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Role of KU70, SIRT1, and SIRT6 Proteins in Diabetic Rat Testis Tissue

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Abstract: Diabetic male infertility/subfertility is an important complication of diabetes. The molecular mechanisms responsible for this complication have not been thoroughly investigated. We aimed to elucidate the role of KU70, SIRT1, and SIRT6 proteins in diabetic testis. Sprague–Dawley male rats were maintained under stable laboratory conditions. The rats were divided into a control group (n=8) and a diabetes group (n=8 treated with a single dose of 50 mg/kg streptozotocin). At the end of the 1-month experimental period, animals were sacrificed under anesthesia. Both testicles were removed, processed lightly, and studied through electron transmission microscopy and western blotting. Blood samples were collected for biochemical analysis. Histopathological analysis revealed that, in the diabetes group, the diaphragmatic tubule diameters and serum testosterone levels were decreased. KU70 immunoreactivity was statistically significantly increased, whereas SIRT1 and SIRT6 expression was significantly decreased compared with that observed in the control group. This is the first study to examine the expression of KU70, SIRT1, and SIRT6 in diabetic testicular tissue for the first time. According to the results, KU70, SIRT1, and SIRT6 may play an important role in cell apoptosis in diabetic testicular tissue. Importance of these proteins should be investigated further in additional quantitative studies.

Diyabetik Rat Testis Dokusunda KU70, SIRT1 ve SIRT6 Proteinlerinin Rolü

Anahtar Kelimeler
Bax/Bcl2-oranı,
TUNEL metodu,
Rat,
Apoptoz,
Histoloji

Öz: Diyabetik erkek infertilite/subfertilitesi, günümüzde diyabetin önemli komplikasyonları arasında yerini almıştır. Diyabetik erkek infertilitesinin altında yatan moleküler mekanizmalar henüz tam olarak aydınlatılamamıştır. Çalışmamızda; KU70, SIRT1 ve SIRT6 proteinlerinin bu mekanizmalar içerisindeki rolünün aydınlatılmasına katkı sağlanması amaçlanmıştır. Stabil laboratuvar koşulları altında tuttuğumuz Sprague Dawley erkek sıçanlardan, kontrol grubu (n=8) ve tek doz 50 mg/kg streptozotosin uygulanan diyabet grubu (n=8) olmak üzere iki grup oluşturulmuştur. Diyabet grubunda; ışık ve elektron mikroskobik incelemelerimizde diyabetik testis dokusunda şimdiye kadar literatürde yer alan tüm histopatolojik bulgular gözlenmiş, morfometrik olarak da seminifer tübül çaplarının azaldığı tespit edilmiştir. Çalışmamızda, KU70, SIRT1 ve SIRT6 ifadeleri incelendiğinde KU70 diyabet grubunda istatistiksel anlamlılıkla artarken, SIRT1 ve SIRT6 ifadelerinin diyabetik grupta, kontrol grubuna göre anlamlı şekilde düştüğü belirlenmiştir. Sonuç olarak, diyabetik erkek hastalarda karşılaşılan subfertilite/infertilite olgularında, KU70, SIRT1 ve SIRT6 proteinlerinin önemli bir rolü olabileceği ve bu proteinlerin, kantitatif başka çalışmalarla da desteklenerek öneminin vurgulanması gerektiği kanısındayız.

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease that occurs due to defects in insulin secretion [1]. Neuropathy, retinopathy, nephropathy, and microvascular and macrovascular pathologies are the main complications associated with diabetes [2, 3]. In addition, abnormal sperm production and sexual dysfunctions in men with diabetes are considered among the long-term complications of diabetes. Studies on diabetic rats revealed a decrease in testicular weight, testosterone levels, and sperm count and motility, along

with an increase in abnormal spermatogenesis [4-7]. The molecular mechanisms underlying testicular dysfunction in individuals with diabetes have not been fully elucidated. However, numerous proteins involved in apoptotic pathways are thought to play a role in this process.

Non-homologous end joining (NHEJ) is one of the two main mechanisms involved in the repair of DNA double-strand breaks. KU70 is a protein involved in this process, and it has been shown that double-strand breaks ends are stabilized by KU70 and KU80 [8]. KU70 and KU80 form the KU heterodimer and, together with the DNA-related protein kinase catalytic subunit, they play a role in the repair mechanism. Apart from DNA repair, KU proteins are involved in cell signal, proliferation, replication, transcription activation, and apoptosis. KU70 is involved in the regulation of apoptosis by suppressing Bax activity. It is localized both in the cytoplasm and nucleus [9].

Sirtuins (SIRT) constitute a family of nicotinamide adenine dinucleotide (NAD+)-dependent, class III deacetylase enzymes. There are seven sirtuins found in mammals (SIRT1–SIRT7). SIRT1 plays an important role in apoptosis by targeting proteins, such as p53, p73, E2 factor-1 (E2F1), and KU70. Studies have shown that it also affects insulin metabolism and, when overexpressed in pancreatic beta (β) cells, it increases insulin secretion and adenosine 3'-triphosphate (ATP) production. KU70 deacetylation by SIRT1 in NHEJ, suppresses Bax and participates in DNA repair. Moreover, KU70 deacetylation by SIRT1 in the cytoplasm leads to KU70/Bax binding and inhibits apoptosis [10-12].

SIRT6 is involved in the repair mechanism of DNA single-strand breaks and base excision repair (BER) pathway. It also plays a role in repairing double-strand breaks by stabilizing the DNA-related protein kinase in NHEJ. Studies investigating the effects of SIRT6 gene suppression demonstrated that this gene plays a role in the DNA repair mechanism in various diseases, such as spinal curvature, metabolic disorders, and aging before maturation [13-15]. SIRT6 is involved in KU70 deacetylation and KU70/Bax binding and suppresses apoptosis [16].

In this study, we investigated the possible roles of KU70, SIRT1, and SIRT6 proteins in diabetic testicular tissues, aiming to contribute to the development of new treatment protocols.

2. MATERIAL AND METHOD

2.1. Experimental Procedure

The present study was approved by the Trakya University Animal Experiments Local Ethics Committee (TUHADYEK-2017/17). Sixteen Sprague–Dawley male rats (age: 3 months; weight: 250–320 g) were obtained from Istanbul University, Institute of Experimental Medicine Research. The rats were maintained under stable laboratory conditions (22 ± 1.0°C, 12 h light/dark cycle) with ad libitum access to water and feed pellets containing crude 21% protein (Purina, Istanbul, Turkey).

The rats were divided into two groups: control and diabetes (n=8, respectively). We intraperitoneally injected 50 mg/kg body weight streptozotocin (STZ) (Sigma–Aldrich, Taufkirchen, Germany) dissolved in 0.1 M citrate buffer, pH 4.2, into rats of the latter group to induce diabetes. Blood glucose levels were measured using a glucometer (IME-DC, Hof, Germany) in blood samples obtained from the tail vein of the rats prior to initiating the experiment, 48 h after STZ administration, and once weekly for 1 month. Following STZ administration, the rat with blood glucose levels >250 mg/dl were considered “diabetic” [17]. At the end of the experimental period, all animals were sacrificed under anesthesia; both testes were removed, processed for light microscopy and transmission electron microscopy and western blotting. Blood samples were collected for biochemical analysis.

2.2. Enzyme-linked Immunosorbent Assay (ELISA)

Testosterone levels were measured in supernatants obtained from cardiac blood samples collected prior to sacrifice. The rat testosterone ELISA kit (Bioassay Technology Laboratory, Shanghai, China) was used in accordance with the instructions provided by the manufacturer.

2.3. Histopathological Evaluations

Light microscopic routine staining and immunohistochemical examinations were conducted. After fixing the testis tissues with Bouin’s fixative, they were washed, dehydrated, and embedded in paraffin. Tissue sections (thickness: 5 µm) were stained with hematoxylin-eosin (H+E) (Sigma-Aldrich, Missouri, USA) and subjected to histological and morphometric analyses to reveal the structural features of the testes. After sealing with Entellan™ (Merck Millipore, Darmstadt, Germany), the slides were examined under an Olympus BX-51 (Olympus Corporation, Tokyo, Japan) microscope and photographed at different magnifications. Spermatogenesis was evaluated using the Johnsen scoring system for five areas/sections for each animal in 10 different fields/seminiferous tubules which were randomly selected. The evaluation was performed using an Olympus BX51 microscope (magnification: ×400) [6].

2.4. Transmission Electron Microscopy

Testicular tissues were pre-fixed for 1.5 h with 2.5% glutaraldehyde and post-fixed for 1 h with 1% osmium tetroxide (OsO4). Subsequently, the tissues were passed through a graded alcohol series and propylene oxide, and blocked using araldite. Semi-thin sections taken by an ultramicrotome (RMC-MTX, Tucson, USA) were stained with toluidine blue to determine the cutting regions of the blocks. Thin sections were obtained from the determined regions and stained with uranyl acetate

and Reynold's lead citrate. Subsequently, the sections were examined using a Jeol 1010 (Jeol Ltd., Tokyo, Japan) transmission electron microscope.

2.5. Terminal Deoxynucleotidyl Transferase UTP Nick End Labeling (TUNEL) Assay

The TUNEL assay was used to determine the apoptosis in testicular tissue sections. Tissue sections (thickness: 5 µm) were incubated overnight at 37°C. The sections were passed through toluene and a graded alcohols series, washed with phosphate-buffered saline (pH 7.4; Invitrogen, Carlsbad, CA, USA), and treated with proteinase K subsequently, they were examined using the ApopTaq Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Darmstadt, Germany) according to the instructions provided by the manufacturer. Finally, the sections were passed through an ascending alcohol series and toluene, and mounted with Entellan™.

2.6. Immunohistochemistry and Western Blotting

Bax, Bcl-2, and Caspase-3 (CASP3) were evaluated immunohistochemically. KU70, SIRT1, and SIRT6 were evaluated through both immunohistochemistry and western blotting. Five µm sections from paraffin blocks were deparaffinized, rehydrated, exposed to antigen retrieval through boiling in citrate buffer (pH: 6.0), and H2O2 for inhibiton of endogenous peroxidase activity. Samples were incubated with blocking solution to prevent non-specific binding at ambient temperature then antibodies of Bax (1/100 dilution; Abcam, Cambridge, MA, USA), Bcl-2 (1/200 dilution; LifeSpan BioSciences, Seattle, WA, USA), CASP3 (1/500 dilution; Abcam, Cambridge, MA, USA), KU70 (1/100 dilution; FnTest, Hubei, China), SIRT1 (1/500 dilution; Novus Biologicals, Centennial, CO, USA), SIRT6 (1/1,000 dilution; Novus Biologicals, Briarwood Avenue, CO, USA) at 4°C for overnight. The following steps were performed with a ready to use detection kit and DAB chromogen (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The samples counterstained with hematoxylin, mounted with entellan and examined under a light microscope.

The frozen tissue samples were homogenized in RIPA buffer with protease and phosphatase inhibitors. The prepared lysates were centrifuged at 10,000 rpm at 4°C for 10 min and supernatants were used for Western blotting. Total protein concentration was measured with Nanodrop device (Optizen NanoQ; Mecasy, Daejeon, South Korea). Equal volume of proteins was loaded and run on Nupage Novex 4–12% Bis-tris (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) precast gels. The separated proteins were transferred on nitrocellulose membranes and the membranes were incubated with antibodies of KU70 (LS-C358891), SIRT1 (LS-B1564), SIRT6 (NB100-2522), and β-Actin (NB600-503) as internal control. The chemiluminescence developed bands were examined under ChemidocTM MP Imaging System Biorad (Model No: Universal Hood 3; Biorad, California, USA) and captured images were analyzed with Image J 1.48v software (Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).

2.7. Quantitative and Statistical Analysis

Measurement of seminiferous tubule diameters (MSTD) in H+E stained slides was carried out using an ocular micrometer at ×200 magnification. Round or nearly round tubules were randomly selected and assessed non-blinded and blinded investigators by selecting the cross section of 10 seminiferous tubules in each slide [18, 19]. In the evaluation of TUNEL slides, the 'apoptotic tubule index' was calculated by counting the seminiferous tubules that contained at least three apoptotic cells in an average of 100 seminiferous tubules. The 'apoptotic cell index' was evaluated by counting apoptotic cells in an average of 1,000 cells in the seminiferous tubules in ×200 magnification [20].

The immunoreactivity of Bax, Bcl-2, KU70, SIRT1, and SIRT6 was evaluated semiquantitatively using histological scoring methods. Changes between the groups were determined by calculating the Bax/Bcl-2-ratio. Immunoreactivity was assessed in five areas which were randomly selected in the tissue section for each animal through ×200 magnification. Immunoreactivites were scored as the number of immunepositive cells using H-score. [19, 21, 22]. In the evaluation of CASP3 slides, assessed non-blinded and blinded investigators, scoring was performed through ×200 magnification, according to the number of immunopositive cells in 1,000 cells in each slide [20].

Statistical analysis was conducted using the SPSS 20.0 software (IBM, New York, USA) Values are presented as the mean ± standard deviation. P-values <0.05 denoted statistically significant differences. Student's t-test was used to analyze glucose levels in blood. MSTD, Johnsen scoring, apoptotic tubule and apoptotic cell indices, and biochemical and immunohistochemical data were evaluated by applying the Mann–Whitney U test.

3. RESULTS

3.1. Blood Glucose and Testosterone Results

There was no difference in the blood glucose levels measured prior to the experiment between the control and diabetes groups (P=0.584). At the end of the experiment, the levels of the diabetes and control groups remained stable (P<0.001) (Table 1).

The ELISA method was used to measure the levels of testosterone in blood samples obtained by cardiac puncture following the experiment. Testosterone levels in the diabetes group were significantly decreased compared with measured for the control group (P<0.001) (Table 1).

Table 1. Blood glucose, testosterone, MSTD, and Johnsen scoring.

	Initial blood glucose levels (mg dl^{-1})	End of experiment blood glucose levels (mg dl^{-1})	Testosterone (mIU dl^{-1})	MSTD (μm)	Johnsen scoring
Control group	102.25 ± 5.17	106.50 ± 7.19	16.25 ± 1.67	345.63 ± 14.77	9.12 ± 0.83
	P=0.584				
Diabetes group	110.13 ± 4.64	463.75 ± 118.91	7.73 ± 1.14	275.38 ± 1.14	7.37 ± 0.51
		P<0.001*	P<0.001*	P<0.001*	P<0.001*

Note: Results are shown as the means ± standard deviation.
*Statistically significant compared with the control group.
MSTD, measurement of seminiferous tubule diameters.

3.2. Morphometry

Comparison of the MSTD measurements between the two groups showed that the values of the diabetes group were significantly decreased compared with those of the control group (P<0.001). In addition, Johnsen's score was significantly decreased in the diabetes group versus the control group (P=0.002) (Table 1).

3.3. Histopathology

3.3.1. Light and electron microscopy

The control group exhibited a normal seminiferous tubule epithelium structure. In seminiferous tubules, spermatogenic serial cells from the basement membrane to the lumen, and respectively as spermatogonium, spermatocyte-I, spermatocyte-II, early and late spermatid. Sperms are in the lumen, while Sertoli cells are located in the basement membrane (Figures 1A, 1B, 1E, 1F). Spermatids were observed close to the lumen with their darkly stained nuclei, and the sperms were located in the lumen with their long, spindle-shaped nuclei (Figures 1A, 1B, 1E, 1F). Leydig cells, which exhibit a polygonal-shaped structure in interstitial connective tissue, demonstrated oval-shaped nuclei and eosinophilic-stained cytoplasms (Figure 1B). In addition to the seminiferous tubules formed by Sertoli cells and few spermatogenic serial cells in the diabetes group, tubules containing spermatogonia, spermatocytes-I, spermatids, and sperms were also observed (Figure 1C). Due to the degeneration of Sertoli and spermatogenic cells, large vacuoles appeared in the germinal epithelium in varios locations (Figures 1C, 1D, 1G, 1H). The accumulation of electron-dense bodies in the cytoplasm of spermatogenic cells was remarkable. Particularly with spermatids located close to the lumen and containing residual bodies, there was electron-dense body accumulation and large vacuoles observed in the sperm (Figures 1G, 1H). Irregular nuclei, extensive vacuole formation, loss of crystals in mitochondria, and reduction in smooth endoplasmic reticulum (SER) were detected in Sertoli cells (Figures 1G, 1H). Vacuole formation, thickening of the outer membrane in mitochondria, SER reduction, and changes in the appearance of chromatin in the nuclei were observed in the cytoplasm of some spermatogonia and spermatids (Figures 1G, 1H). It is suggested that the corrugations observed in the seminiferous tubule basement membranes also occurred due to the degenerations noted in the tubular epithelial cells (Figures 1C, 1D, 1G).

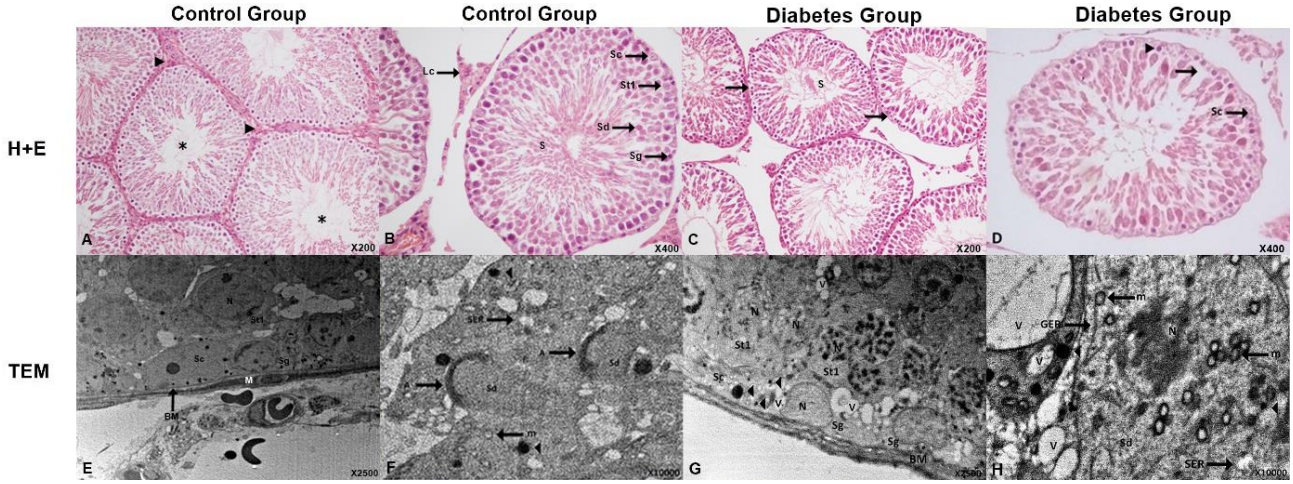

Figure 1. Sections with H+E stains (A–D), TEM micrographs (E–H). A. Interstitial connective tissue (►), seminiferous tubules (*). B. Spermatogonia (Sg), spermatocytes-I (St1), spermatids (Sd), sperms (S), Sertoli cells (Sc), and Leydig cells (Lc) C. Sperms (S), seminiferous tubules (→). D. Disorganization in cells (→) and picnotic cell nuclei (►). E. Basal membrane (BM), peritubular myoid cell (M), Sertoli cell (Sc), spermatogonium (Sg), spermatocyte-I (St1), and nucleus (N). F. Spermatids (Sd), acrosom (A), mitochondria (m), smooth endoplasmic reticulum (SER), and electron-dense bodies (►). G. Sertoli cell (Sc), spermatogonium (Sg), vacuole (V), spermatocyte-I (St1), and electron-dense bodies (►) with irregular basement membrane (BM). H. The cytoplasm contains degenerated nucleus (N), multiple vacuoles (V), mitochondria (m), granular endoplasmic reticulum (GER), smooth endoplasmic reticulum (SER), and spermatids (Sd) containing electron-dense bodies (►). Magnifications: x200 (A&C), x400 (B&D), x2500 (E&G), x10000 (F&H). Abbreviations: TEM, transmission electron microscopy.

3.3.2. TUNEL assay

In the tissue sections of the control group, TUNEL-positive cells were observed in a small number of cells in seminiferous tubules, especially in spermatogonia (Figure 2A). However, in the seminiferous tubules of the tissue sections of the diabetes group, a large number of TUNEL reactions was observed in other cells (besides spermatogonia) belonging to the spermatogenic series (Figure 2B). The apoptotic cell index in the diabetes group (34.75 ± 3.37) was significantly increased compared with that recorded in the control group (2.75 ± 1.48) (P<0.001) (Figure 2C). In addition, the apoptotic tubule index in the diabetes group (14.13 ± 2.32) was significantly increased compared with that noted in the control group (3.25 ± 1.83) (P<0.001) (Figure 2D).

3.3.3. Immunohistochemistry and western blotting

The examination of CASP3 immunoreactivity showed that the cellular localization was nuclear and cytoplasmic in both groups. In the control group, a small number of cells were stained, and mild staining was observed in spermatogenic serial cells and Sertoli cells (Figure 2E). However, in the diabetes group, a large number of immunopositive cells were detected in the seminiferous tubules (Figure 2F). CASP3 immunoreactivity was generally observed only in spermatogonia and Sertoli cells in both groups. CASP3 immunoreactivity in the diabetes group (16.75 ± 1.9) was significantly higher than that recorded in the control group (7.5 ± 1.3) (P<0.001) (Figure 2E).

Bax immunoreactivity was cytoplasmic for both groups. In the control group, weak staining was observed in the spermatogenic cell lines in seminiferous tubules. Of note, staining was not observed in Sertoli cells, while spermatogonia and other spermatogenic serial cells exhibited weak and moderate staining, respectively (Figure 2H). In the diabetes group, Sertoli cells were not stained, spermatogonia were weakly stained, and spermatocytes-I and other spermatogenic serial cells were moderately stained (Figure 2I). Bax immunoreactivity was significantly higher in the diabetes group (234.38 ± 18.48) versus the control group (121.13 ± 7.18) (P<0.001) (Figure 2J).

Considering that staining showed cytoplasmic Bcl-2 immunoreactivity in cells in seminiferous tubules, moderate and strong cytoplasmic staining was observed in testicular tissue sections of the control group.

Spermatogonia showed weak and spermatocyte-I cells moderate cytoplasmic immunoreactivity, while there was no staining observed in Sertoli cells (Figure 2K). In the diabetes group, there was no staining observed in spermatogonia and Sertoli cells, whereas other serial cells showed weak staining (Figure 2L). Bcl-2 immunoreactivity in the diabetes group (113.13 ± 5.93) was significantly decreased compared with that noted in the control group (204.38 ± 4.95) (P<0.001) (Figure 2M). The Bax/Bcl-2-ratio is a frequently used method to determine whether cells in tissues will undergo apoptosis. An increase in Bax and Bcl-2 expression indicates cell apoptosis and survival, respectively. In this study, the Bax/Bcl-2-ratio was significantly higher in the diabetes group (2.07 ± 0.204) versus the control group (0.59 ± 0.38) (P<0.001) (Figure 2N). This finding indicates that the number of cells which are prone to apoptosis were significantly increased in the diabetes group.

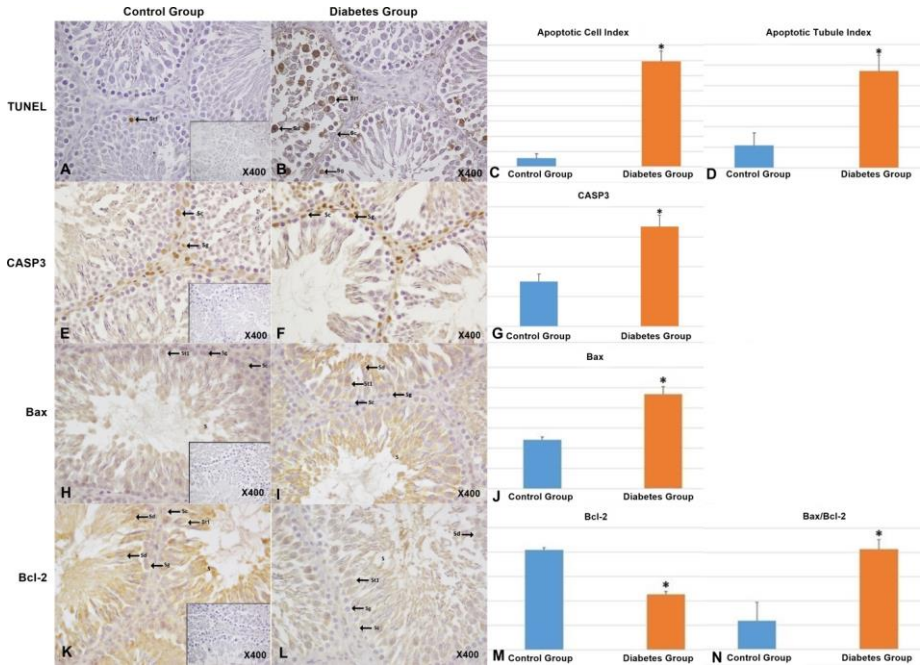

Figure 2. TUNEL sections (A, B), apoptotic cell and apoptotic tubule index (C, D). Immune-positive cells of all groups are monitored (→). Caspase 3 (CASP3) sections (E, F), CASP3 immunoreactivity values in G. Bax sections (H, I), Bax immunoreactivity values in J. Bcl-2 sections (K, L). Bcl-2 immunoreactivity values in (M) and Bax/Bcl-2-ratio in (N). Inner shape; negative control. *P<0.05 denotes statistically significant difference compared with the control group.
Abbreviations: S, sperms; Sc, Sertoli cells; Sd, spermatids; Sg, spermatogonium; St1, spermatocytes-I; TUNEL, terminal deoxynucleotidyl transferase UTP nick end labeling.

Of note, the cellular localization of KU70 in the sections was both nuclear and cytoplasmic. KU70 immunoreactivity examination revealed that, in the control group, spermatogonia and Sertoli cells showed very limited staining. Spermatocytes-I and other spermatogenic serial cells exhibited weak and moderate staining (Figure 3A). In the diabetes group, cells in the seminiferous tubules generally demonstrated moderate and strong staining. Notably, Sertoli cells showed weak staining, whereas spermatogonia, spermatocytes-Is and other spermatogenic serial cells exhibited moderate and strong staining (Figure 3B). KU70 immunoreactivity was significantly higher in the diabetes group (248.75 ± 5.82) versus the control group (106.88 ± 5.30) (P<0.001). Western blotting analysis determined that KU70 protein expression was significantly higher in the diabetes group (1.17 ± 0.16) versus the control group (0.64 ± 0.17) (P=0.004) (Figures 3C, 3D). The results of the present immunohistochemistry and western blotting analyses were consistent, clearly demonstrating that the expression of KU70 increases in diabetic testicular tissues.

The immunoreactivity examination revealed that the cellular localization of SIRT1 in tissue sections was both nuclear and cytoplasmic. In the control group, immunoreactivity was exhibited by a large number of cells. There was no staining observed in Sertoli cells, while spermatogonia, spermatocytes-I, and other spermatogenic serial cells demonstrated moderate and strong staining (Figure 3E). In the diabetes group, weakly immunoreactivity was observed in the seminiferous tubules. Staining was not detected in Sertoli cells, while spermatogonia were weakly stained, (Figure 3F). SIRT1 immunoreactivity in the diabetes group (98.75 ± 7.44) were found to be significantly lower than that in the control group (193.13 ± 4.58) (P<0.001). Moreover, western blotting analysis revealed that the expression of SIRT1 was significantly lower in the diabetes group (0.68 ± 0.19) versus the control group (1.33 ± 0.48) (P=0.004) (Figures 3G, 3H). The results of the immunohistochemistry and western blotting analyses were consistent, demonstrating that SIRT1 expression was significantly lower in diabetic testicular tissues versus control tissues.

The cellular localization of SIRT6 in the tissue sections was both nuclear and cytoplasmic. In the control group, there were numerous SIRT6-immunopositive cells observed. Sertoli cells exhibited weak and moderate staining, while spermatogonia, spermatocytes-I, and other spermatogenic cells showed moderate and strong staining (Figure 3I). In the diabetes group, the staining intensity was weak. Spermatogonia and Sertoli cells showed weak immunoreactivity, while other serial cells exhibited weak and moderate staining (Figure 3J). SIRT6 immunoreactivity was significantly lower in the diabetes group (64.63 ± 7.44) versus the control group (145.88 ± 8.56) (P<0.001). Western blotting analysis revealed that the expression of SIRT6 protein was significantly lower in the diabetes group (0.70 ± 0.22) versus the control group (1.88 ± 0.40) (P <0.001) (Figures 3K, 3L). The results of both immunohistochemistry and western blotting analyses demonstrated that SIRT6 expression was significantly lower in diabetic testicular tissues versus control tissues.

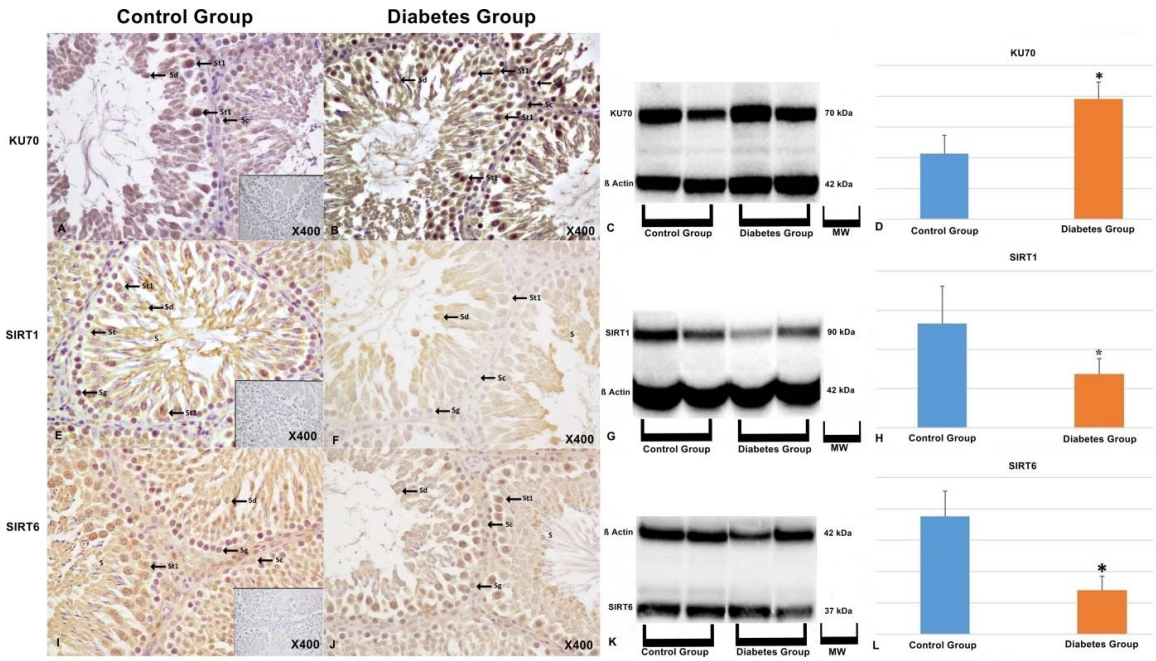

Figure 3. KU70 sections, western blotting bands, and graph of analysis (A–D). SIRT1 sections, western blotting bands, and graph of analysis (E–H). SIRT6 sections, western blotting bands, and graph of analysis (I–L). Inner shape; negative control. *P<0.05 denotes statistically significant difference compared with the control group.
Abbreviations: MW, molecular weight; S, sperms; Sc, Sertoli cells; Sd, spermatids; SIRT1, sirtuin 1; Sg, spermatogonium; St1, spermatocytes-I.

4. DISCUSSION AND CONCLUSION

Studies have shown that diabetes affects the hypothalamus-pituitary-testicular axis and decreases testosterone synthesis and secretion. In addition, conditions such as increased prostate glandular volume, decreased spermatogenesis and sperm quality, erectile dysfunction, retrograde ejaculation, and decreased libido

have also been reported in diabetes studies of diabetes [5, 7, 23]. Numerous studies have also recorded serious changes in the diameters of diaphragmatic tubules as a result of the decreased number of spermatogenic cells in the seminiferous tubules and their degeneration, accompanied by a decrease in the serum testosterone levels of diabetic rats [4, 24]. Cameron et al. [25] showed that tubule alimentation is impaired by thickening of capillary and seminiferous tubule basement membranes due to diabetes. Öztürk et al. [26] argued that diabetes led to Leydig cell dysfunction and a consequent decrease in testosterone. Owing to the thickening of the tubule basement membranes, the difficulty of diffusion of testosterone into the tubule may prevent the continuity of spermatogenesis.

Through electron microscopic studies, Trindade et al. [7] reported an irregular basement membrane structure, disruption of the seminiferous tubules, disruption of the structure of Sertoli cells and spermatogonia, and vacuole formation in the cytoplasm of Sertoli cells. In addition to degeneration of spermatogenic cells, Koroglu et al. [27] observed deformation of the acrosomal structures and tails of sperms. Other studies showed thickening and irregularity in seminiferous tubular basement membranes, vacuolization in Sertoli cells, and disruption of the structure of the SER [28-32]. In the nuclei of spermatogenic serial cells, condensation, and disruptions of mitochondria and the SER were also noted. Along with a decrease in the number of mitochondria, changes in the shape of the organelle and irregularities in the cristae were observed [28-32]. Trindade et al. [7] and Kianifard et al. [28] suggested that vacuolization and reduction in SER negatively affect the function and spermatogenesis process in Sertoli cells. Mitochondria degeneration and decrease in number due to diabetes has been shown. It has been stated that inadequate ATP production and increased reactive oxygen species production in spermatogenic cells may cause infertility [7, 28]. When the underlying causes of infertility were examined, it was observed that degeneration in mitochondria plays an important role [7]. Owing to mitochondria degeneration, the accumulation of electron-dense bodies and lipofuscin in the cytoplasm of spermatogenic serial cells leads to an increase in reactive oxygen species, thereby causing a degenerative effect and lipid peroxidation in cells [7, 28, 29].

In this study, the findings of apoptotic cell and apoptotic tubule indices were consistent with those of previous studies [33, 34]. Ghosh et al. [24] and Ding et al. [30] reported that the expression of Bax was significantly increased in diabetic testicular tissues, and Bax played a role in the apoptotic process. Liu et al. [35] suggested that the expression of Bax was increased in diabetic testicular tissues, and that this change was important in the damage mechanism involved in diabetic testicles. Other studies confirmed that Bax expression in diabetic testicular tissues was significantly increased and played a role in the apoptotic pathway [3, 36, 37]. Collectively, these studies showed that Bax protein plays an important role in the apoptotic process, especially in diabetes. A previous study revealed a significant increase and decrease in the expression of the Bax and Bcl-2 genes, respectively, in testicular tissues of diabetic rats [19]. It was also shown that the Bax/Bcl-2-ratio was significantly increased [19]. Another study reported that Bcl-2 expression was significantly decreased in diabetic testicular tissues versus control tissues [38]. In a study investigating the expression of proteins belonging to the Bcl-2 family (Bcl-2, Bcl-XL, Bax, and Bad) in testicular tissues of diabetic rats was, the expression of anti-apoptotic proteins was decreased in the diabetes group [37]. Khamis et al. [39] showed that CASP3 mRNA expression was significantly increased in diabetic testicular tissue. In another study investigator showed that CASP3 expression was significantly increased in the diabetic testicular tissues; increased CASP3 expression induced caspase-activated DNase activation, leading to DNA fragmentation [36].

This was the first study to investigate the expression of KU70 in diabetic testicular tissues through immunohistochemistry and western blotting. Ahmed et al. [40] showed that expression and localization of KU70 in the nucleus of Sertoli cells, type A spermatogonia, late spermatocytes I, secondary spermatocytes and round spermatids as well as in the cytoplasm of metaphases I and II cells in mice testis by immunofluorescence, and this findings consistent with our study. They observed that KU70 impairment leads to reduced testis size, limited spermatogenesis and DNA repair, limited sperm production and increased metaphase I apoptosis. In a diabetic nephropathy study conducted by Tuncdemir et al. [41] the investigators stated that the expression of KU70 was significantly increased in the diabetes group versus the control group. In another study examining KU70, the anesthetic substance bupivacaine was administered to mouse dorsal root ganglion neurons cultured in a hyperglycemic environment. The results suggested that hyperglycemia leads to inactivation of the DNA-related protein kinase catalytic subunit and causes KU70 inhibition, thereby resulting in DNA damage and apoptosis [42]. Another study examined the expression of KU70 in cultured cardiomyocyte cells in a hyperglycemic environment. The results demonstrated that hyperglycemia increased KU70 acetylation and, accordingly, increased Bax activity. They found that KU70 acetylation was decreased, KU70/Bax binding was increased, and apoptosis was inhibited in the treated groups [43]. It is thought that KU70, which shows increased expression in diabetes, may play an important role in the apoptotic pathway. Other studies reported that KU70 binds to Bax in the cytoplasm, thereby isolating Bax from mitochondria, preventing the formation of pore, and blocking apoptosis [44, 45].

A mouse study showed that spermatocytes expressed high levels of SIRT1 during the spermatogenesis process. Interestingly, sperm anomalies and infertility were observed after silencing the SIRT1 gene [46]. It has been stated that SIRT1 is expressed in numerous tissues during embryonic development. In a study, SIRT1 expression was demonstrated during the fetal period in the neuroepithelium, dorsal root ganglion, trigeminal ganglion, eye, heart, kidney, testis, liver and lung tissues.

These findings suggested that SIRT1 expression is important in fetal development. Strong SIRT1 immunoreactivity has been demonstrated, especially in the testes, during embryonic development [47]. In a study investigating SIRT1 immunoreactivity in testicular tissues of rats with type 1 diabetes, SIRT1 expression was decreased in the diabetes group compared with the control group. SIRT1 increases insulin sensitivity and decreases glucotoxicity in testicles. Poly (ADP-ribose) polymerase-1 (PARP)/SIRT1 interaction is thought to be involved in the testicular apoptotic pathway [48, 49]. Several studies have shown that SIRT1 activation is reduced in diabetic testes, similar to our findings [50-52]. Wang et al. [53] has reported that, in Type 2 diabetes rats testes, SIRT1 expression decreased compared to control groups and SIRT1 expression increased in melatonin treated groups. In a recent study, researchers were fed mice with high fat diet and showed that, long-term calorie restriction increase obese male fertility, likely by relieves oxidative stress via activation of SIRT1 signaling [54]. Consistent with our findings, another two studies revealed that SIRT1 expression was decreased in diabetic testes. They have been suggested that, in diabetic testes, miR-34a expression increases, whereas SIRT1 expression decreases; therefore miR-34a may suppress SIRT1 and induce apoptosis [55, 56]. Zhao et al. [57] examined the effects of zinc deficiency on testes and measured SIRT1 protein expression in mouse testicular tissues with type 1 diabetes. The results indicated that SIRT1 expression was significantly decreased in the diabetes groups. The investigators suggested that zinc regulates SIRT1 activity in diabetic testicular tissues.

Currently, there is a limited number of research studies examining the expression of SIRT6. SIRT6 and SIRT1 are involved in the maturation processes of spermatids [58]. A study of mouse testicles showed that SIRT6 was expressed in both the nuclei and cytoplasms of spermatogonia, spermatocytes, and early spermatids [59]. An obesity study based on a high-fat diet did not find a significant difference in SIRT6 expression between the control and diet groups, showing nuclear immunoreactivity in early and late spermatids. A significant decrease in SIRT6 expression led to a decrease in sperm protamination. Of note, in testicular germ cells, a decrease in SIRT6 expression was associated with an increase in H3K9 acetylation and DNA damage [60]. Fan et al. [61] stated that, in diabetic nephropathy, apoptosis increases due to a decrease in SIRT6 expression in podocytes; particularly in adenosine 5′ monophosphate-activated protein kinase (AMPK) dephosphorylation and mitochondrial dysfunction. Liu et al. [62] suggested that SIRT6 is the regulator of the Wnt signal and exerts anti-inflammatory and antiapoptotic effects by inhibiting H3K9 deacetylation and Notch1/Notch4 transcription factors. However, Tao et al. [16] revealed for the first time that SIRT6, similarly to SIRT1, deacetylated KU70 and inhibited KU70/Bax-related apoptosis in a hepatocellular carcinoma cell culture model. Zhang et al. [63] examined the expression of SIRT6 and KU70 in human dental pulp cells undergoing apoptosis induced by bacterial lipopolysaccharides. They reported that SIRT6 increased KU70/Bax binding and inhibited apoptosis by deacetylation of KU70. A recent study has reported that SIRT6 expression on mice testes. It has shown that SIRT6 expression may be associated with fertility [64].

In conclusion, the results of the present study may contribute to the knowledge regarding the possible roles of KU70, SIRT1, and SIRT6 proteins in diabetic testicular tissues, apoptosis, and infertility, as well as provide directions for the development of new treatment protocols.

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