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INVESTIGATION OF THE EFFECT OF *IN PUNICA GRANATUM* STREPTOZOTOCIN INDUCED DIABETIC RAT TESTIS

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1.İntroduction

Diabetes mellitus (DM) is one of the most common metabolic disorders effecting on world population. Researches on diabetes which become a major health problem in the community in recent years is increasing rapidly (1). DM is a disease linked to low blood insulin level or insensitivity of target organs to insulin (2). Sexual dysfunction is associated with diabetes in men. Investigations of diabetic humans and animal models have revealed abnormal spermatogenesis, degenerative and apoptotic changes in the testis, increased thickness of seminiferous epithelium, decreased total area of testis, decreased serum testosterone levels and changes in the blood-testis barrier, all of which contribute to infertility (3,4). Beside, oxidative stress induced by diabetes mellitus leads to damages in the testis, as a consequence of which cognitive functions is impaired. Reactive oxygen species (ROS) accumulate in the cell and stimulate apoptosis, which compromises sperm quality and function (4). Therefore, for the treatment of infertility, in addition to antidiabetics, antioxidants are used to cope with oxidative stres (5). *Punica granatum* (PG) scientifically known as Pomegranate have been used in the herbal medicine of different populations (6). PG has become more famous due to its important pharmacological properties, including anti-cancer, antiproliferative, apoptotic, antimicrobial, antioxidant activity and free radical scavenger effect. PG is found to be rich in vitamin C and polyphenolic compounds such as ellagic, punicalagin, anthocyanins, gallic acid flavonoids namely luteolin, apigenin, and quercetin (7). Besides diabetes, ıt has also been shown to be effective in diseases such as cancer, cardiovascular, azlheimer (8). The aim of this study is investigation effects of PC on decreasing the damage induced by DM.

2.Materials and Methods

Preparation of in *Punica granatum*

Pomegranate seeds were dried and pulverized. The dry powder shell (1g) was extracted with 0.1 hydro alcohol solvent and kept at room temperature for 48 hours. After shaking, the extract was filtered through filter papers three times and concentrated under vacuum at 37 ° C to obtain a concentrated extract.

Induction of diabetes

In the control group, a single dose of 55 mg / kg will be given to saline and diabetes group. Streptozotocin prepared with 0.1mol / L sodium citrate buffer (pH: 4.7) (STZ) was injected intraperitoneally. Blood measurement by hand glucometer from tail vein over 250 mg/dl subjects with blood-glucose levels were accepted as diabetes.

Experimantal design Twenty-one male Wistar rats were randomly divided into three groups (n=7/each) according to the experimental protocol: (Group I) Control, (Group II) Diabetic, (Group III) Punica granatum-treated diabetic rats. For Group II local injection of salin while Group III injection of (500 mg/kg) PC was performed on diabetic animal testis under local anesthesia. After four hours, the testis were removed for histological examination.

Tissue processing

Histochemistry

For morphological analysis, hemotoxylin and eosin staining routine procedure was performed.

Immunocytochemistry

To detect the expression Enos and İnos specific monoclonal antibodies were used. The tissue tissues were deparaffinized and passed through the alcohol series. Antigen retrieval with trypsin was performed. Hydrogen peroxide was performed for endogenic peroxidase inhibition. Then the tissue were blocked with blocking solution and incubated with primary antibodies at 4°C overnight. Then the secondary antiboy incubation were done and the cells stained with DAP and counterstained with mayer's hematoxylin.

Tunel

In the determination of apoptotic cell death, Terminal Transferase dUTP Nick End Labeling (TUNEL) staining method was used. 20 µg / ml Proteinase-K was diluted with PBS at a rate of 1/500. It was treated with 3% hydrogen peroxide for 5 minutes. Samples were kept at room temperature with 5 minutes equilibration buffer and plastic slides were kept at 37 °C for 60 minutes in a humid atmospheric environment. With Stop Wash Buffer after 10 minute after standing for 30 min with Antidioxygenin Peroxidase Conjugate was treated. Then, staining with DAB (Diaminobenzidine) was performed and background staining was done with Mayer's Hematoxilene. TUNEL positive cells were determined by the blind method and the results were evaluated statistically.

Statistical analysis

For the evaluation of the testis histology, Johsen score was performed with at least 100 tubules, the level of sperm maturation is graded between 1 and 10, according to the most advanced germ cell in the tubule. The total Johnsen score is then determined by dividing the total score by the number of evaluated tubules. İmmunohistochemical evaluation was evaluated as weak (+), moderate (++) and severe (+++). The amount of cell stained was calculated by H-score method and analyzed statistically.

3.Results and Discussion

In this study, histological examination of testis tissue showed that diabetes causes degenerative changes in the seminiferous tubules. Tubular cell disintegration, reduction of seminiferous tubules diameter, thickness of epithelium and intertistitial tissue, sertoli and spermatogonia cell vacuolization in seminiferous tubules were seen in diabetic groups in comparison with control groups (Figure:1) Moderate damage was observed according to johnson scoring Table 1).While the oxidative stress and apoptosis increased in the diabetic group, there was a significant decrease in the treatment group (Table:3). The treatment of diabetic groups was increase diameter seminifourus tubules and spermatogenesis according to diabetic groups. Thanks to the antioxidant properties of pomegranate extract, a significant result was obtained by reducing the oxidative stress. These data demonstrated that *Punica granatum* significantly improved diabetes complication in rat testis. As a result, PC effecting improvement of morphological condition of seminiferous tubules in testis. PC would be beneficial for infertility in diabetic male testes.

Histochemical Results

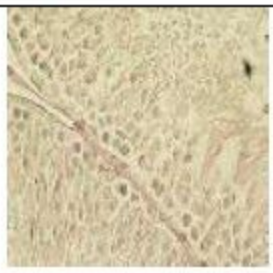
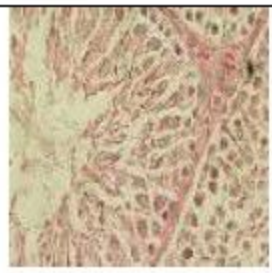
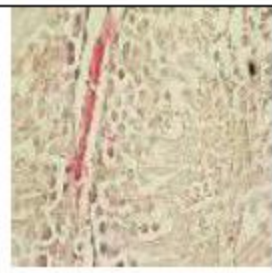
Control	Diabetes	Diabetes +Punica
		

Figure 1: Hematoxylin and eosin stainning of control, diabetes and diabetes+punica

Table 1: Johson scor analysis

	Difference	q	P value
Control vs Diabetes	3.080	5.497	** P<0.01
Kontrol vs Diabetes + Punica	0.9000	1.606	ns P>0.05
Diabetes vs Diabetes +Punica	2.180	3.891	* P<0.05

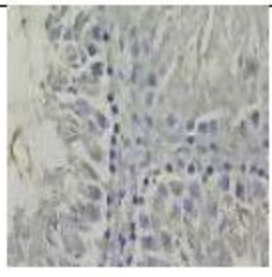
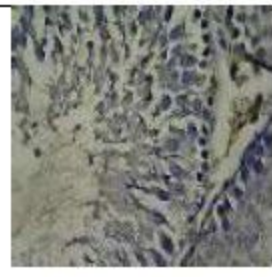
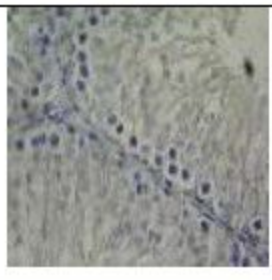
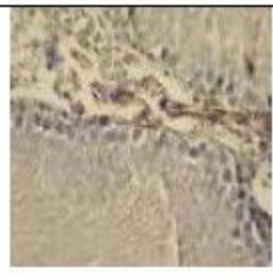
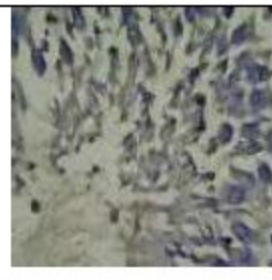
	Control	Diabetes	Diabetes +Punica
Enos			
İnos			

Figure 2: Immunocytochemistry for Enos and İnos in control, diabetes and diabetes+punica groups.

h score analysis of Enos			
	Difference	q	P value
Control vs Diabetes	71.940	9.571	P<0.001
Kontrol vs Diabetes + Punica	-42.260	5.623	P<0.01
Diabetes vs Diabetes +Punica	29.680	3.949	P<0.05

h score analysis of İnos			
	Difference	q	P value
Control vs Diabetes	67.940	11.36	P<0.001
Kontrol vs Diabetes + Punica	-37.940	6.344	P<0.01
Diabetes vs Diabetes +Punica	30.000	5.017	P<0.05

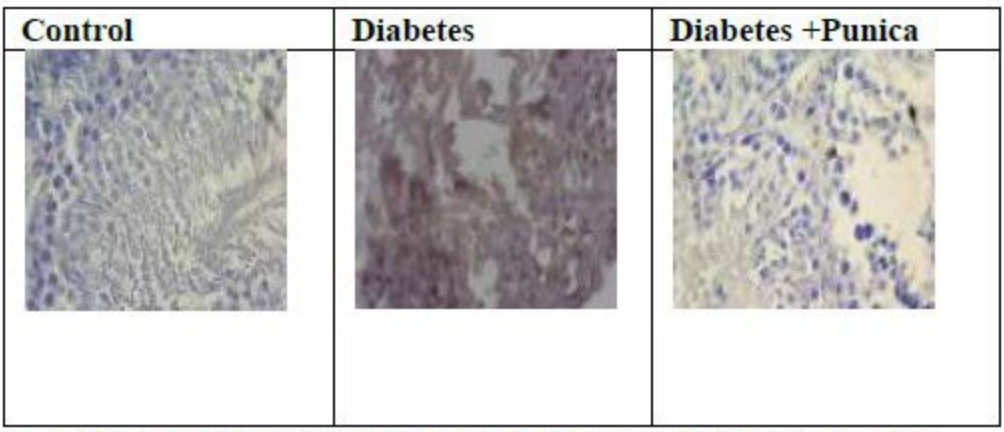


Figure 3: TUNEL staining for control, diabetes and diabetes+punica groups.

	Difference	q	P value
Control vs Diabetes	-41.110	8.885	***P<0.001
Kontrol vs Diabetes + Punica	-17. 110	3.698	* P<0.05
Diabetes vs Diabetes +Punica	24.000	5.187	** P<0.01

Table 3 : Analysis of apoptotic index

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