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Histological Investigation of Experimentally Induced Diabetes Effects on the Distribution of Transforming Growth Factor (TGFβ), Nuclear Factor Kappa B (Nf-κB), Heat Schock 90β (Hsp90β) and E-cadherin Proteins in Testicular Tissue

Investigación Histológica de los Efectos de la Diabetes Inducida Experimentalmente en la Distribución del Factor de Crecimiento Transformante (TGFβ), Nuclear Factor Kappa B (Nf-κB), y Proteinas Heat Schock 90β (Hsp90β) y E-Cadherina en Tejido Testicular

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SUMMARY: Diabetes is a metabolic disorder characterized by high blood sugar levels and it causes complications in many systems, including the reproductive system. As a result of diabetic conditions, one of the mechanisms that can cause repression of reproductive activity is testicular oxidant stress. The identification of diabetes on the cell signaling molecules axis is still under discussion. The aim of this study was to determine the effect of Transforming Growth Factor (TGFβ), Nuclear Factor kappa B (NF-κB), Heat-schock 90β (HSP90β) signal pathways and E-cadherin cell adhesion molecule on infertility in diabetic rat testicular tissue. In our study, includes histological, molecular and biochemical analysis of testicular tissue removed at the end of the 2 weeks experiment period. A total of 14 adult male rats were divided as control and diabetes. No intervention was given to 7 male rats in the control group. For the diabetic group, 7 male rats were injected by intraperitoneal with a single dose of 55 mg/kg streptozotocin (STZ). TGFβ, NF-κB, HSP90β and E-cadherin proteins were immunohistochemically studied to investigate possible tissue damage, inflammatory process, cell stabilization and integrity due to diabetes. In order to determine oxidant stress, lipid peroxidation product malondialdehyde (MDA), glutathione (GSH) and glutathione peroxidase (GPx) analyzes were performed. Fibrosis, inflammatory changes and loss of spermatogenetic series are prominent findings in the diabetic group. On analysis of all the samples with immunostaining, in the diabetic group, TGFβ and NF-κB immunoexpression significantly increased, while Hsp90β and E-cadherin immunoexpression significantly decreased compared with control groups. Experimental diabetes was found to cause fibrosis, inflammation, disrupting cell adhesion and stabilization in testicular tissue. These results suggest that cellular therapy studies are needed for possible damage.

KEY WORDS: Diabetes; Testis; TGFβ; Hsp90β; NF-κB; E-cadherin.

INTRODUCTION

Diabetes is a metabolic disease characterized by insufficient insulin release from the pancreas and leading cause disorders in carbohydrate, fat and protein metabolism. The prevalence of diabetes disease is increasing day by day worldwide (Ozougwu *et al*., 2013). Diabetes causes complications in many organs and triggers health problems such as oxidative stress, inflammation and apoptosis that cause infertility in the male reproductive system as one of the long term consequences (Samie *et al*., 2018).Recent studies have focused on the molecules and mechanisms

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responsible for diabetes-induced changes in the male reproductive system (Zhou & Griswold; Maresch *et al*., 2018). Spermatogenesis is regulated by three different events as cell differentiation, proliferation and loss of cells. In this arrangement, the specific expression of many genes/ proteins is effective, and many are still unknown. The disruption of one of these molecules can affecting multiple mechanisms and causing cell damage (Zhou & Griswold). The aim of this study was to determine the effect of Transforming Growth Factor (TGFβ), Nuclear Factor κappa B (NF-κB), Heat-schock 90β (HSP90β) signal pathways and E-cadherin cell adhesion molecule on infertility in diabetic rat testicular tissue.

TGF-β family is the main regulator of extracellular matrix protein synthesis and its transcription is provided by Smads in the cytoplasm. It acts as a cellular stimulant by suppressing inhibitors of certain collagen, protease and integrin regulators to increase protein synthesis in the matrix (Khan & Marshall, 2016). Heat-shock protein (HSP) family are molecular chaperones that regulate cell hemoastasis and survival in acute or chronic stress situation in almost all living creature. Thus, it prevents the cell from death by destroying misfolded or damaged proteins by the ubiquitin-proteosome system, endoplasmic reticulum-related degradation or different autophagic pathways (Penke *et al*., 2018). NF-κB transcription factor regulates by activating target genes that play a critical role in apoptosis regulation, immune and stress response and cell differentiation and proliferation, as a result of environmental changes such as cytokines, radiation and some chemotherapeutic agents (Oeckinghaus & Ghosh, 2009).

E-cadherins are calcium-dependent transmembrane proteins that bind to intracellular actin filaments via binding proteins, such as α-, β-, and γ-catenins, which play a role in cell adhesion and differentiation (Aktug *et al*., 2013). It increases the stability of cell-cell and extracellular-matrix structures together with other cell adhesion molecules for proper physiological function of tissues.

Diabetes-induced hyperglycemia increases mitochondrial glucose oxidation, causing more hydrogen peroxide (H_2O_2) and superoxide anion radical release of reactive oxygen species (ROT) into the cell plasma, and ultimately oxidative stress (OS).OS develops in the reproductive organs in diabetes, is one of the mechanisms underlying the negative effects of the disease on the reproductive function (Jangir & Jain, 2014; Maresch *et al*.). OS occurs when the balance between ROT and protective antioxidant systems increases in favor of oxidation. In addition to the presence of ROT producing reproductive system, the plasma membranes of the cells in the testicular tissue are rich in polyacited fatty acids and the low antioxidant capacity makes testicular germ cells more sensitive to OS than somatic cells (Majd *et al*., 2019). Malondialdehyde (MDA) are lipid peroxidation products formed by the peroxidation of polyac saturated fatty acids in cell membranes with ROT. Since the increase of ROT increases the formation of MDA, and MDA is one of the most frequently used biomarkers of OS. Glutathione (GSH) and glutathione peroxidase (GPx) are protective antioxidants that play a role in removing ROT and lipid peroxidation products such as MDA. In the light of this information, we examined the level of OS and its effects on cell damage by measuring MDA, GSH and GPx at tissue level.

MATERIAL AND METHOD

Induction of Type I Experimental Diabetes Model and Measurement of Glucose Levels. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. A total of 14 male Wistar albino rats aged 3- 4 months, weighing 250–350 g were used in the study. Experimental study was carried out with the approval of Ege University, Local Ethics Committee for Animal Experiments (Number 2014-008). The rats were fed *ad libitum* and housed in steel cages at 24°C ± room temperature with 12 h light/dark cycles. Study rats were equally distributed into control and diabetes group. Control group (n = 7): No intervention was given to the control group. Diabetes group (n = 7): Streptozotocin (STZ) (Santa Cruz, sc-200719) prepared with 0.1mol/L sodium citrate buffer (pH:4.7)was injected intraperitoneally as a single dose of 55 mg / kg. Experimental rats were not fed 12 hours prior to the experiment. After STZ injection, 30 % dextrose was added to drinking water to prevent hypoglycaemia in rats. Before the experiment, the blood glucose levels of the rats were determined by measuring digital handheld glucometer (Accu Chek Glucometer-Roche) from the tail veins. By making clinical and biochemical observations, 48 hours after STZ injection, glucose level was measured from the tail veins and blood sugar values of 250 mg / dl and above rats were considered as diabetes. At the end of two weeks, 10mg/kg xlasine (Alfazyne 2 %, Ege Vet, Izmir) and 60 mg/kg ketamine hydrochloride (Alfamine 10 %, Ege Vet, Izmir) were given intramuscularly for anesthesia. Experimental rats were sacrificed and testicular tissue was removed. For histopathological and immunohistochemical analysis, tissues were taken into 10 % neutral buffered formol (Merck, USA) for fixation. For biochemical analysis, tissues were immediately frozen on dry ice and stored at -80 °C until assay.

Histopathological Examination of Testes. For histological and immunohistochemical studies, 5µm sections were taken from the fixed tissues using the microtome (LeicaRM2145) device and were stained with hematoxylin and eosin (H&E). All stained sections were photographed on C-5050 digital camera mounted on Olympus BX51 microscope.

Immunoexpressions of TGFβ, Nf-κβ, HSP90β and E-cadherin. The slide were heated in a incubator for overnight at 58°C and dewaxed in xylene for 1h, rehydrated through decreasing alcohol series. Afterwards, sections were treated with citrate buffer (0.01 M, pH 6.0) at 90 °C for 30 min, and then incubated at room temperature for 15 min to let cooled. Gently washed in distilled H_2O for 5 min and Phosphate-buffered saline (PBS, pH 7.4) containing 0.1 % triton for 5 min H_2O_2 (30 %) was applied to eliminate endogenous activity for 10 min. Washed three times with PBS again and blocked any non-specific binding with 5 % serum blocking solution (ScyTec Inc.USA) for 1h at room temperature. The slides were incubated with primary antibodies rabbit Anti-HSP90 beta (Bioss-USA, bs-0135R; dilution 1/100), rabbit anti-TGF beta1 (Bioss-USA, bs-0086R; dilution 1/100), rabbit anti-E cadherin (Bioss-USA, bs-10009R;dilution 1/100) and rabbit anti- NF-κB p65 (Bioss-USA, bs-0465R; dilution 1/100) at 4°C in a humidified chamber for overnight followed by washing with PBS. The biotinylated secondary antibody (Anti Rabbit-Mouse Boster-USA, SA1020) solution was applied for 30 min and washed three times with PBS before incubating with streptavidin peroxidase conjugate for 30 min. Washed three times with PBS. DAB (3,3-diaminobenzidine) solution was used to visualize the final product. Washed with distilled H_2O again. As a last step, counterstaining was performed with mayer's hematoxylin for 2 min and washed three times with tap water. Added enthallan to slides and toped with coverslips.

Determination of tissue lipid peroxidation(MDA), glutathione(GSH) and glutathione peroxidase(GPX). Oxidative stress in testicular tissues was detected by measuring lipid peroxidation (BioVision,Catalog#K739-100), glutathione (BioVision, Catalog#K261-100), glutathione peroxidase (BioVision, Catalog#K762-100). All applications were made according to the manufacturer's instructions.

Statistical analysis. Statistical Package for the Social Sciences (SPSS) 15.0 Windows software (IBM corp. Armonk, NY) program was used for statistical analysis. In order to compare blood-glucose levels, animal weights and biochemical parameters, intra-group evaluation was performed with the Wilcoxon Test. Mann Whitney U test was used to determine the significance inter-group evaluation. Values were expressed as mean ± standard error of mean (SEM). A value of $p < 0.05$ was accepted statistically significant.

RESULTS

Blood glucose levels. There were a significant difference between the before and after glucose levels in the diabetes and control groups with [1]Wilcoxon test. As a result of the [2]Mann Whitney U test, the before and after glucose levels were statistically significant between the groups. Higher blood sugar levels were determined in the diabetic group compared to the control group. The increase in glucose levels was observed 48 hours after the STZ injection. While glucose levels decreased in the diabetes group during the experiment period but final levels could not reach the control group (Table I).

Rat weight. As a result of the intra-group evaluation with the [1]Wilcoxon test, a significant difference was found between the weights of rats before and after the experiment. At the end of the experiment, in the control group weights of rats increased significantly, while in the diabetes group significantly decreased.When evaluated inter-group with the [2]Mann Whitney U Test, there was no statistical significance between the weights before the experiment, it was found significant after the experiment (Table II)

Biochemical oxidative stress markers. As a result of Mann Whitney U test, when glutathione (GSH), glutathione peroxidase (GPX) and lipid peroxidation (MDA) data were evaluated, no statistical significance was found between the groups (Table III).

Table I. Blood glucose levels of rats before and after the experiment (mg/dL).

Groups (n=7)	Before Experiment	After Experiment	p value[1]
Control	143,33±12,04	189,00±22,12	0,028*
Diabetes	446,71±103,90	286,00±32,93	0,028*
p value[2]	0,001*	0,002*	

Values are present as Mean ± SD.* P <0.05 values were accepted statistically significant.

Table II. Weights of rats before and after the experiment.

Groups (n=7)º	Before Experiment	After Experiment	p value[1]
Control	223,17±22,27	248,67±32,62	0,028*
Diabetes	211,29±15,92	179,83±12,29	0,046*
p value[2]	0,366	0,002*	

Values are present as Mean ± SD. * P <0.05 values were accepted statistically significant

Table III. GSH, GPX and MDA values of rat testes tissue.

Groups (n=7)	Glutathione (U/mg)	Glutathione peroxidase (mU/mL)	Lipid peroxidation (nmol/mg)
Control	113,55±21,02	5,09±1,61	0,49±0,32
Diabetes	129,50±35,76	5,25±0,89	0,66±0,35
p value	0,352	0,905	0,476

Values are present as Mean ± SD. * P <0.05 values were accepted statistically significant

Histological findings. As a result of the histological examination, there was no disorganization in the spermatogenetic series cells in the control group. While there were no ınflammation and fibrosis found in the interstitial area, the interstitial cells were seen morphologically normal. It was observed that the seminiferous tubules were properly structured and the germ cells were arranged according to the developmental series, while the sustentacular cells (Sertoli cells) and spermatogenic series cells in tubules of different stages maintained their normal histological appearance. Vascular structure endothelial cells and myoepithelial cells were observed that had a normal histological structure. In the diabetes group, degeneration of some seminiferous tubule structures, fibrotic and scarring changes, interstitial space and loss in the spermatogenetic series cell were observed. Degenerated tubule structures due to dilatation were observed in seminiferous tubules. Spermatogenetic series cells in the basal and adluminal compartments of the seminiferous tubule were disrupted. Atypical cells in the tubule lumen were seen in many areas. In addition, dense stromal fibrosis was observed in the interstitial area. In some areas, was detected edema characterized by expansion of the interstitial area, especially starting from the hilum. Interstitial cells (Leydig cells) were not clearly seen in the interstitial area (Fig. 1).

Immunohistochemical findings. For immunohistochemical evaluation, 100 cells were counted in 40x objective under light microscope, and were evaluated as negative (0), weak (+), moderate (++) and strong (+++) according to the intensity of staining spermotogenetic series cells. TGFβ expression was weak (+) staining in spermatogonium, spermatocyte, spermatid and sustentacular cells in the control group, showed moderate (++) staining in the diabetic group. Hsp90β expression was showed moderate (++) staining in the control group, and negative (0) staining in spermatids, weak (+) staining in spermatogonium, spermatocyte and sustentacular cells in the diabetic group.NF-κB expression was showed weak (+) staining in the control group and strong (+++) staining in the diabetic group. E-cadherin was showed strong (+++) staining in spermatagonium, moderate (++) staining spermatocyte, spermatid and sustentacular cells in the control group, weak (+) staining in spermatogonium, spermatocyte, spermatid and sustentacular cells (Fig. 2).

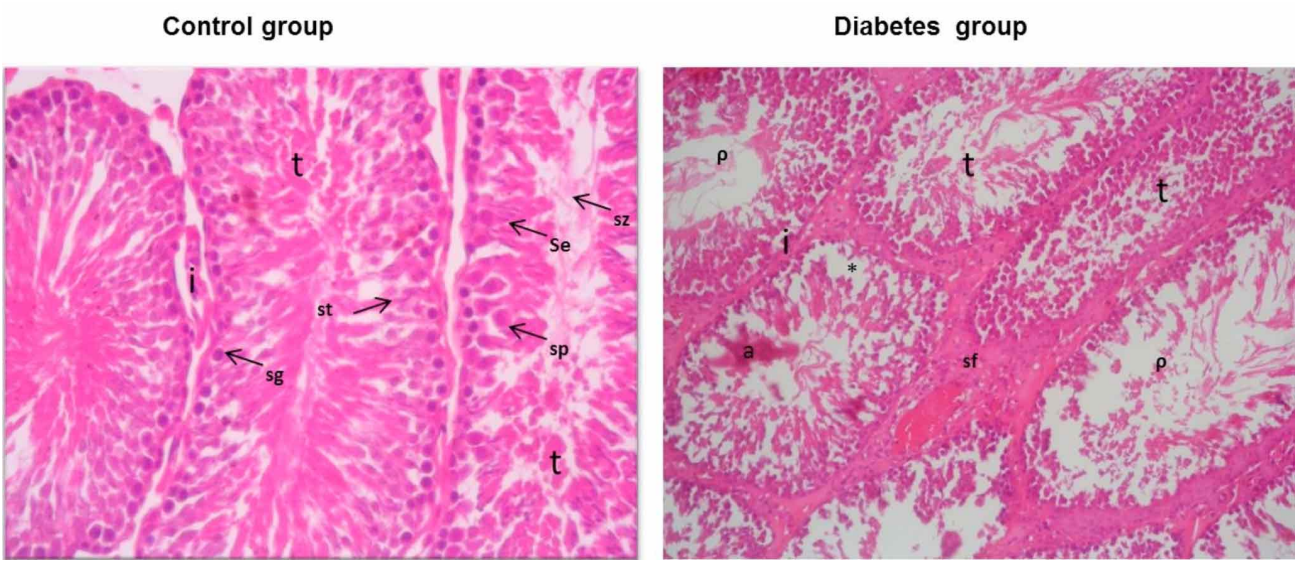

Fig.1. Hematoxylin and Eosin (H&E) staining in experimental groups. In the control group, normal seminiferous tubule structure (t), interstitial space (i) and spermatogonium (sg), spermatocyte (sp), spermatid (st), spermatazoa (sz), Sustentacular cells (Se) were shown (x40 magnification). Tubule degeneration (r) in the diabetes group, stromal fibrosis (sf), edema (*) and atypical (a) cells in the tubule lumen were observed in the interstitial area (x20 magnification)

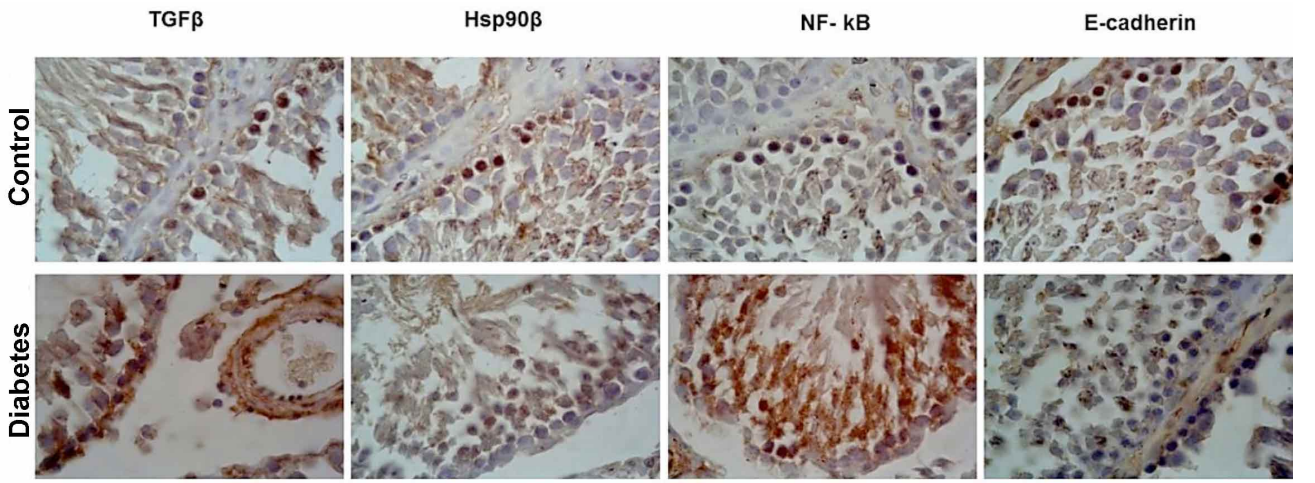

Fig. 2. Immunostaining of TGFβ, Hsp90β, NF- κB and E-cadherin proteins (x100 magnification).

DISCUSSION

The problem of infertility in diabetic men is characterized by many dysfunctions such as degeneration in the testicles, apoptotic defect in the cells of the spermatogenetic series, decreased testosterone synthesis, impaired semen quality, abnormal sperm morphology, and decreased motility (Jangir & Jain). In our study, histological examination of the testis in the STZ-induced diabetic rat model was detected loss of spermatogenic series cells and somatic sustentacular cells, disorganization characterized by intercellular degradation and atypical cells in the lumen in many tubule.In most of the short and long term diabetes studies have shown, decreased numerically in spermatogenic series cells and sustentacular cells tubule degenerations and interstitial edema in the diabetes group (Cameron *et al*., 1985; Jangir & Jain). From the data of the current study, similar results were obtained with the findings of the literature. Cameron *et al*. observed that testicular degeneration in diabetes was due to decreased testosterone and the finding in this study on humans, interstitial cell degeneration occurs due to vascular thickening in the interstitial area (Cameron *et al*.).

In this study, MDA, GSH and GPX total antioxidant capacities were analyzed as the oxidative stress marker. In the diabetes group, MDA, GSH and GPX levels were increased compared to the control group, but this increase was not statistically significant (P> 0.05).In the literature, it was observed that different results were obtained in studies investigating the effects of oxidant stress on testicular tissue in STZ-induced type 1 diabetes model (Gobbo *et al*., 2015; Nna *et al*., 2019). In one of these studies, it was reported that MDA levels increased significantly and GSH levels and GPx activity decreased in testicular tissue samples taken after 4 weeks in diabetic rats compared to control (Nna *et al*.). However, consistent with our results, Gobbo *et al*. did not observe any significant change in these parameters at the end of the eight-week diabetes period and this situation may be caused by the histophysiological and antioxidant expression features of the testicular tissue compared to the prostate. Gobbo *et al*. suggested that the microenvironment formed by sustentacular cells in the testis protects the sperm cell against oxidative stress to a certain extent and may be better protected from oxidant stress than prostate (Gobbo *et al*.). In addition, it should be taken into consideration that the differences between the results of the studies in the literature may be due to the duration of diabetes, the dose of STZ given and the age of the rats.

TGF-β1 expression provides inflammatory response, angiogenesis, granulation tissue formation, re-epithelization and temporary collagen accumulation for wound repair in many organs (Penn *et al*., 2012). However, excessive accumulation of the temporary extracellular matrix results in fibrosis and can lead to morbidity and mortality. To avoid this situation, prevention of overexpression of TGF-β1 is controlled by a feedback mechanism thanks to many signal pathways (Kim *et al*., 2018). In our study; TGFβ1 immunoexpression was significantly increased in diabetes, which was considered to be related fibrosis in the interstitial areas in some seminiferous tubules. Salama *et al*. (2001) evaluated the expression of TGF-β1 in diabetic rats depending on age. However, TGF-β expression was

significantly increased in diabetic groups of different ages compared to the control group. Roy *et al*. (2014) over-expression of TGF-β1 and IL-1 in diabetic testes was significantly reduced by ferulic acid. Another marker, the NF-κB is present in the cytoplasm bound to the inhibitor κB (IκB) protein while inactive, and its translocation is regulated by the enzyme kB kinase (IKK). Under the influence of external stimulation, IκB protein is phosphorylated by the IKK enzyme, NF-κB is released and translocated from the cytoplasm to the nucleus. In the nucleus, NF-κB triggers the expression of proinflammatory cytokines, chemokines, and adhesion molecules, such as TNF-α (Oeckinghaus & Ghosh). In diabetes, pancreatic cells are the target of an autoimmune attack mediated by cytokines such as interleukin-1 and interferon (Cnop *et al*., 2006). Therefore, NF-kB activation plays a critical role in the pathogenesis of inflammation and provides ROS detoxification by various mechanisms (Morgan & Liu, 2011). Based on this study, NF-kB immunoexpression increased all spermatogenetic series cells and significantly in sustentacular cells. Xu *et al*. (2014) in their study showed that spermatogenetic cell apoptosis, NF-κB and Wnt4 expressions increased significantly in diabetic rat testis compared to the control group. Heat-shock proteins are cellular damage markers which are encoded by two separate genes in humans has two cytosolic isoforms, as Hsp90α and Hsp90β, and play a role in cellular processes such as apoptosis, cell cycle control, cell viability, misfolding of proteins (Hoter *et al*., 2018). Mitochondria are the primary target of the aging process and they also play an important role in the spermatogenesis process. It has been concluded that some of them, including Hsp90, function as apoptotic inhibitors and can be used as molecular markers in mitochondrial mediated apoptosis, aging and male infertility (Rajender *et al*., 2010). Kim *et al*. (2014) showed that, hsp90 expression was found to decrease in diabetes in kidney tissue compared to the control group. The findings obtained in the current study, expression of hsp90β was decreased significantly in all spermatogenic cells, especially dramatic in spermatids. Previous studies have shown, expression of hsp90 protein decreased in cadmium toxicity and testicular damage due to varicocele (Hassanpour *et al*., 2017; Song *et al*., 2018). E-cadherin is the cell adhesion molecule for necessary normal epithelial function and is important in signal transduction cells in diabetes. As it is known that the blood-testis barrier system is present in the testicular tissue, and cell adhesion molecules are important on this system. According to this study, E-cadherin was found to be decreased in the diabetic group in sustentacular cells in particular. This decrease is considering weakness in the blood-testis barrier and can be due to cell loss of spermatogenetic series cells in basal-adluminal compartments. Aktug *et al*., investigated the expression of cell adhesion molecules and related proteins, which play an important role in germ cell maturation and fertilization, principally in diabetes and in early embryogenesis. It was demonstrated that expressions of e-cadherin protein and similarly b-catenin decreased significantly in germ cells (Aktug *et al*.).

CONCLUSION

In conclusion, the results of this study showed that the seminiferous tubule structures, interstitial areas and spermatogenetic series cells of the testis were evaluated as collectively. In addition, using intracellular signal pathway markers has been demonstrated that diabetes progressed in a base that disrupted cell-cell interaction by triggering the inflammatory and oxidative stress. Compensation of oxidative damage is very difficult in diabetes, and there is an intense need for the development of molecular targeted therapeutic drugs through these intracellular signaling pathways.

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TOROS, P.; OLTULU, F.; TUGLU, I.; UYSAL, A.; ÖZÇINAR, E.; TURGAN, N.; ROUHRAZI, H. & AKTUG, H. Investigación histológica de los efectos de la diabetes inducida experimentalmente en la distribución del factor de crecimiento transformante (TGFβ), nuclear factor kappa B (Nf-κB) y proteínas heat schock 90β (Hsp90β) y E-cadherina en tejido testicular. *Int. J. Morphol., 39(1)*:18-24, 2021.

RESUMEN: La diabetes es una enfermedad metabólica caracterizada por niveles altos de azúcar en sangre y causa complicaciones en muchos sistemas, incluido el sistema reproductivo. Como resultado de las condiciones diabéticas, uno de los mecanismos que puede causar alteraciones en la actividad reproductiva es el estrés oxidativo testicular. La identificación de la diabetes en el eje de las moléculas de señalización celular aún está en discusión. El objetivo de este estudio fue determinar el efecto del factor de crecimiento transformante (TGFβ), el factor nuclear kappa B (NF-κB), las vías de señalización de Heat-Schock 90b (HSP90β) y la molécula de adhesión celular de E-cadherina sobre la infertilidad en testículo de rata diabética. Al término de dos semanas se realizaron análisis histológico, molecular y bioquímico del tejido testicular extraído. Las 7 ratas macho del grupo control no fueron intervenidas. Para el grupo de diabéticos, 7 ratas macho fueron

inyectadas por vía intraperitoneal con una dosis única de 55 mg / kg de estreptozotocina (STZ). Se estudiaron inmunohistoquímicamente las proteínas TGFβ, NF-κB, HSP90β y E-cadherina para investigar el posible daño tisular, el proceso inflamatorio, la estabilización celular y la integridad debido a la diabetes. Para determinar el estrés oxidativo, se realizaron análisis del producto de peroxidación lipídica malondialdehído (MDA), glutatión (GSH) y glutatión peroxidasa (GPx). La fibrosis, los cambios inflamatorios y la pérdida de series espermatogenéticas son hallazgos destacados en el grupo de ratas diabéticas. En el análisis de todas las muestras con inmunotinción, en el grupo diabético, la inmunoexpresión de TGFβ y NF-κB aumentó significativamente, mientras que la inmunoexpresión de Hsp90β y e-cadherina disminuyó significativamente en comparación con los grupos control. Se encontró que la diabetes experimental causa fibrosis, inflamación, alteración de la adhesión celular y estabilización en el tejido testicular. Estos resultados sugieren que son necesarios estudios de terapia celular para verificar posibles daños.

PALABRAS CLAVE: Diabetes; Testículo; TGFβ; Hsp90b; NF-κB; E-cadherina.

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