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Research Article

IMMUNOHISTOCHEMICAL ANALYSIS OF SFLT-1 AND ADAMTS-8 EXPRESSION IN DIABETIC RAT TESTIS TISSUE

Seval Kaya*[1], Yusuf Nergiz[1], Fırat Asır[1], Ebru Gokalp Ozkorkmaz[1], Gamze Erdogan[1], Engin Deveci[1]

[1]Dicle University, Faculty of Medicine, Department of Histology& Embryology, Diyarbakır, Turkey.
* Corresponding author: seval.kaya@hotmail.com

Abstract*: The aim of this study was to determine the testicular inflammation and angiogenetic effect of diabetes with ADAMTS-8 and SFlt-1 proteins immunohistochemically. Wistar albino male rats (n: 12) were used for the study, Group 1: Control group (n: 6), only 1 ml i.p. saline injection was performed. Group 2: Diabetes group (n: 6) received a single intraperitoneal dose of streptozotocin (STZ) of 60 mg/kg. The glucose value measured above 250 mg/dl was considered as diabetic. Under anesthesia, dissection was performed at the lower part of abdominal clearance and testicular tissue was removed. Testis tissues were fixed in 10% neutral formalin, followed by the routine paraffin protocol and cut with a microtome. Then, primary antibodies (ADAMTS-8, sFlt-1) were applied by the immunohistochemistry method and incubated at +4 ˚C overnight. The sections were then examined under a light microscope. The diabetes group showed that Leydig cells in the intertubular area had vacuolization and capillary dilatations in histopathological examinations. sFlt-1 staining of the control group showed positive expression in capillary endothelium between Leydig chambers of the intertubular area. Sflt-1 expression of diabetes group was observed in degenerative spermatic cells and Sertoli cells of the basement membrane facing tubules. The expression of the ADAMTS-8in control group was positive in some Leydig cells in the interstitial area of Sertoli cells in seminiferous tubule but, spermatogenetic cells were negative. In the ADAMTS-8 staining of the diabetes group, the expression of ADAMTS-8 was increased in the stromal cells and some inflammatory cells in the intertubular space. SFlt-1 plays a crucial role in angiogenesis as well as in diabetic testes and is marked as a precursor for the disruption of vascular structure and blood flow due to degenerative changes. It is thought that the distribution of ADAMTS-8 may be a determinant protein in the development of the extracellular matrix and in damage to testicular tissue of diabetic testis.***

Keywords*: Diabetes, testis, ADAMTS-8, sFlt-1, immunohistochemistry*

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1. Introduction

Diabetes mellitus (DM) is a common metabolic disease characterized by increased blood sugar in the long term.DM causes complications in various organs of the retina, heart and reproductive system in both sexes of humans and animals [1]. Patients with diabetes diagnosed with libido, impotence and

fertility impairments in sexual function including declines emerge [2]. Diabetes also disrupts spermatogenesis and reduces sperm count, sperm motility, seminal fluid volume, and testosterone levels [3-5]. Diabetes oxidative stress and damage to cell components it is known to cause an increase in free radical production [6-7].

ADAMTSs, a disintegrin and thrombospondin motif metalloproteinase, is found in the M12B (adamalysin) subgroup [8]. ADAMTS-8 has cell adhesion, cell fusion, proteolysis and signal transduction functions [9]. This protease has been shown to separate proteoglycans such as aggrecan, versican and brevican [10]. The substrate aggregate ADAMTS-8 is expressed in macrophage-rich regions in atherosclerosis [11]. ADAMTS-8 has been reported to inhibit VEGF-mediated angiogenesis in vitro endothelial cells [12].

The association of diabetes with different types of ADAMTS families was investigated and it was reported that ADAMTS-4 and ADAMTS-5 increased in diabetes but decreased with insulin therapy [13].

Soluble Fms-Like Tyrosine kinase-1 (sFlt-1), also known as Vascular Endothelial Growth Factor Receptor-1, is an endothelial receptor for PlGF and VEGF [14] and is a tyrosine kinase protein with anti-angiogenic properties [15].

The aim of this study was to demonstrate the testicular inflammation and angiogenetic effect of diabetes as a result of ADAMTS-8 and sFlt-1 proteins immunohistochemically.

2. Material and Methods

2.1. Experimental Animals

Our study was approved by Dicle University Health Sciences Research Center Local Ethics Committee.12 Wistar albino male rats weighing 200-250 gr were taken from Dicle University Experimental Animals Unit. Group 1: Control group (n: 6), 1 ml i.p. the saline injection was performed. Group 2: Diabetes group (n: 6), streptozotocin (STZ) with a single dose intraperitoneally (60 mg/kg) was prepared in 0.1 M pH: 4.5 citrate buffer and i.p. diabetes was induced by injections (STZ, Sigma Aldrich, USA).72 hours after STZ injection, glucose was measured as diabetic with a drop of blood taken from the tail vein of the rats using a glucometer. Rats were anesthetized by intraperitoneal injection of 5 mg/kg xylazine HCl and 40 mg/kg ketamine HCl. Dissection of the lower abdomen was performed to remove testicular tissue.

2.2. Histopathological procedure

Testicular tissues were fixed with 10% neutral formalin. Routine paraffin tissue follow-up protocol was applied.4-6 μm sections were obtained from paraffin blocks. It was then incubated in xylene for 2x30 minutes and the sections were brought to distilled water. Some of the sections were stained with routine Hematoxylin and Eosin, the remainder were incubated for 3x5 minutes in PBS for immunostaining.

2.3. Immunohistochemical procedure

Following the routine paraffin protocol, 4-6 μm paraffin sections were cut with a microtome (Leica, Germany) Antigen release was carried out twice (5 min and 3 min) in citrate buffer solution (pH: 6.0) in a 700 W microwave oven. The sections were allowed to cool for 20 minutes at room temperature

and washed twice with distilled water for 4 minutes. The endogenous peroxidase blockade was performed in a 10% hydrogen peroxide solution for 7 minutes. The washed samples were incubated in Ultra V block (catalog no. TA-015UB, Thermo Fischer, USA) for 8 minutes. Blocking solution was removed from the sections and allowed to incubate overnight at +4 ° C with primary antibodies (ADAMTS-8 PA5-64274, Thermofischer, USA), sFlt-1 (ab9540, Abcam).After washing the sections in PBS, the secondary antibody (TP-015-BN, ThermoFischer, USA) was applied for 20 min. The sections were washed in PBS for 2x5 min and then exposed to streptavidin-peroxidase (TS-015-HR, ThermoFischer, USA) for 20 min. Sections washed with PBS were allowed to react with DAB (TA-001-HCX, ThermoFischer, USA) chromogen. Counterstaining with hematoxylin was applied and after washing, the preparations were closed. Sections were examined under a light microscope (Carl Zeiss Imager A2, Germany).

3. Results

Table 1. Glucose values of control and diabetes mellitus groups

	Control (n=6)	**Diabetes (n=6)**	*p
Glucose (mg/dL) ± SD	95,22±5,34	402,12± 28,72	<0.001
Values are given as mean ± SD.			

Glucose values of the control and diabetes groups were compared (Table 1). Blood glucose concentration was significantly increased in diabetic rats ($p < 0.001$).

3.1. Histopathological results

The control group, the basement membrane of the seminiferous tubules was of normal thickness and the sperm cells were placed in the tubule towards the surface regularly and the apical were normal in appearance. Leydig cells in the inter-tubular area showed solitary and regular localization around the blood vessels in groups (Figure 1).

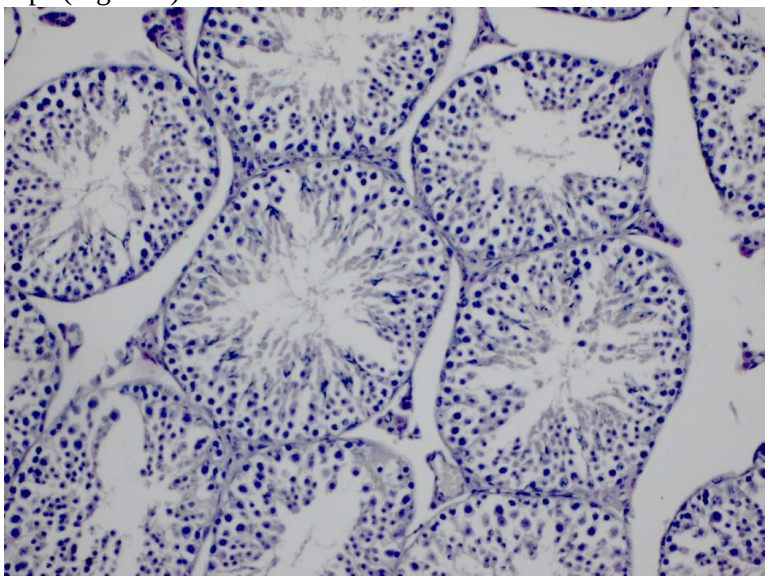

Figure 1. Control group testis section normal view. Hematoxylin & Eosin Bar: 50µm

Diabetic group, thickening of the basement membrane, degeneration of spermatic cells, pyknosis and loss of nuclei of cells towards the surface, deterioration of spermatid formation and degenerative change of sertoli cells were observed. The occurrence of vacuolization and capillary dilatations were observed in Leydig cells in the intertubular area (Figure 2).

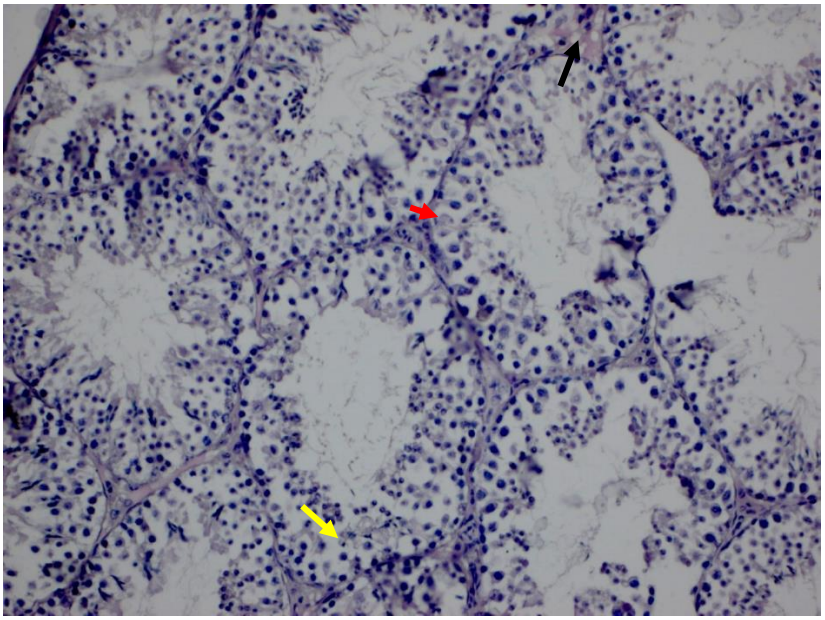

Figure 2. Diabetes group testis section. pyknosis (yellow arrow), sertoli cell (red arrow), vacuolization (black arrow). Hematoxylin & Eosin Bar: 50µm

3.2. Immunohistochemical results

The ADAMTS-8 control group showed positive outward ADAMTS-8 expression in the seminiferous tubular basement membrane, while the expression of ADAMTS-8 was positive in some Leydig cells in the interstitial space in the Sertoli cells in the tubule, while the spermatogenetic cells were negative (Figure 3).

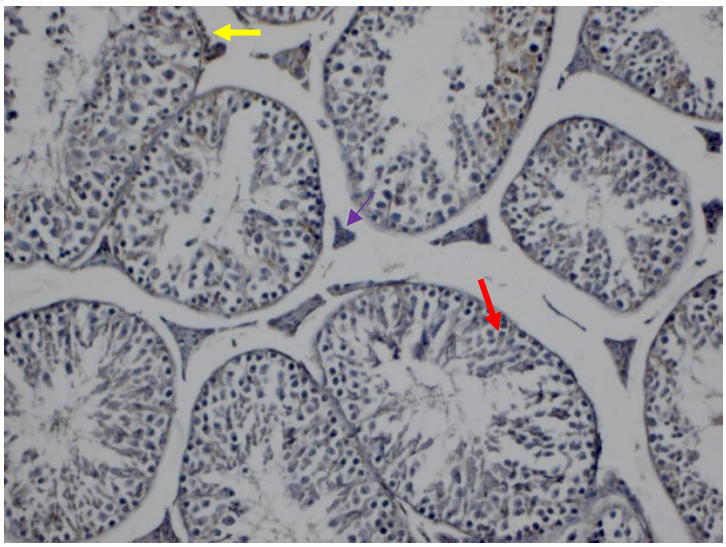

Figure 3. Testis section of the ADAMTS-8 control group. Outwardly, ADAMTS-8 expression was positive (yellow arrow) in the seminiferous tubular basement membrane, ADAMTS-8 expression was positive (purple arrow) in Leydig cells, and spermatogenetic cells were negative (red arrow). Immuno-staining. Bar: 50µm

The ADAMTS-8 diabetes group showed increased ADAMTS-8 expression in the membrane outside the tubules, but increased expression of ADAMTS-8 in stromal cells and some inflammatory cells in the interstitial space. Spermatids located in the apical side of some sertoli cells in the tubule also showed ADAMTS-8 positive reaction (Figure 4).

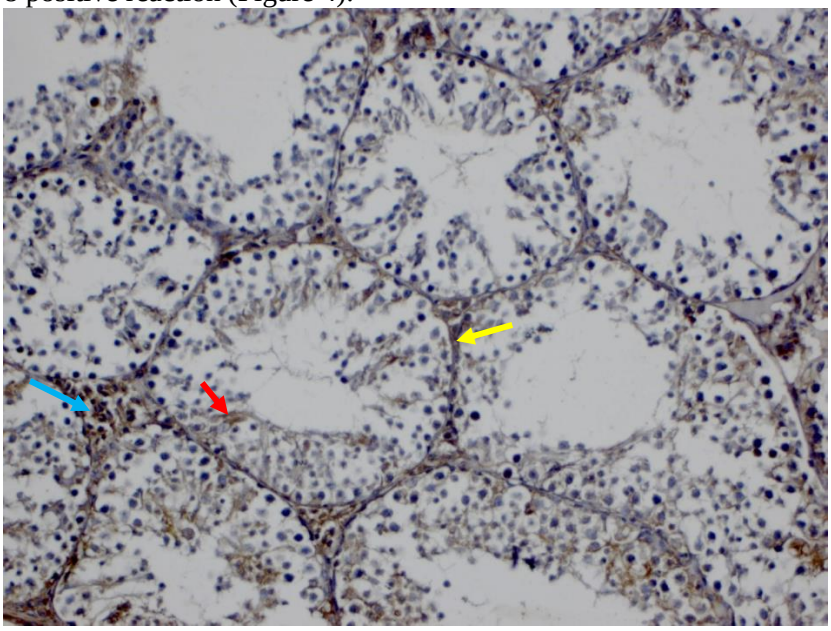

Figure 4. Testicular section of ADAMTS-8 diabetes group, increased ADAMTS-8 expression in the membrane (yellow arrow), ADAMTS-8 expression in stromal cells and some inflammatory cells (blue arrow), ADAMTS-8 positive reaction in spermatids (red arrow). Immuno-staining. Bar: 50µm

SFlt-1 control group, sFlt-1 positive reaction was observed in some spermatids whose negative sFlt-1 expression did not mature towards the surface in the majority of spermatic cells in Seminiferous tubules. Capillary endothelium sFlt-1 showed positive expression among Leydig cells in the intertubular area (Figure 5)

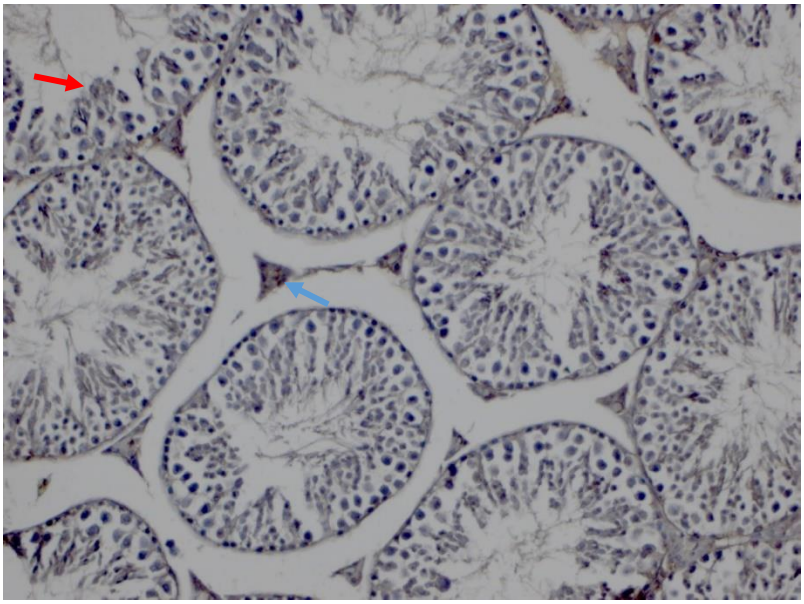

Figure 5. Testis section of the SFlt-1 control group, immature spermatids (red arrow), capillary endothelium (blue arrow). SFlt-1 immunostaining Bar: 50µm

In the SFlt-1 diabetes group, the expression of sFlt-1 was positive in the degenerative spermatic cells and sertoli cells on the tubular side of the basement membrane. In addition, sFlt-1 reaction was evaluated as positive in immature spermatids and sperm (Figure 6).

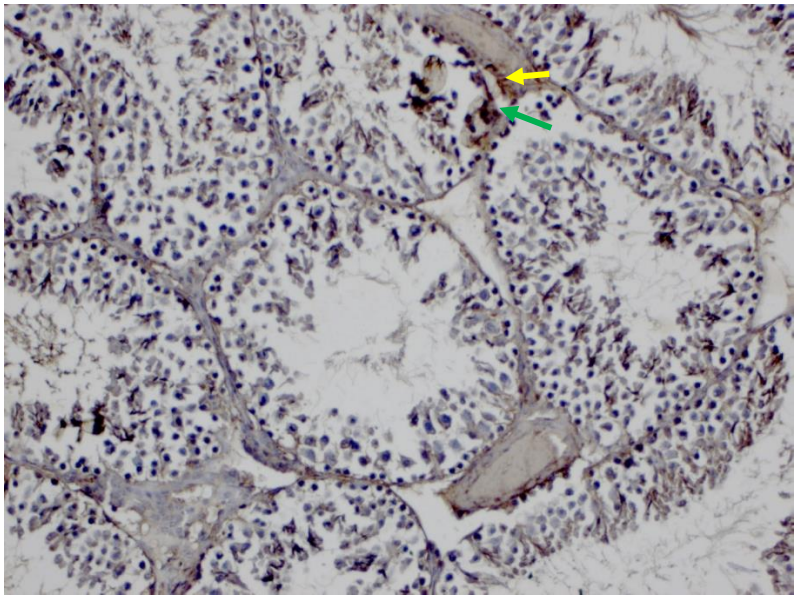

Figure 6. Cross-section of the testis of the SFlt-1 diabetes group, degenerative spermatic cell (green arrow), dilated blood vessels endothelial cell (yellow arrow). Immuno-staining Bar: 50µm

4. Discussion

Diabetes is known to cause decreased androgen receptors in rats, impaired hormone synthesis and sexual function, as well as abnormalities such as sperm count, motility and quality, as well as decreased testicular weight [16].

The ADAMTS family is an important protein in angiogenesis that is found in the connective tissue as aggregates and versicans. Versican is a protein that leads to remodeling of the vascular structure. ADAMTS-8 protein has an angiogenetic inhibitory effect in the structure of the extracellular matrix in the regulation of various cancers that have the ability to suppress tumor genes in tumor proliferation [17].

ADAMTS-8 is a large molecule that carries an enzyme that binds to lipoproteins from proteoglycans in the extracellular matrix to versican cytokines. It has been reported that ADAMTS-8 protein may be under the influence of extracellular matrix damage due to diabetes and changes in the basement membrane structure [18].

Although ADAMTS-8 expression was increased in our study, ADAMTS-8 expression was increased in stromal cells and some inflammatory cells in the intertubular space. It is thought that the inflammation process may increase with cytokine activity due to alteration of the vertical molecule structure in ADAMTS-8.

The soluble VEGF receptor, SFlt-1 protein, has been reported to cause dysfunction of endothelial cells and decreased angiogenesis in patients with renal insufficiency [19].

Irtegün and Deveci in their study, VEGF expression in the testicular tissues of the DM group is almost undetectable compared to the control group, the decrease in VEGF expression as a result of the decrease in the endothelial permeability and may lead to insufficient angiogenesis may occur. [20].

It was seen that sFlt-1 effect was similar to VEGF reaction in diabetic endothelial cell dysfunction and sFlt-1 expression was increased in endothelial cells of dilated blood vessels in interstitial space in SFlt-1 treated group.

5. Conclusion

Due to diabetes damage, degenerative changes in the spermatic cells of the testis with Sertoli cells induce apoptosis-induced inflammation between the tubules and increased vascular dilatation has been observed. It is thought that steroid secretion changes in Leydig cells and testosterone secretion are adversely affected by increasing vacuolar structure.

SFlt-1 plays an important role in the regulation of angiogenesis, and it has been shown to lead to the deterioration of the vascular structure and blood flow due to degenerative changes in diabetic testes. ADAMTS-8 distribution is thought to be a determinant protein in the development of extracellular matrix in diabetic testis and testicular injury due to inflammation.

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