Is Losartan a Promising Agent for the Treatment of Type 1 Diabetes-Induced Testicular Germ Cell Apoptosis in Rats?

Aylin Buhur
Ege Universitesi
Çevik Gürel (cevikgurel@gmail.com)
Ege University https://orcid.org/0000-0003-0266-2115
Gökçe Ceren Kuşçu
Ege University: Ege Universitesi
Gürkan Yiğittürk
Mugla Sitki Kocman University: Mugla Sitki Kocman Universitesi
Fatih Oltulu
Ege University: Ege Universitesi
Nefise Ülkü Karabay Yavaşoğlu
Ege University: Ege Universitesi
Ayşegül Uysal
Ege University: Ege Universitesi
Altuğ Yavaşoğlu
Ege University: Ege Universitesi

Research Article

Keywords: Apoptosis, Diabetes, Losartan, Oxidative Stress, Testis

Posted Date: November 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2203719/v1

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. 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.

1. 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, which is expected to affect 642 million people by 2040, increases the risk of comorbidities such as cardiovascular diseases, nephropathy, retinopathy and neuropathy via triggering defects in carbohydrate, protein and lipid metabolism [2, 3]. Also, growing evidence showed that adverse effects of DM can lead to male reproductive system dysfunctions by including abnormal spermatogenesis, apoptotic alterations in the testes, low testosterone level, changes in sperm count and defective sperm morphologies [4].

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 [5]. Numerous study 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. 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 antiapoptotic molecules such as Bcl-2 in germ cells under diabetic condition [6–8].

Lipid peroxidation (LPO) is another important phenomenon of DM-related male reproductive system dysfunctions. In fact, LPO induced by ROS attack caused malfunction of oxidant/antioxidant balance through LPO products accumulation such as malondialdehyde (MDA) and inhibition of the antioxidant

enzymes activity such as superoxide dismutase (SOD) in testicular tissue. Most importantly, today it is known that LPO causes plasma membrane degeneration, DNA fragmentations and apoptosis in spermatogonic cells [9–11].

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 [12]. Additionally, losartan protects podocytes, pancreatic and retinal cells from apoptosis via reducing ROS and cleaning LPO products [13–15]. 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 diabetic rats' testis. In addition, biochemical analyzes were carried out to evaluate changes in oxidative stress and LPO.

2. Materials And Methods

2.1. 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.

2.2. 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 [16].
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 [10]. 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.

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.

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.

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.

2.3. Biochemical Analyses

2.3.1. Determination of LPO in Plasma Samples

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

2.3.2. Analysis of Serum SOD Activity in Plasma Samples

Blood samples collected at the end of the experimental procedures were centrifuged at + 4°C and 1000 xg for 15 minutes to obtain plasma samples. 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).

2.3.3. Evaluation of Testosteron Levels in Plasma Samples

Blood samples collected at the end of the experimental procedures were centrifuged at + 4°C and 1000 xg for 15 minutes to obtain plasma samples. Plasma samples, suitably frozen on dry ice, were stored at -80°C until testosteron levels analyses. To determination of testosteron levels, the instructions of Rat Testosteron ELISA Kit (CUSABIO, Wuhan, PRC) were followed 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, which were kept in paraformaldehyde (PFA) for 48 hours for fixation, were washed in phosphate buffer solution (PBS) for 24 hours after fixation and embedded in paraffin blocks using routine protocols. Sections of 5 µm were taken from paraffin embedded tissues. Sections were deparaffinized with xylene and stained with Hematoxylin-Eosin (H&E). Tissues were photographed after staining with a digital camera (C-5050, Olympus, Tokyo, Japan) mounted on a microscope (BX5, Olympus, Tokyo, Japan).

2.4. Immunoexpresions of the Bax, Bcl-2 and cleaved-Caspase 3

5 µm-thick sections were deparaffinized with xylene and hydrated by a series of graded alcohols. To endogenous peroxidase blockade, sections were kept in 10% H_2O_2 (Sigma-Aldrich, Inc.; Saint Louis, MO, USA) for 10 min. Sections were treated with Super Block (ScyTec Inc., USA) for 1 hour at room temperature for prevent non-specific antibody-antigen binding and washed with PBS. Next, incubation of sections with at appropriate dilution of primary antibodies (Bax. Bcl-2 and cleaved-Caspase 3, Santa Cruz, CA, USA) was performed. After primary antibody incubation, sections were respectively incubated with biotinylated secondary antibody (ScyTec Inc., USA) and horseradish peroxidase (HRP) conjugated streptavidin (ScyTec Inc., USA). In the last step, sections incubated with diaminobenzidine (DAB) and stained with Mayer Hematoxylin (Merck, Germany) [18].

2.5. Terminal-deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

TUNEL analysis was performed to determine apoptosis [19] 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 [20]. Counting was repeated by three histologists blinded to each other and recorded numbers were averaged to determine AI.

2.6. 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 [21]. The primer sequences are given in Table 1 [22–24].

Table 1
Primer sequences of genes used in Real Time PCR analysis

Gene	Primer Sequences	Reference
Bax	**Forward**: 5'-AGGGTGGCTGGGAAGGC-3' **Reverse**: 5'-TGAGCGAGGCGGTGAGG-3'	27
Bcl 2	**Forward**: 5'-ATCGCTCTGTGGATGACTGAGTAC-3' **Reverse**: 5'-AGAGACAGCCAGGAGAAATCAAAC-3'	27
Caspase 3	**Forward**: 5'-CCTCAGAGAGACATTCATGG-3' **Reverse**: 5'-GCAGTAGTCGCCTCTGAAGA-3'	29
GAPDH (House Keeping Gene)	**Forward**: 5'-GGATGCAGGGATGATGTTCT-3' **Reverse**: 5'-AAGGGCTCATGACCACAGTC-3'	28

2.7. Sperm Parameters

2.7.1. 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 minutes. Supernatant was removed and the pellet dissolved in 1 ml of saline [25]. Samples were used for sperm morphology, total and apoptotic sperm count analyzes.

2.7.2. Determination of Epididymal Sperm Count

Sperm counting was performed with a hemocytometer under a phase contrast microscope using the calculation system proposed by Wang [25]. Counting was repeated by three histologists blinded to each other and recorded numbers were averaged to determine the sperm counts of the groups.

2.7.3. Sperm Morphology Analysis

Epididymal sperm was spread on clean glass slides and slides air dried, fixed in methanol and stained with Giemsa for 35 minutes. To remove excess stain, slides were washed under running tap water and slides air dried [26]. 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 [23].

2.7.4. Determination of Apoptotic Sperm Count by Annexin-V Method

Apoptosis analysis was performed with the Muse ™ Cell Analyzer using the Muse ™ Annexin V & Dead Cell Kit. 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.

2.8. 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.

3. Results

3.1. 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 2.

Table 2
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($p < 0.05$).

3.2. 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 2).

3.3. 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)[27] are shown in Table 3 with p values.

Table 3
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 ($p < 0.05$).

3.4. 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 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) [28] and p values are shown in Table 3.

3.5. 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 3.

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).

3.6. 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 5.

Table 5
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.					

3.7. 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).

Table 4
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 ($x10^6$)	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 ($p < 0.05$).						

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.

4. Discussion

DM is a metabolic disease that poses a serious risk to public health and causes severe damage to a number of organs. This disease also regards as one of the important cause of morbidity and mortality worldwide Moreover, growing evidence suggests that DM is closely associated with fertility problems,

particularly in the male [29]. In fact, previous clinical and pre-clinical studies have reported that DM causes serious disruptions in male reproductive function by paving the way for problems such as testicular tissue defects [29, 30]. 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 such as seminiferous tubular atrophy, tubular degeneration, interstitial edema and Leydig cells losses. Another facts frequently reported in studies examining the effects of diabetes on male infertility is that DM causes a decrease in testosterone level and total epididymal sperm count [31–35]. Taken together with literature, it can be argued that Leydig cell losses 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 in the male reproduction system. DM-induced oxidative stress causes LPO in mammalian sperm, which contains abundant unsaturated fatty acids in their membranes [36]. Also, oxidative stress causes an increase in LPO products such as MDA and a decrease in activation of antioxidant enzymes such as SOD [37–39]. More importantly, LPO triggers sperm plasma membrane degeneration and DNA fragmentation and causes sperm morphology abnormalities in mammals [9]. In this study, we found that DM caused SOD activity to decrease but MDA levels and abnormal sperm morphology increased. In this context, our study supports the previous studies and points out that LPO may caused contribute to male reproductive dysfunction by causing sperm morphology abnormalities in diabetic conditions.

DM-induced oxidative stress triggers apoptotic cell death in spermatogonia and spermatocytes [40]. However, molecular mechanism of DM-induced germ cell apoptosis is not fully understood. Nevertheless, there is strong evidence that ROS accumulating with increased oxidative stress activates the intrinsic apoptosis pathway by increasing mitochondrial membrane permeability and promoting cytochrome C release [10]. 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 [41]. Also, different studies have shown that TUNEL positive germ cell count [42] and caspase (3 and 9) [43] 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 to these analyzes, Annexin V method which we use to determine early and late apoptotic sperm cell count showed that the number of late apoptotic cells increases dramatically in diabetic rats.

Today, it is known that drugs used to treat hyperglycemia in DM patients have devastating effects on many other organs, such as the 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 [44]. Because of these undesirable effects, scientists began to investigate the use of alternative drugs to control the complications of diabetes. One of these drugs is losartan. Clinical and pre-clinical studies have shown that this drug has a delaying effect on DM-related complications such as diabetic cardiomyopathy [45]. 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. Present study was planned to determine the effect of losartan against DM-induced testicular germ cell apoptosis, oxidative stress, and histopathological change. Results of the present study pointed out that mid-dose losartan treatment may mitigate the testicular tissue degeneration and significantly regulate LPO and SOD activity via restoring the histology/biochemistry of testis. Previous studies have shown that losartan administering to diabetic rats has a protective effect by cleaning LPO products in pancreatic and retinal cells [13, 15]. These reports support to results of this study and result of this study suggest that mid-dose losartan may have an antioxidant effect on diabetic testicular cells such as spermatogonia, spermatocyte and Leydig cells. 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.

The immunohistochemical and Real-Time PCR analysis of the study suggested that mid-dose losartan could attenuate apoptotic cell death in diabetic testicular germ cells. The analysis showed that pro-apoptotic Bax and cleaved-Caspase 3 protein and mRNA expression decreased in the diabetic group administering mid-dose losartan, whereas antiapoptotic Bcl-2 protein and mRNA expression increased. In this group, TUNEL (+) testicular germ cell number was found to significantly lower compared to other diabetic groups. In a study with similar results, Yu et al. revealed that Ang-II-induced apoptosis was suppressed in the glomerular podocytes of diabetic rats treated with losartan [14]. Recently, Wang et al. showed that losartan treatment in cavernous nerve injury rat model produced a protective effect by suppressing the expression of pro-apoptotic molecules such as Bax and Caspase 3 in cavernous cells and supporting the expression of antiapoptotic molecules such as Bcl-2 [46]. Furthermore, Bolat et al. reported that losartan administration in the experimental varicocele model reduced TUNEL (+) testicular germ cell count [47]. When the results of our study and the literature are evaluated together, it can be suggested that losartan supports survival of testicular germ cells by suppressing apoptosis. Besides, decrease in the number of late apoptotic cells despite the increase in the number of early apoptotic cells in medium and high dose losartan groups observed in Annexin V tests is another noteworthy finding. This finding supports the argument that moderate dose losartan administration can increase the chances of survival of these cells by suppressing testicular germ cell apoptosis 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 inhibition may be one of these mechanisms. On the other hand, there are clues that Ang-II inhibition may be one of these mechanisms. For example, Ang-II activates apoptosis under diabetic conditions and causes damage to many organs, including the testis [48, 49]. Moreover, Ang-II overactivation causes inactivation of Leydig cells and inhibition of steroidogenesis in testes [50]. 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.

Declarations

Acknowledgement: The experimental procedures on the animals in this study were performed in Ege University, Drug Research and Development and Pharmacokinetic Applications (ARGEFAR).

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. NUK and ÇG performed real time-PCR analyses. GY; FO and AB performed Annexin V analyses. NUK 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.

Funding: This study was supported by the Ege University Research Fund [grant number 16-TIP-092 (to Altuğ Yavaşoğlu)].

Data availability: Data available on request from the authors

Declarations

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

Consent to participate: All authors have given permission to participate in this publication.

Consent for publication: The publication is approved by all Authors.

Ethical approval: The protocol was approved by Ege University, Local Ethics Committee for Animal Experiments (Approval no: 2016-085).

References

1. Banday MZ, Sameer AS, Nissar S (2020) Pathophysiology of diabetes: An overview. Avicenna J Med 10:174
2. Xu G, Liu B, Sun Y, Du Y, Snetselaar LG, Hu FB, Bao W (2018) Prevalence of diagnosed type 1 and type 2 diabetes among US adults in 2016 and 2017: population based study. BMJ 362:k1497
3. Petrie JR, Guzik TJ, Touyz RM (2018) Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. Can J Cardiol 34:575–584
4. Pourmasumi S, Sabeti P, Rahiminia T, Mangoli E, Tabibnejad N, Talebi AR (2017) The etiologies of DNA abnormalities in male infertility: An assessment and review. Int J Reprod Biomed 15:331

5. HE Z, YIN G, LI QQ, ZENG Q (2021) J. DUAN, Diabetes Mellitus Causes Male Reproductive Dysfunction: A Review of the Evidence and Mechanisms, In Vivo (Brooklyn)., 35 2503
6. Koh P-O (2007) Streptozotocin-induced diabetes increases apoptosis through JNK phosphorylation and Bax activation in rat testes. J Vet Med Sci 69:969–971
7. Koh P-O (2007) Streptozotocin-induced diabetes increases the interaction of Bad/Bcl-XL and decreases the binding of pBad/14-3-3 in rat testis. Life Sci 81:1079–1084
8. Sadik NAH, El-Seweidy MM, Shaker OG (2011) The antiapoptotic effects of sulphurous mineral water and sodium hydrosulphide on diabetic rat testes. Cell Physiol Biochem 28:887–898
9. Karimi J, Goodarzi MT, Tavilani H, Khodadadi I, Amiri I (2011) Relationship between advanced glycation end products and increased lipid peroxidation in semen of diabetic men. Diabetes Res Clin Pract 91:61–66
10. Yigitturk G, Acara AC, Erbas O, Oltulu F, Yavasoglu NUK, Uysal A, Yavasoglu A (2017) The antioxidant role of agomelatine and gallic acid on oxidative stress in STZ induced type I diabetic rat testes. Biomed Pharmacother 87:240–246
11. Nasr AY (2017) The impact of aged garlic extract on adriamycin-induced testicular changes in adult male Wistar rats. Acta Histochem 119:648–662
12. Katsiki N, Tsioufis K, Ural D, Volpe M (2018) Fifteen years of LIFE (Losartan Intervention for Endpoint Reduction in Hypertension)-Lessons learned for losartan: An “old dog playing good tricks”. J Clin Hypertens 20:1153–1159
13. Kamper M, Tsimpoukidi O, Chatzigeorgiou A, Lymberi M, Kamper EF (2010) The antioxidant effect of angiotensin II receptor blocker, losartan, in streptozotocin-induced diabetic rats. Transl Res 156:26–36
14. Yu SY, Qi R, Zhao H (2013) Losartan reverses glomerular podocytes injury induced by AngII via stabilizing the expression of GLUT1, Mol. Biol Rep 40:6295–6301
15. Silva KC, Rosales MAB, Biswas SK, Lopes de Faria JB (2009) Lopes de Faria, Diabetic retinal neurodegeneration is associated with mitochondrial oxidative stress and is improved by an angiotensin receptor blocker in a model combining hypertension and diabetes. Diabetes 58:1382–1390
16. Tannenbaum J, Bennett BT (2015) Russell and Burch’s 3Rs then and now: the need for clarity in definition and purpose. J Am Assoc Lab Anim Sci 54:120–132
17. Gurel C, Kuscu GC, Buhur A, Dagdeviren M, Oltulu F, Karabay Yavasoglu NU, Yavasoglu A (2019) Fluvastatin attenuates doxorubicin-induced testicular toxicity in rats by reducing oxidative stress and regulating the blood-testis barrier via mTOR signaling pathway. Hum Exp Toxicol 38:1329–1343
18. Oktem G, Uysal A, Oral O, Sezer ED, Olukman M, Erol A, Akgur SA, Bilir A (2012) Resveratrol attenuates doxorubicin-induced cellular damage by modulating nitric oxide and apoptosis. Exp Toxicol Pathol 64:471–479
19. Akarca Dizakar S, Saribas GS, Tekcan A (2021) Effects of ellagic acid in the testes of streptozotocin induced diabetic rats,Drug Chem. Toxicol.,1–8

20. Kong W-Y, Tong L-Q, Zhang H-J, Cao Y-G, Wang G-C, Zhu J-Z, Zhang F, Sun X-Y, Zhang T-H, Zhang L-L (2016) The calcium-sensing receptor participates in testicular damage in streptozotocin-induced diabetic rats. Asian J Androl 18:803
21. Rao X, Huang X, Zhou Z, Lin X (2013) An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath 3:71–85
22. Behroozaghdam M, Hashemi M, Javadi G, Mahdian R, Soleimani M (2015) Expression of bax and bcl2 Genes in MDMA-induced Hepatotoxicity on Rat Liver Using Quantitative Real-Time PCR Method through Triggering Programmed Cell Death,Iran Red Crescent Med J,17
23. Lee K-M, Lee I-C, Kim S-H, Moon C, Park S-H, Shin D-H, Kim S-H, Park S-C, Kim H-C, Kim J-C (2012) Melatonin attenuates doxorubicin-induced testicular toxicity in rats. Andrologia 44(1):796–803
24. Xu W, Guo G, Li J, Ding Z, Sheng J, Li J, Tan W (2016) Activation of Bcl-2-caspase-9 apoptosis pathway in the testis of asthmatic mice. PLoS ONE 11:1–14
25. Wang Y (2003) Epididymal Sperm Count. In: Costa LG, Hudgson E, Lawrence DA, Reed DJ (eds) Curr. Protoc. Toxicol. John Wiley & Sons, Inc., Hoboken, NJ, USA. Unit16.6.
26. Akbarsha MA, Murugaian P (2000) Aspects of the male reproductive toxicity/male antifertility property of andrographolide in albino rats: effect on the testis and the cauda epididymidal spermatozoa. Phytother Res 14:432–435
27. Johnsen SG (1970) Testicular biopsy score count – a method for registration of spermatogenesis in human testes: Normal values and results in 335 hypogonadal males,Horm. Res. Paediatr.,
28. Gui Y, Zhang J, Yuan L, Lessey BA (1999) Regulation of HOXA-10 and its expression in normal and abnormal endometrium. MHR Basic Sci Reprod Med 5:866–873
29. Rakhshandeh H, Rajabi Khasevan H, Saviano A, Mahdinezhad MR, Baradaran Rahimi V, Ehtiati S, Etemad L, Ebrahimzadeh-bideskan A, Maione F, Askari VR (2022) Protective Effect of Portulaca oleracea on Streptozotocin-Induced Type I Diabetes-Associated Reproductive System Dysfunction and Inflammation,Molecules,27
30. Naas H, de Oliveira AA, Karpova T, Nunes KP (2019) Toll-like receptor 4 (TLR4) as a possible pathological mechanism in hyperglycemia-associated testicular dysfunction, Med, vol 127. Hypotheses, pp 116–119
31. Rato L, Alves MG, Dias TR, Lopes G, Cavaco JE, Socorro S, Oliveira PF (2013) High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology 1:495–504
32. El-Behery EI, El-Naseery NI, El-Ghazali HM, Elewa YHA, Mahdy EAA, El-Hady E, Konsowa MMH (2019) The efficacy of chronic zinc oxide nanoparticles using on testicular damage in the streptozotocin-induced diabetic rat model. Acta Histochem 121:84–93
33. Ahangarpour A, Oroojan AA, Khorsandi L, Arzani G, Afshari G (2016) Effects of Betulinic Acid on the Male Reproductive System of a Streptozotocin-Nicotinamide-Induced Diabetic Mouse Model. World J Mens Health 34:209

34. Atta MS, Almadaly EA, El-Far AH, Saleh RM, Assar DH, Al Jaouni SK, Mousa SA (2017) Thymoquinone Defeats Diabetes-Induced Testicular Damage in Rats Targeting Antioxidant, Inflammatory and Aromatase Expression,Int. J. Mol. Sci.,18
35. Simas JN, Mendes TB, Paccola CC, Vendramini V, Miraglia SM (2017) Resveratrol attenuates reproductive alterations in type 1 diabetes-induced rats. Int J Exp Pathol 98:312–328
36. John Aitken R, Clarkson JS, Fishel S (1989) Generation of Reactive Oxygen Species, Lipid Peroxidation, and Human Sperm Function. Biol Reprod 41:183–197
37. Barrera G (2012) Oxidative stress and lipid peroxidation products in cancer progression and therapy, ISRN Oncol., (2012) 137289
38. Kurutas EB (2016) The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr J 15:71
39. Colagar AH, Pouramir M, Marzony ET, Jorsaraei SGA (2009) Relationship between seminal malondialdehyde levels and sperm quality in fertile and infertile men. Brazilian Arch Biol Technol 52:1387–1392
40. Nna VU, Bakar ABA, Ahmad A, Eleazu CO, Mohamed M (2019) Oxidative Stress, NF-κB-Mediated Inflammation and Apoptosis in the Testes of Streptozotocin–Induced Diabetic Rats, vol 8. Combined Protective Effects of Malaysian Propolis and Metformin, p 465
41. Zhao L, Gu Q, Xiang L, Dong X, Li H, Ni J, Wan L, Cai G, Chen G (2017) Curcumin inhibits apoptosis by modulating Bax/Bcl-2 expression and alleviates oxidative stress in testes of streptozotocin-induced diabetic rats, Ther. Clin Risk Manag 13:1099–1105
42. Kanter M, Aktas C, Erboga M (2013) Curcumin attenuates testicular damage, apoptotic germ cell death, and oxidative stress in streptozotocin-induced diabetic rats. Mol Nutr Food Res 57:1578–1585
43. Rashid K, Sil PC (2015) Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum-dependent apoptotic death. Biochim Biophys Acta - Mol Basis Dis 1852:70–82
44. Adaramoye O, Akanni O, Adesanoye O, Labo-Popoola O, Olaremi O (2012) Evaluation of toxic effects of metformin hydrochloride and glibenclamide on some organs of male rats. Niger J Physiol Sci 27:137–144
45. Wang L, Li J, Li D (2015) Losartan reduces myocardial interstitial fibrosis in diabetic cardiomyopathy rats by inhibiting JAK/STAT signaling pathway. Int J Clin Exp Pathol 8:466–473
46. Wang Y, Meng X-H, Zhang Q-J, Wang Y-M, Chen C, Wang Y-C, Zhou X, Ji C-J, Song N-H (2019) Losartan improves erectile function through suppression of corporal apoptosis and oxidative stress in rats with cavernous nerve injury,Asian J. Androl.,
47. Bolat D, Oltulu F, Uysal A, Kose T, Gunlusoy B, Yigitturk G, Turk NS, Turan T (2016) Effects of losartan on experimental varicocele-induced testicular germ cell apoptosis. Andrologia 48:840–846
48. Hanes DS, Nahar A, Weir MR (2004) The tissue renin-angiotensin-aldosterone system in diabetes mellitus. Curr Hypertens Rep 6:98–105

49. Fletcher EL, Phipps JA, Ward MM, Vessey KA, Wilkinson-Berka JL (2010) The renin–angiotensin system in retinal health and disease: Its influence on neurons, glia and the vasculature. Prog Retin Eye Res 29:284–311
50. Leung PS, Sernia C (2003) The renin-angiotensin system and male reproduction: new functions for old hormones. J Mol Endocrinol 30:263–270

Figures

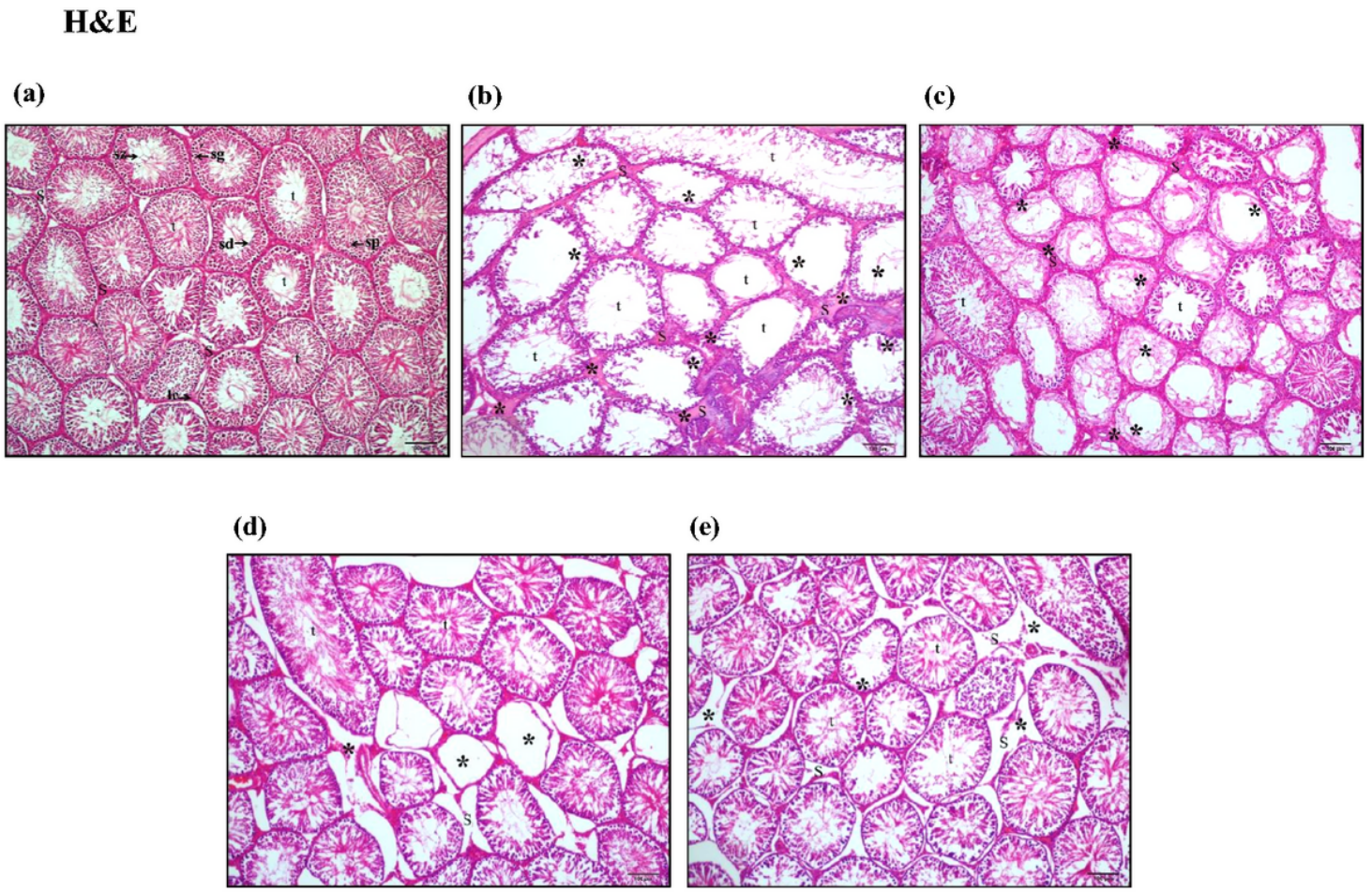

Figure 1

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)

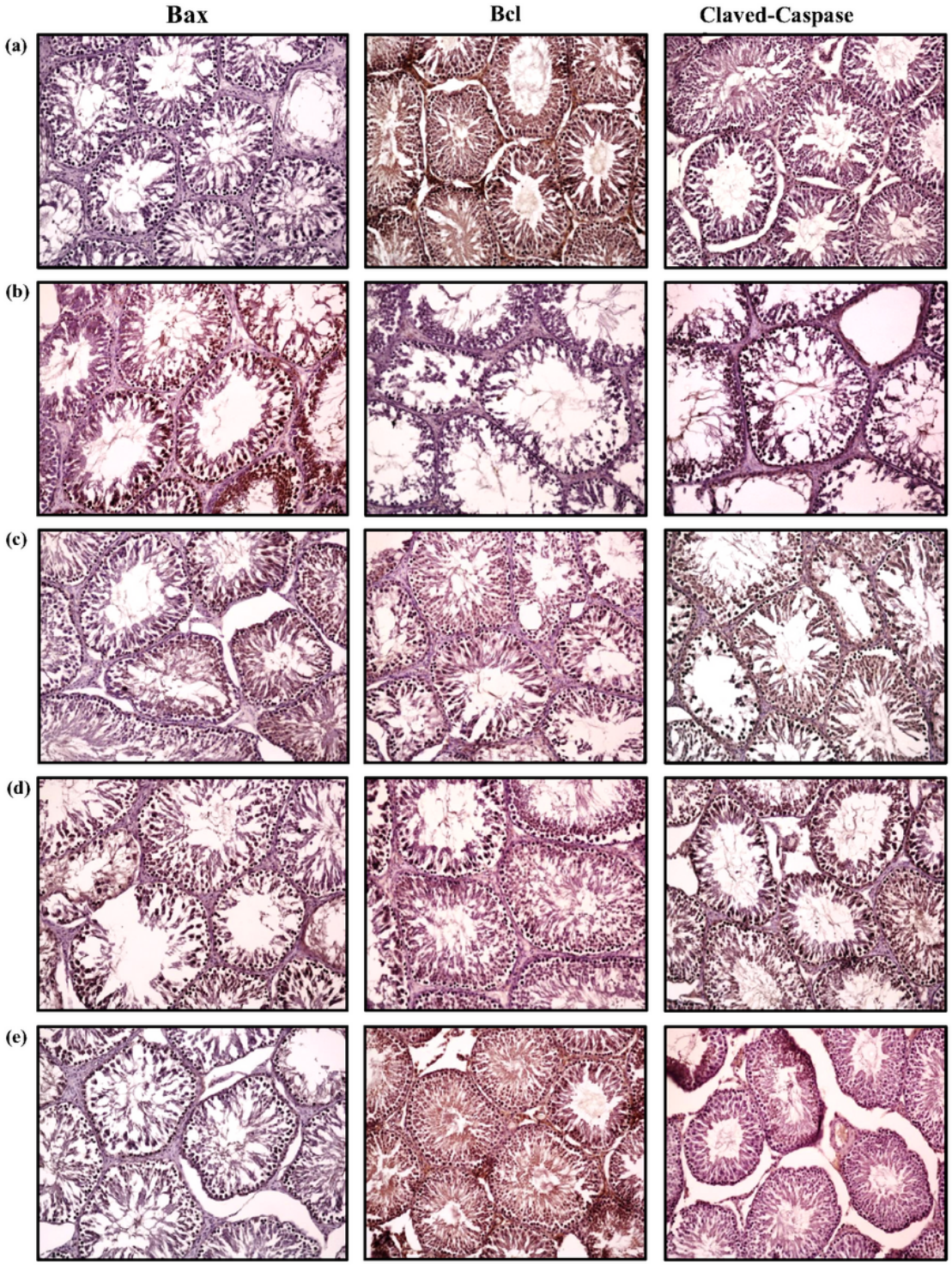

Figure 2

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).

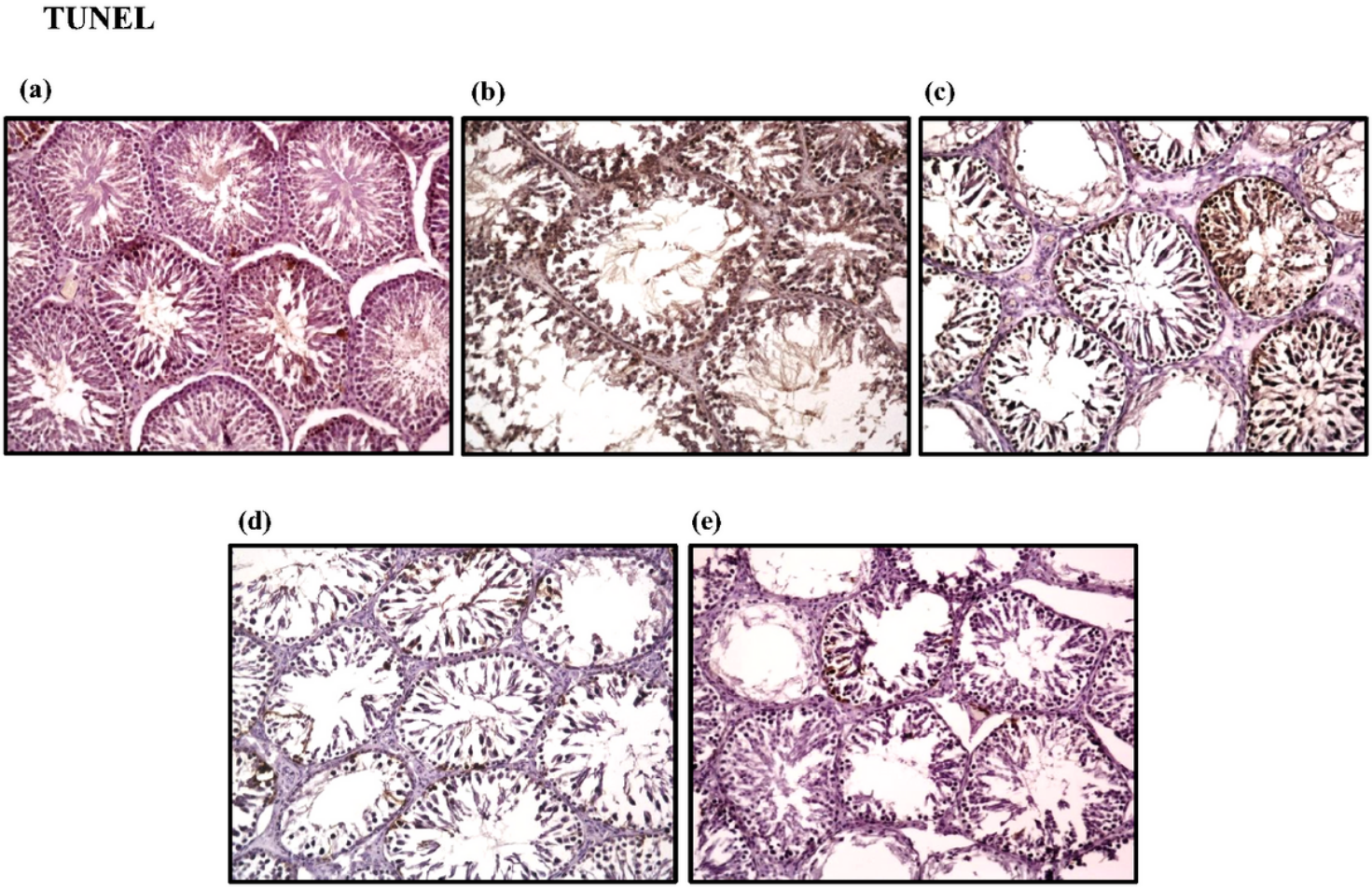

Figure 3

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 ($p<0.05$).

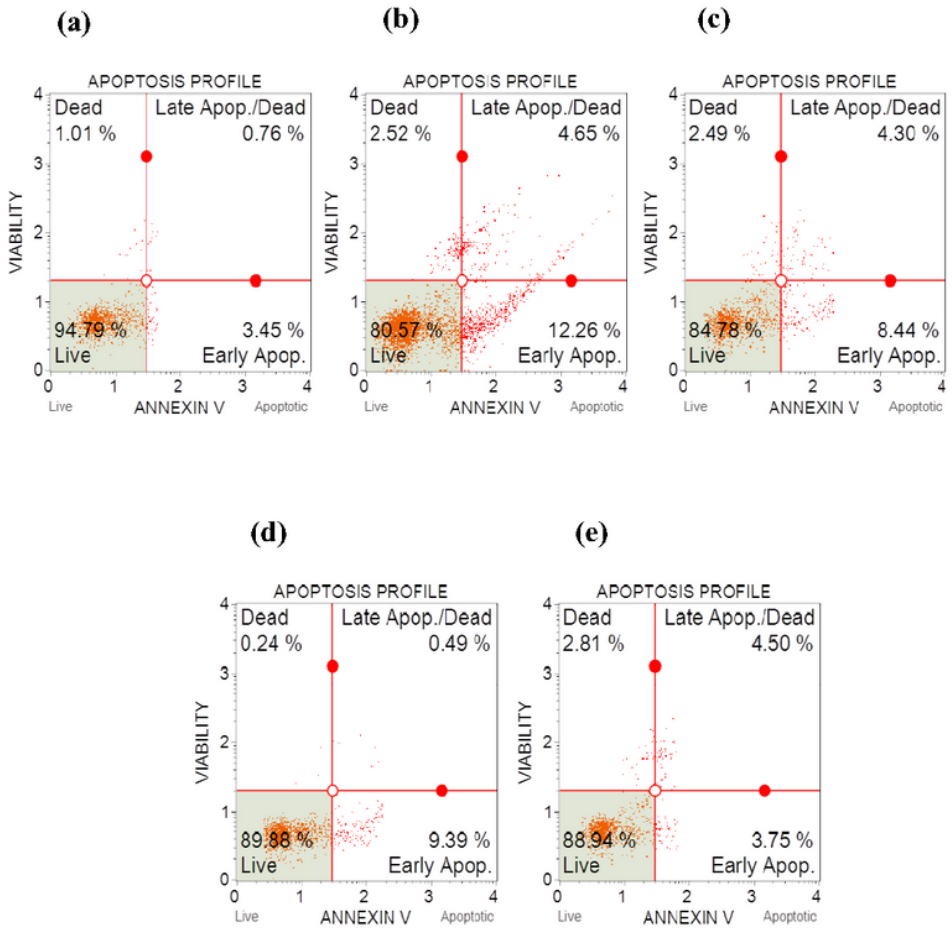

Figure 4

Annexin V analyses of epididymal sperm sample.