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ORIGINAL ARTICLE

Is losartan a promising agent for the treatment of type 1 diabetes-induced testicular germ cell apoptosis in rats?

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Abstract

Background Diabetes mellitus (DM) is common metabolic disease that poses a major risk to public health and fertility. Previous studies indicate that DM may cause male infertility by triggering oxidative stress and germ cell apoptosis in the testis. Due to the undesirable effects of known antidiabetic drugs, scientists have begun to investigate the use of alternative drugs to control infertility complications observed in men. In this context, present study aimed to investigate the possible antiapoptotic effect of losartan against DM-induced testicular germ cell apoptosis.

Methods and results Expreimental DM model was induced by intraperitoneal injection of streptozocin (STZ, 55 mg/kg) to 28 rats, which were then randomly assigned to 4 groups; 1 mL saline solution was given to DM + saline group by oral gavage, 5 mg/kg/day oral losartan was given to DM + low-dose losartan, 20 mg/kg/day oral losartan was given to DM + mid-dose losartan and, 80 mg/kg/day oral losartan was given to DM + high-dose losartan group for 4 weeks. Bax, Bcl-2 and cleaved-Caspase 3 immunoexpression, terminal-deoxynucleotidyl transferase dutp nick end labeling (TUNEL), Annexin-V and Real Time PCR analyses performed to evaluate antiapoptotic effects of losartan on diabetic rats' testis. In addition, biochemical analyzes carried out to evaluate change in oxidative stress.

Conclusion The results showed that losartan may have dose-related antiapoptotic effects on rats' testis via decreasing oxidative stress.

Keywords Apoptosis · Diabetes · Losartan · Oxidative stress · Testis

Introduction

Diabetes mellitus (DM) is one of the common chronic disorders characterized by inappropriately elevated blood glucose level (hyperglycemia) caused by insufficient insulin secretion due to pancreatic β cells dysfunction [1]. This disease affects approximately 422 million people worldwide today and increases the risk of comorbidities such as cardiovascular diseases, nephropathy, retinopathy and neuropathy via triggering defects in carbohydrate, protein and lipid metabolism [1]. Also, growing evidence showed that adverse effects of DM can lead to male reproductive system dysfunctions by abnormal spermatogenesis, apoptotic alterations in the testes, low testosterone level, changes in sperm count and defective sperm morphologies [2].

Basic molecular mechanisms of DM-induced male reproductive system dysfunction is increased oxidative stress arising from chronic hyperglycemia-induced protein glycosylation and auto-glucose oxidation [3]. Numerous studies have indicated that the intrinsic (mitochondrial) apoptosis pathway activated in response to excess reactive oxygen species (ROS) production is a key paradigm in testicular injury induced by DM-associated oxidative stress [4]. These studies suggested that an increased in expression of intrinsic apoptosis pathway activator molecules such as Bax and cleaved-Caspase 3, whereas a decreased in expression of

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antiapoptotic molecules such as Bcl-2 in germ cells under diabetic condition [5].

Lipid peroxidation (LPO) is another important phenomenon of DM-related male reproductive system dysfunctions [6]. In fact, LPO induced by ROS attack caused malfunction of oxidant/antioxidant balance through LPO products accumulation such as malondialdehyde (MDA). Moreover, LPO inhibits the antioxidant enzymes activity such as superoxide dismutase (SOD) in testicular tissue [6]. Most importantly, it is known that LPO causes plasma membrane degeneration, DNA fragmentations and apoptosis in spermatogonic cells [7].

During recent years, the use of both natural and synthetic antioxidants to cure of DM-induced testicular damage have grown into one of the frequently investigated topics. In this context, therapeutic potential of losartan, an anti-hypertensive drug, on diabetic rat testes evaluated in the present study. Losartan is an angiotensin (Ang) II type I receptor antagonist that has a delaying effect on comorbidities such as nephropathy in hypertensive diabetic patients [8]. Additionally, losartan protects podocytes, pancreatic and retinal cells from apoptosis via reducing ROS and cleaning LPO products [9–11]. However, there is a big gap in the literature about the antiapoptotic activity of losartan in diabetic testes.

This study aimed to investigate the possible antiapoptotic effect of losartan on DM-induced testicular germ cell apoptosis. In this context, Bax, Bcl-2 and cleaved-Caspase 3 immunoexpression, terminal-deoxynucleotidyl transferase dutp nick end labeling (TUNEL), Annexin-V and Real Time PCR analyses were carried out to evaluating the antiapoptotic effects of losartan. In addition, biochemical analyzes were carried out to evaluate changes in oxidative stress and LPO.

Materials and methods

Animals

35 male Wistar rats weighing between 200 and 250 g at sexual maturity were used in the experimental procedure of present study. Until the experimental procedures were completed, rats were housed in rooms with 22 ± 3 ºC temperature, 45–75% humidity and 12 h dark/light cycle. They were fed orally with standard rat chow and tap water *ad libitum*. Experimental procedures of this study were performed strict accordance with international guidelines for the care and use of laboratory animals.

Induction type 1 DM model in rats and experimental Desing

Rats were divided into five groups, each contained seven rats. In determining this value, the principle of 3R (Replacement, Reduction and Refinement) proposed by Russell and Burch was taken into consideration [12].

Beginning of study, control group was formed with randomly selected 7 rats. Type 1 DM was induced in 28 rats by a single dose of 55 mg/kg STZ (Sigma-Aldrich, Inc.; Saint Louis, MO, USA) injection. STZ was dissolved in 0.1 M citrate buffer with pH 4.5 and injected via intraperitoneal route. Diabetes was verified after 24 h by evaluating the blood glucose levels. Rats with blood glucose levels of > 250 mg/dl were included in the study as the diabetic [13]. After then, diabetic rats were randomly separated into 4 groups, each contained seven rats:

DM + saline Rats belongs to this group were administered 1 mL/kg/day 0.9% NaCl via for 4 weeks.

DM + low-dose losartan Rats belongs to this group were treated with 5 mg/kg/day losartan (Cozaar 50 mg, Merck Sharp & Dohme, USA) diluted in 1 mL saline for 4 weeks [14].

DM + mid-dose losartan Rats belongs to this group were treated with 20 mg/kg/day losartan diluted in 1 mL saline for a 4 weeks period [15].

DM + high-dose losartan Rats belongs to this group were treated with 80 mg/kg/day losartan diluted in 1 mL saline for a 4 weeks period [16].

When the experimental protocols were completed, rats were anesthetized with combined ketamine (60 mg/kg, Ege Vet, Alfamine®, Alfasan International B.V., Holland) and xylazine (10 mg/kg, Ege Vet, Alfazyne®, Alfasan International B.V., Holland). After then, 1 ml of blood collected from all rats for biochemical analysis. After blood collection, dissection of testis and epididymis tissues were performed and animals were euthanized by cervical dislocation. To ensure standardization in analyses, right testes were fixed by 4% paraformaldehyde (PFA) for histopathological examination, while the left testes were preserved at -80 °C without fixation for real time PCR analyses.

Biochemical analyses

Isolation of plasma samples for biochemical analyses

Blood samples collected at the end of the experimental procedures were centrifuged at +4 °C and 1000 xg for 15 min to obtain plasma samples. Plasma samples, suitably frozen on dry ice, were stored at -80 °C until biochemical analyses.

Determination of LPO and antioxidant enzyme activation in plasma samples

LPO was determined by measuring MDA levels in plasma samples [17]. To determination of plasma MDA levels, the instructions of Lipid Peroxidation Colorimetric/Fluorometric Assay (BioVision®, CA, USA) were followed and plasma samples were measured at 532 nm with an ELISA plate reader (PolarSTAR Omega, BMG LABTECH, Germany).

Antioxidant enzyme activation was determined by measuring SOD activation levels in plasma samples [18]. Plasma samples, suitably frozen on dry ice, were stored at -80 °C until SOD levels analyses. To determination of SOD activity levels, the instructions of Superoxide Dismutase (SOD) Activity Assay Kit (BioVision®, CA, USA) were followed and plasma samples were measured at 450 nm with an ELISA plate reader (PolarSTAR Omega, BMG LABTECH, Germany).

Evaluation of Testosteron levels in plasma samples

To determination of testosteron levels, the instructions of Rat Testosteron ELISA Kit (CUSABIO, Wuhan, PRC) were followed [17] and plasma samples were measured at 450 nm with an ELISA plate reader (PolarSTAR Omega, BMG LABTECH, Germany).

Histopathological evaluation of testicular tissues

Right testis samples fixed for 48 h 4% PFA and tissues embedded in paraffin blocks using routine protocols. Afterward, tissues were sectioned at a thickness of 5-µm with a microtome. Tissue sections deparaffinized with xylene were rehydrated with a decreasing alcohol series and hematoxylin-eosin (H&E) staining was performed following the routine protocol [17]. Tissues were photographed after staining with a digital camera (C-5050, Olympus, Tokyo, Japan) mounted on a microscope (BX5, Olympus, Tokyo, Japan).

Immunoexpresions of the bax, Bcl-2 and cleaved-caspase 3

To endogenous peroxidase blockade testicular tissues were incubated with 10% hydrogen peroxide (H_2O_2) solution (Sigma Aldrich, Inc., St Louis, Missouri, USA) for 30 min. Next, Super Block (Scytec Consulting Inc., Greenwood Village, Colorado, USA) solution were dropped on the sections and incubated for 1 h at room temperature. After, sections washed with PBS and were incubated with 1:200 diluted primary antibodies (Bcl-2, Bax and cleaved Caspase-3; Santa Cruz, California, USA) for 24 h at +4 °C. Afterward, sections were respectively incubated with biotinylated secondary antibody (Scytec Consulting Inc.) and horseradish peroxidase (HRP)-conjugated streptavidin (Scytec Consulting Inc). Ultimately, sections incubated with diaminobenzidine (DAB) were performed with Mayer Hematoxylin (Merck, Germany) [19].

Terminal-deoxynucleotidyl transferase dUTP Nick End labeling (TUNEL) assay

TUNEL analysis was performed to determine apoptosis [20] in testicular tissues belonging to groups. To determination of apoptotic index (AI) of all groups, TUNEL assay carried out according to instructions of the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Merck, Germany). AI of all groups was established by TUNEL positive cell count on the photographs of testicular tissue sections applied TUNEL assay [21]. Counting was repeated by three histologists blinded to each other and recorded numbers were averaged to determine AI.

RNA isolation and real time PCR analysis

50 mg of left testis specimens removed were taken into 1 ml of TriPure Isolation Reagent(Roche Applied Science, Germany) with guanidinium thiocyanate and specimen stored at -20 °C until used. RNA isolation was performed according to instructions of the TriPure Isolation Reagent Kit. After, cDNA synthesis was performed by following routine protocols. After cDNA synthesis, Real Time PCR analysis was carried out according to instructions of SYBR® Green PCR Master Mix (ThermoFisher, Waltham, USA) and Light Cycler 480 (Roche, Germany). Changes in gene expression were calculated by $2^{-\Delta\Delta Ct}$ method [22]. The primer sequences are given in Table S1 [23–25].

Table 1 SOD, MDA and testosteron values of rat blood plasmas

	Control	DM + Saline	DM + Low-Dose Losartan	DM + Mid-Dose Losartan	DM + High-Dose Losartan
SOD Activation (% Inhibition Rate)	120.15 ± 7.39	75.37 ± 5.28[I]	138.81 ± 6.33[I,II]	97.01 ± 8.44 [I,II,III]	86.57 ± 2.11 [I,II,III]
MDA Levels (nmol/ml)	56.98 ± 8.22	122.09 ± 8.22[I]	81.4 ± 3.29[I,II]	81.4 ± 6.58[I,II]	82.56 ± 1.64[I,II]
Testosteron Levels (ng/ml)	27.090 ± 3.996	10.810 ± 0.697[I]	15.647 ± 1.865[I,II]	15.869 ± 3.771 [I,II,III]	19.251 ± 2.188[I,II,III]

Values are presented mean ± SEM. I: Statistically significant compared to control group ($p < 0.05$). II: Statistically significant compared to DM + saline group ($p < 0.05$). III: Statistically significant compared to DM + low-dose losartan group($p < 0.05$)

Sperm parameters

Preparation of sperm samples

Cauda of left epididymis were minced in 10 mL of Ham's F10 medium and incubated for 15 min at 37 °C to release sperm into medium. The incubated samples were mixed several times with Pasteur pipette to obtain a homogenous sperm suspension. 0.5 ml of suspension was then transferred to Falcon's tubes containing 2 ml of saline and centrifuged at 1000 xg for 5 min. Supernatant was removed and the pellet dissolved in 1 ml of saline [26]. Samples were used for sperm morphology, total and apoptotic sperm count analyzes.

Determination of Epididymal sperm count

Sperm counting was performed with a hemocytometer under a phase contrast microscope using the calculation system proposed by Wang [26]. Counting was repeated by three histologists blinded to each other and recorded numbers were averaged to determine the sperm counts of the groups.

Sperm morphology analysis

Epididymal sperm was spread on clean glass slides and slides air dried, fixed in methanol and stained with Giemsa for 35 min. To remove excess stain, slides were washed under running tap water and slides air dried [27]. For each slide prepared in this way, 250 spermatozoa were randomly examined by three histologists blinded to each other and recorded numbers were averaged to determine percentage of sperm with abnormal morphology [24].

Determination of apoptotic sperm count by Annexin-V method

Apoptotic sperm count analysis was performed with Annexin-V method [28]. To determination of apoptotic sperm count activity the instructions of Muse ™ Cell Analyzer using the Muse ™ Annexin V & Dead Cell Kit were followed. 100 µl Muse ™ Annexin V & Dead Cell reagent took to the sterile microcentrifuge tubes and add 100 µl sperm sample. These solution incubated for 20 munite in the dark and room temperature. At the end of the incubation sample analysed with Muse ™ Cell Analyzer. Analysis was repeated three times for each group.

Statistical analysis

SPSS version 15.0 for Windows software (IBM Corp., Armonk, NY) was used for statistical analysis. Then, statistical comparison between control and other groups were analysed by using one-way analysis of variance (ANOVA) and Tukey post hoc test. Data were expressed with mean standard errors (SEM) and $p < 0.05$ was considered statistically significant.

Results

Losartan reduced lipid peroxidation and increased antioxidant enzyme activity

The increased MDA level in DM + saline group was significantly decreased in DM + mid-dose losartan and DM + high-dose losartan. In addition to the decrease in MDA level, SOD activity was significantly increased in these groups. Changes in blood MDA levels and SOD activity are shown in Table 1.

Losartan regulated testosterone level in diabetic conditions

The finding showed that STZ administration caused a significant decrease in plasma testosterone level. When diabetic groups were compared, testosterone levels were significantly higher in the DM + mid-dose losartan group (Table 1).

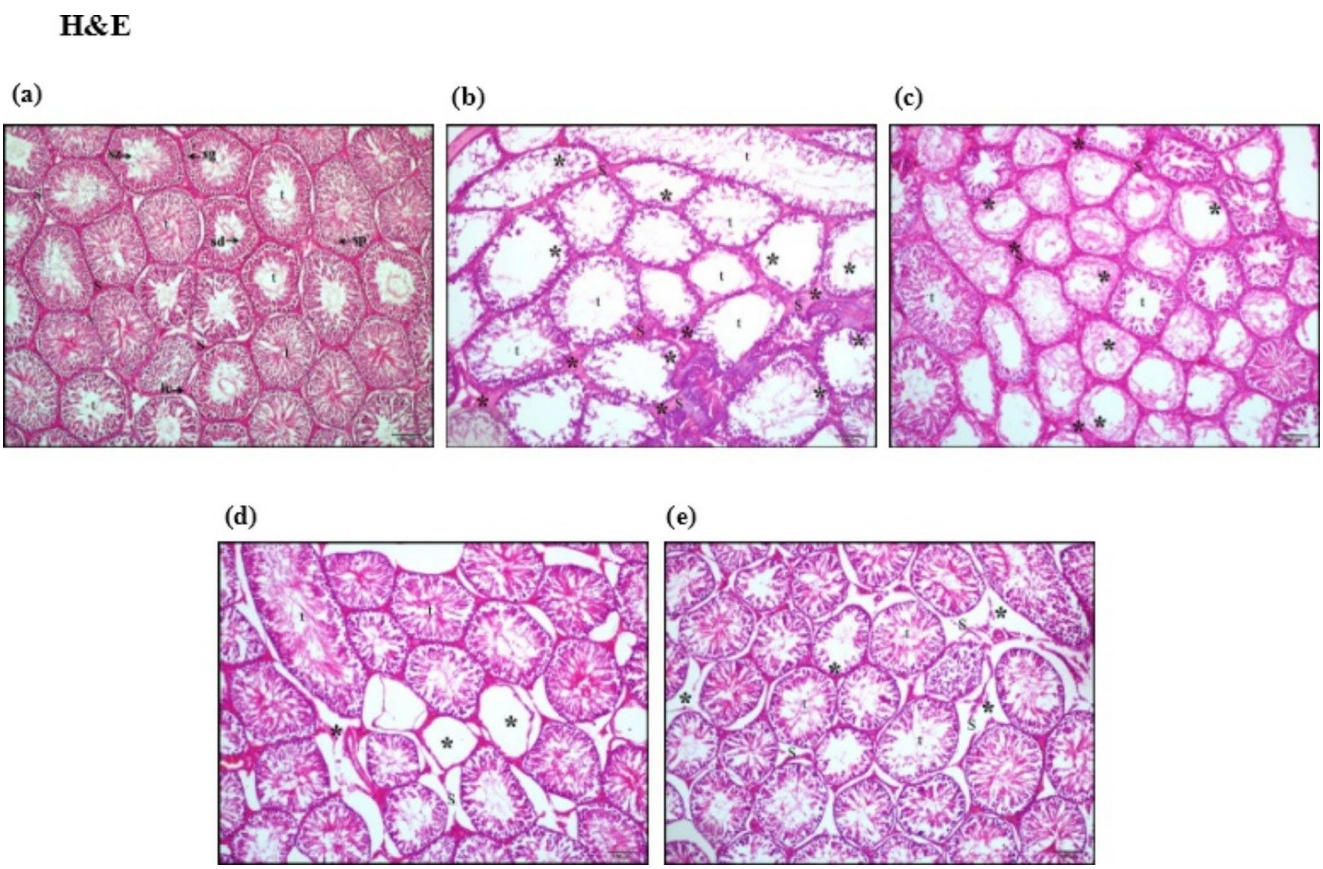

Fig. 1 Hematoxylen & Eosine (H&E) staining of sections from control and other experimental groups. Control **(a)** groups testes showed normal seminiferous tubules. Diabetes + salin administered**(b)** group testes showed number of histopathological changes such as spermatogenic and Leydig cells degeneration, seminifer tubule vacuolization and inflammation. Diabetes + low dose losartan administered**(c)** group testes showed decrease in spermatogenic and Leydig cells degeneration, seminifer tubule vacuolization but can showed some pathologies such as interstitial edema, inflammation and narrowing of capillary compared to the control group. Diabetes + mid dose losartan administered**(d)** group testes showed significantly decrease in spermatogenic and Leydig cells degeneration, disorganization in seminiferous tubules compared to other diabetic groups. Diabetes + high dose losartan administered**(e)** group testes showed seminiferous tubules degeneration higher than mid dose losartan group (x10 magnification)

Losartan alleviated DM-induced testicular damage

When the testes tissues were histopathologically examined, disorganization in seminiferous tubules and intense losses in spermatogenic cells were determined in DM + saline group. Additionally, losses in Leydig cells, extensive inflammatory cell infiltration, inflammation, narrowing of capillaries and hyperemia are other pathological changes observed in the interstitial connective tissue of this group.

Loss of spermatogenic cells was slightly decreased in the DM + low-dose losartan group compared to DM + saline group. However, pathologies such as Leydig cells defects, interstitial edema, inflammation and narrowing of capillary were maintained a great extent when compared to the control group.

Histopathological findings such as interstitial edema, cellular dissociation, Leydig cells losses, spermatogonial cells defects and disorganization in seminiferous tubules were significantly decreased in DM + mid-dose losartan group compared to other diabetic groups. In an other saying, the general histological parameters of this group were close to the control group.

Most of the histopathological changes in DM + saline group were not detected in DM + high-dose losartan group. In contrast, the number of degenerate seminiferous tubules was higher than DM + mid-dose losartan group (Fig. 1). Johnsen testicular biopsy scores (JTBS)[29] are shown in Table 2 with p values.

Losartan had an effect on apoptosis-related protein expressions

Significant increase detected in Bax and cleaved-Caspase 3immunoreactivity in DM + saline and DM + low-dose losartan groups, whereas there was a significant decrease in

Table 2 Histological scores and immunoexpression levels of control and other experimental groups

Group	Control	DM + Saline	DM + Low-Dose Losartan	DM + Mid-Dose Losartan	DM + High-Dose Losartan	F	p value
Histological Scores							
JTBS	9.53 ± 0.10	3.28 ± 0.32	3.75 ± 0.47	7.40 ± 0.28	7.83 ± 0.37	65.86	0.0001
Immunoexpresion levels							
Bax	33 ± 0.81^{II}	148 ± 1.17^{I}	126 ± 1.63^{I,II}	49 ± 1.29 ^{I,II}	50 ± 1.40 ^{I,II}	1615.13	0.0001
Bcl 2	153 ± 1.31^{II}	25 ± 1.06^{I}	88 ± 1.18 ^{I,II}	140 ± 1.35 ^{I,II}	105 ± 1.34 ^{I,II}	1629.85	0.0001
claved-Caspase 3	45 ± 1.46^{II}	168 ± 1.46^{I}	106 ± 1.46 ^{I,II}	60 ± 1.35 ^{I,II}	78 ± 1.34 ^{I,II}	1169.33	0.0001
TUNEL Scores (%)							
TUNEL Positive Cells	30 ± 1.34^{II}	150 ± 1.35^{I}	132 ± 1.46 ^{I,II}	47 ± 1.54 ^{I,II}	50 ± 1.34 ^{I,II}	1516.82	0.0001

Values are presented mean ± SEM. I: Statistically significant compared to control group ($p < 0.05$). II: Statistically significant compared to DM + saline group ($p < 0.05$)

Bcl-2 positive cell number and expression intensity in these groups.

On the other hand, the number of Bcl-2 positive spermatogenic and Leydig cells in the DM + mid-dose losartan group decreased compared to the control group, while Bcl-2 expression intensity was significantly higher than DM + saline group testicular tissues. Moreover, Bax and cleaved-Caspase 3expression were lower in the DM + mid-dose losartan group compared to DM + saline and DM + low-dose losartan groups.

In the evaluation of DM + high-dose losartan group, the Bcl-2, Bax and cleaved-Caspase 3immunoexpression patterns were found similar to the DM + mid-dose losartan group. When these two groups were compared, cleaved-Caspase 3 and Bax expression were found to be slightly higher in the DM + high-dose losartan group (Fig. 2).

The immunoexpression scores (H-scores) [30] and p values are shown in Table 2.

Losartan reduced DM-induced apoptosis in spermatogenic cells

The number of TUNEL positive cells in the testicular tissues of the control group was quite low compared to the other groups. In DM + saline group, TUNEL positive cells were dramatically higher in primary spermatocytes, spermatids and myoepithelial cells compared to control group ($p < 0.05$).

Similar to DM + saline group, high levels of TUNEL positive cells was observed in DM + low-dose losartan group, particularly in the spermatogenic cells. Furthermore, the TUNEL positive cells was significantly higher in Leydig cells compared to the control group ($p < 0.05$). The number of TUNEL positive cells was lower in the spermatogenic cells compared to DM + saline group.

TUNEL positive cell counts were higher in DM + mid-dose losartan group compared to the control group. On the contrary, less TUNEL positive cells were detected in comparison with DM + saline and DM + low-dose losartan group ($p < 0.05$).

Evaluation of high-dose losartan group, the TUNEL positive cells count was higher compared to the control and DM + mid-dose losartan group. Compared with other groups, a significantly reduced TUNEL positive cell was detected (Fig. 3). TUNEL scores and p values of the groups are shown in Table 2.

Apoptosis was also assessed by Annexin V method in epididymal sperm samples. As a result of this analysis, apoptosis was observed in diabetic groups at a higher rate than the control group. The number of apoptotic sperm decreased in the DM + mid-dose losartan group. In addition, the decrease in early apoptotic values indicates that sperm apoptotic orientation of sperm cells was reduced (Fig. 4).

Losartan regulated apoptosis-related gene expressions

Findings of Real Time PCR analysis indicated that Bcl-2 mRNA expression significantly decreased while Bax and Caspase 3 mRNA expression significantly increased in the DM + saline group compared to other groups. Real Time PCR showed that Bax and Caspase 3 mRNA expressions were downregulated in DM + mid-dose losartan group compared to other diabetic groups. In fact, mRNA expression pattern of the DM + mid-dose losartan group was closest to the control group among the diabetic groups. The results of Real Time PCR analysis are shown in Table 3.

Losartan increased epididymal sperm count

When total epididymal sperm counts were compared, there was a significant decrease in DM + saline group compared to control and other diabetic groups ($p < 0.05$). A significant increase in sperm count was observed in DM + medium dose losartan group compared to other diabetic groups ($p < 0.05$) (Table 4).

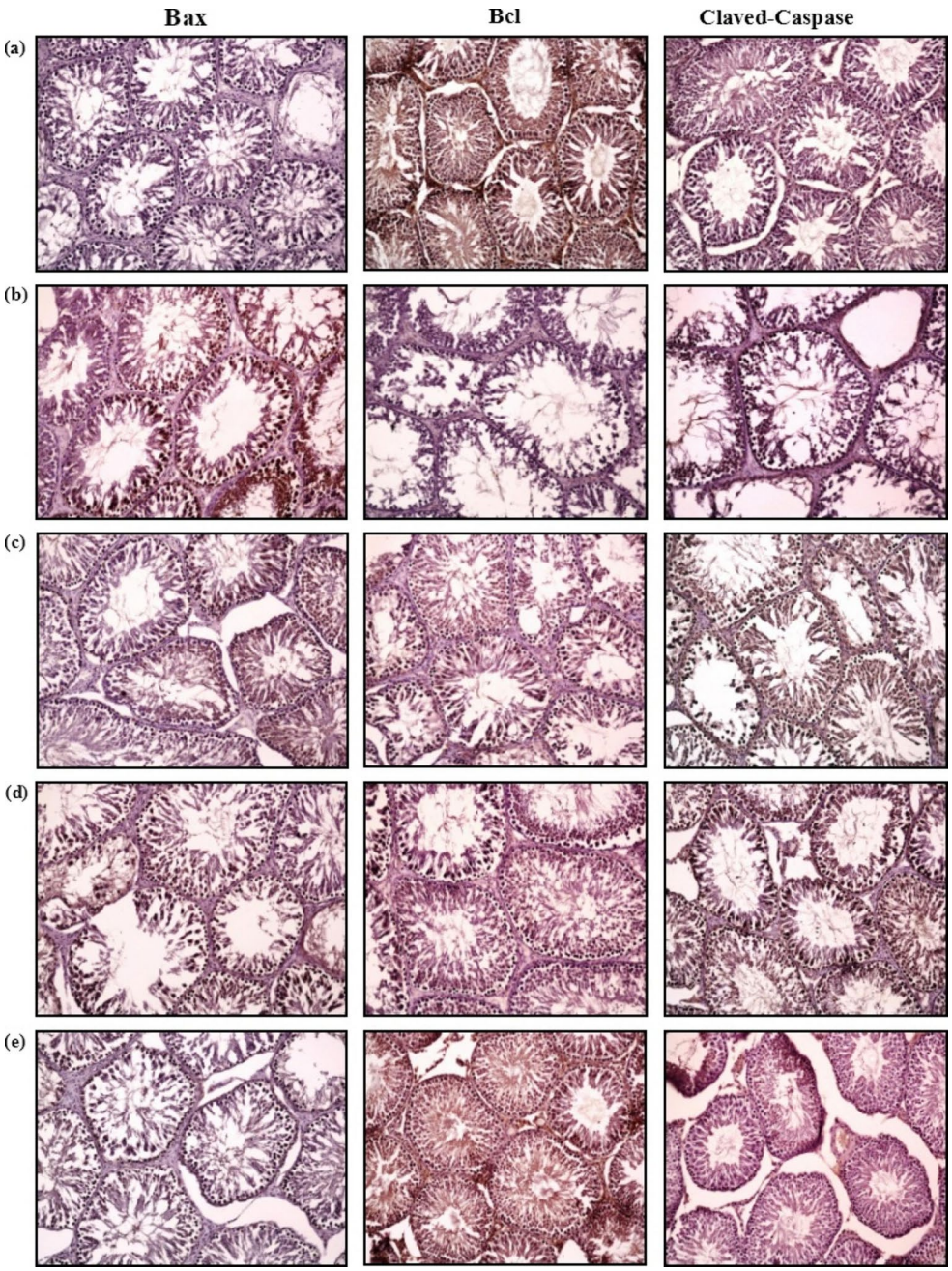

Fig. 2 Bax, Bcl-2 and Caspase 3 immunostaining of all experimental groups testes. There was difference between control **(a)** and other diabetic groups (diabetes + salin group**(b)**, diabetes + low dose losartan group**(c)**, diabetes + mid dose losartan group**(d)** and diabetes + high dose losartan group**(e)**) ($p > 0.05$) in terms of Bax, Bcl-2 and Caspase 3 protein expression. On the other hand, Bax and caspase 3 expressions significantly increase compared to the control group in the diabetic groups ($p < 0.05$). Bcl-2 expression decreased compared to the diabetic groups ($p > 0.05$). (x20 magnification)

Sperm morphology analysis indicated that the percentage of abnormal sperm morphology significant increased in DM + saline group compared to control and other diabetic groups ($p < 0.05$). Moreover, the percentage of abnormal sperm in DM + mid-dose and DM + high-dose groups was similar and lower than DM + saline group. The results of sperm morphology analysis are shown Table 4.

Discussion

Growing evidence suggests that DM is closely associated with fertility problems, particularly in the male [31]. In fact, previous studies have reported that DM causes serious disruptions in male reproductive function by paving the way for problems such as testicular tissue defects [31, 32]. Similar to the literature, the results of our histopathological examination (JTBS) revealed that DM was dramatically disrupted histo-architecture of the testicular tissue and caused histopathological alterations. Taken together with literature [33, 34], it can be argued that Leydig cell losses

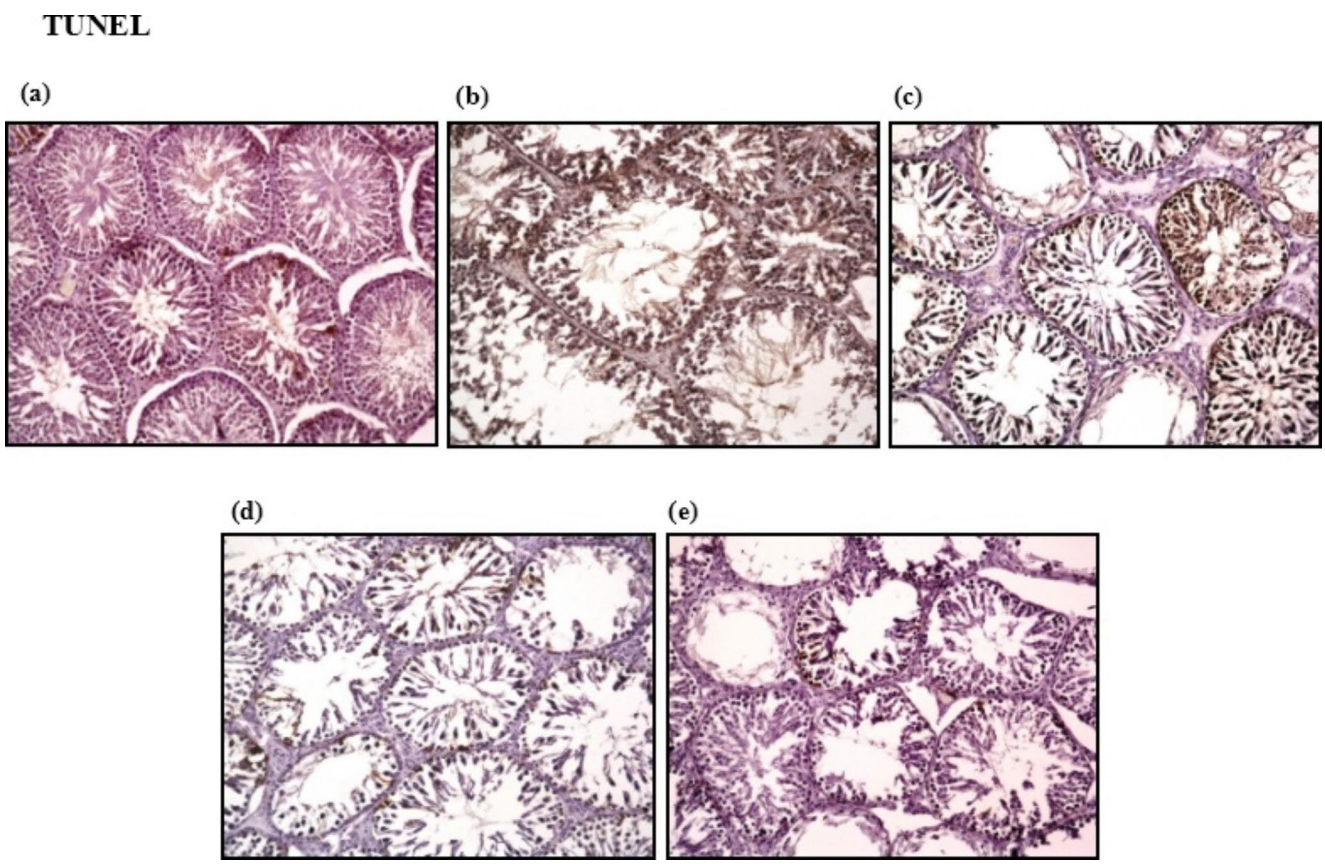

Fig. 3 TUNEL staining of all experimental groups testes. (x20 magnification). Control **(a)** group testes showed a few TUNEL positive cells. Diabetes + salin administered group **(b)** testes showed a large number TUNEL positive cells. In particular, TUNEL positive primary spermatocytes and spermatogonial cells are high compared to control group ($p < 0.05$). TUNEL positive cells in diabetes + low dose losartan administered group**(c)** that was higher compared to the control group, but TUNEL positive cells in diabetes + mid dose losartan group**(d)** decreased significantly compared to the diabetes + salin group ($p < 0.05$). TUNEL positive cell in spermatogenic cells was found higher in diabetes + high dose losartan group**(e)** compared to control group ($p < 0.05$)

are clearly associated with the decrease in testosterone and total epididymal sperm count in diabetic rats. In addition to testicular tissue defects, DM can triggers severe sperm morphology abnormalities by increasing the oxidative stress related LPO products such as MDA. Also, DM-associated oxidative stress causes a decrease in activation of antioxidant enzymes such as SOD [33]. In this study, we found that DM caused a decreasing in SOD activity but increasing in MDA levels and abnormal sperm morphology. In this context, our study supports the previous studies and points out that LPO may caused contribute to male reproductive dysfunction. On the other hand, histopathological and biochemical results of the this study pointed out that mid-dose losartan may mitigate the testicular tissue degeneration and significantly regulate LPO and SOD activity via restoring the histology/biochemistry of testis. Besides, the increase in testosterone level in parallel with the decrease in Leydig cell loss indicates that mid-dose losartan may affect hormonal regulation of spermatogenesis.

DM-induced oxidative stress also triggers apoptotic cell death in spermatogonia and spermatocytes [35]. Studies reveal that ROS accumulated with increasing oxidative stress causes alterations in protein expressions involved in the intrinsic apoptosis pathway [13]. For example, Zhao et al. showed an increase in pro-apoptotic Bax expression and a decrease in antiapoptotic Bcl-2 expression in diabetic rats' testis [36]. Also, different studies have shown that TUNEL positive germ cell count [37] and caspase (3 and 9) [38] activity are increased in diabetic rats. In herein, we determined an increase in pro-apoptotic Bax and Caspase-3 expressions and a decrease in antiapoptotic Bcl-2 expression in diabetic rats' testis. Furthermore, TUNEL positive germ cells count was high in the diabetic rats' testis compared to control. In addition, Annexin V results showed that the number of late apoptotic cells increases dramatically in diabetic rats. However, the decrease observed in the count of apoptotic cells in testicular tissue and epididiymal sperm samples belonging to mid-dose losartan group indicates that losartan may exert antiapoptotic activity in a dose-dependent

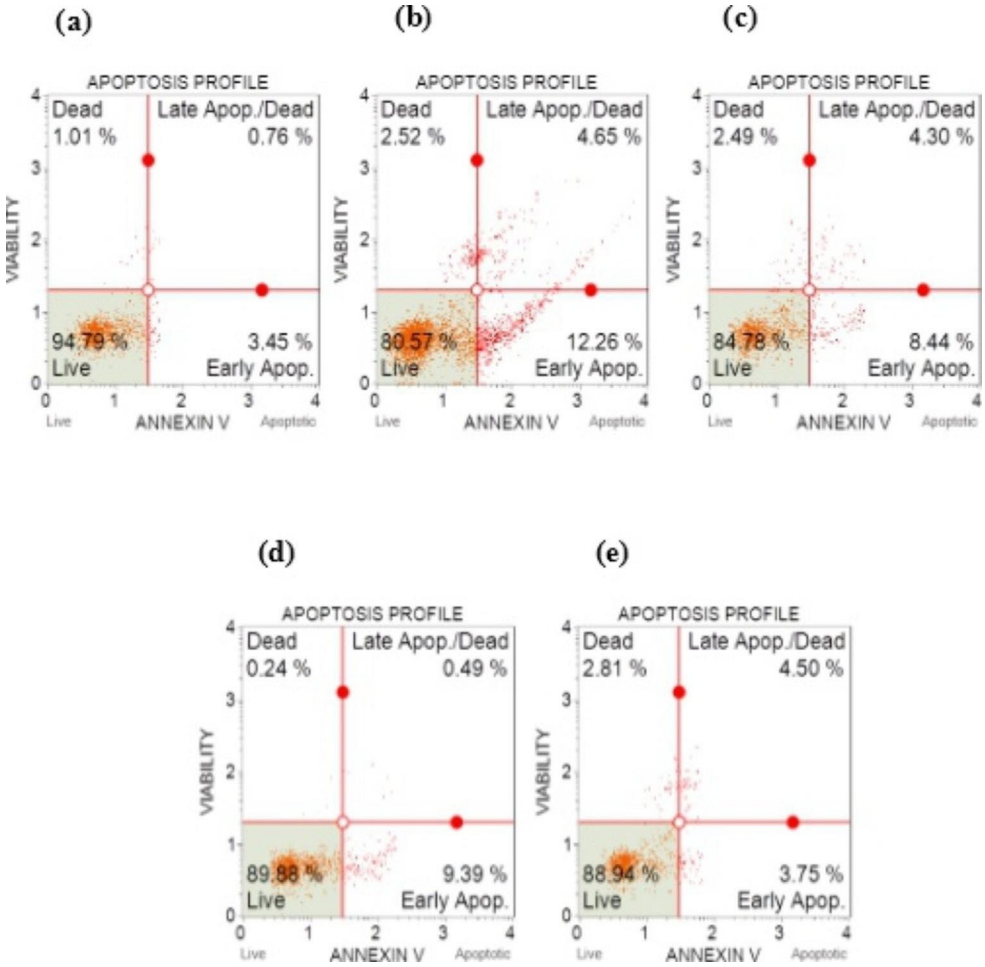

Fig. 4 Annexin V analyses of epididymal sperm sample

Table 3 Findings of epididymal sperm count and sperm morphology analysis of all groups

Group	Control	DM + Saline	DM + Low-Dose Losartan	DM + Mid-Dose Losartan	High Dose	p value
Epididymal Sperm Counts (x10⁶)	23 ± 4,082	8 ± 1.19^{I}	12 ± 1.17^{I}	17 ± 1.46 ^{I,II}	20 ± 1.46 ^{II}	< 0,0001
Number of Sperm with Abnormal Morphology/250	63 ± 1.19^{II}	198 ± 1.35^{I}	141 ± 1.50^{I,II}	84 ± 1.46 ^{I,II}	90 ± 1.29 ^{I,II}	0,0001
Percentage of Sperm with Abnormal Morphology (%)	21 ± 0.40^{II}	66 ± 0.45^{I}	47 ± 0.50 ^{I,II}	28 ± 0.49 ^{I,II}	30 ± 0.43 ^{I,II}	0,0001

Values are presented mean ± SEM. I: Statistically significant compared to control group ($p < 0.05$). II: Statistically significant compared to diabetes + saline group ($p < 0.05$)

Table 4 The fold change of Bax, Bcl-2 and Caspase 3 genes expressions in rat testes

$2^{-(\Delta\Delta Ct)}$ (Fold Change)	Control	Diabetes	Low Dose	Mid dose	High Dose
Bax	1	9.84	2.6	1.01	2.14
Bcl 2	1	3.57	2.14	2.48	1.1
Caspase 3	1	15.24	5.57	1.97	4.05

Values greater than 2 and less than −2 were considered significantly

manner. The existence of publications [10, 39, 40] showing that losartan exerts an antiapoptotic effect in different experimental models supports this idea. In addition to these, our immunohistochemistry and Real Time PCR analyzes indicate that losartan exerts its anti-apoptotic activity through regulation of molecules (Bcl-2, Bax and Caspase 3) in the intrinsic apoptosis pathway.

Today, it is known that drugs used to treat hyperglycemia in DM patients have devastating effects on other organs, such as testis. For example, Adaramoye et al. reported that metformin and glibenclamide cause significant reduction in the sperm count and histopathological alteration in testicular tissue via LPO and antioxidant system disruption [41]. Because of these undesirable effects, scientists began to investigate the use of alternative drugs to control the complications of DM. One of these drugs is losartan. This drug has a delaying effect on DM-related complications such as diabetic cardiomyopathy [39]. On the other hand, there is a

large gap in the literature regarding the effects of losartan on germ cell apoptosis associated with DM-induced testicular toxicity. Our results indicated that mid-dose losartan treatment can increase the chances of survival of germ cells by suppressing intrinsic apoptosis pathway at an early stage. Further studies are required to analyze the mechanisms underlying this possible antiapoptotic effect of losartan. On the other hand, there are clues that Ang-II overactivation causes inactivation of Leydig cells and inhibition of steroidogenesis in testes [42]. Therefore, Ang-II inhibition in diabetic conditions may be a promising target in the prevention or treatment of DM-related male reproductive problems.

In conclusion, findings of present study indicated that mid-dose losartan administration may have a therapeutic potential in diabetic testis by reducing the LPO, supporting the antioxidant system and inhibiting the apoptosis. In addition, inhibition of Ang-II provides a candidate approach in the treatment of male fertility problems associated with DM.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-08172-9.

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Authors' contributions AY; AB and FO desinged to study. AB; ÇG and GCK performed the animal models and in vivo experiments. AB, AU and GCK performed histochemical and immunohistochemical staining. NUKY and ÇG performed real time-PCR analyses. GY; FO and AB performed Annexin V analyses. NUKY and GY performed biochemical analyses. AY; AB and ÇG performed sperm parameters evaluation. AY; NUKY; AU and FO performed statistical analyses. ÇG and GCK wrote manuscript and all authors reviewed manuscript.

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Data availability Data available on request from the authors.

Declarations

Conflict of interest The authors declare that there is no confict of interests.

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