3 expressions.

Conflict of Interest
The authors have no conflicting financial interests.

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