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Research Article

The effects of ethanol extract of *Punica granatum L. peel* on the testis damage induced by diabetes in rats

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ABSTRACT

The aim of this study was to determine the effect of ethanol extract of Punica granatum L. Peel on biochemical and histopathological changes in blood and testicular tissue in rats with experimental diabetes. A total of twenty-eight male Sprague-Dawley rats, 7 rats in each group, were used in this study. Group 1; peros physiological saline to the rat, Group 2; STZ 60 mg/kg/IP single dose, Group 3; PGE 10 mg/kg/20days/peros, Group 4; STZ as 60 mg/kg/IP + PGE 10 mg/kg/20days/peros. After the end of the experimental procedure, the rats were sacrificed, blood and testicular tissues were taken, and biochemical and histopathological examinations were performed. The administration of PGE was shown that the activities of CAT, SOD, GPx and GSH increased and the levels of MDA decreased in diabetic rats. Compared to the diabetic and control groups, the treatment group decreased the LPO level and improved the antioxidant activity in plasma and testicular tissues. Histopathological examination of testicular tissues in the group 2 is revealed edema in the intertubular spaces, thinning of the tubule walls due to diminished spermatocytes in the walls of the seminiferous tubules and severe degenerative and necrotic changes in spermatocytes. These changes were found to be very mild in group 4. According to immunohistochemical findings, ın group 2, caspase 3 expression was intensely expressed in spermatocytes. As a result, it was observed that Punica granatum L. bark extract strengthened antioxidant defense and reduced oxidative stress in diabetic rats.

INTRODUCTION

Diabetes mellitus is one of the metabolic diseases that cause functional disorders in many systems, including male infertility (Akhtar et al., 2015). In rats, diabetes causes a decrease in testicular weight, sperm count, total motility and testosterone levels, and increases apoptosis (Cai et al., 2000; Scarano et al., 2006). Diabetes mellitus causes an increase in the level of oxidative stress in tissues and decreases in the antioxidant defense system (Baynes and Thorpe, 1999).

Streptozotocin (STZ), which is used to cause diabetes in rat, is an alkylating chemotherapeutic including a nitrosurea group (Mythili et al., 2004). Further, after STZ enters the beta cells, it is converted into glucose and methylnitrosourea. Because it has an alkylating structure, changes occur in biological mac-romolecules and DNA is fragmented. As a result, beta cells are deformed and a model of insulin-dependent diabetes is formed. By disrupting the mitochondrial DNA of beta cells, it has been shown that insulin release is inhibited by STZ (Len-zer, 2008).

Pomegranate (Punica granatum) is widely used in medicine(Longtin, 2003). Punicalagins, ellagic acid, anthocyanins, flavonoids and a wide variety of antioxidant phenolic compounds are found in pomegranate (Henning et al., 2019). There are approximately 48 phenolic compounds with antioxidant properties in the pomegranate peel(Benzie and Wachtel-Galor, 2011). In addition, pomegranate peel contains punicalagin and ellagitanens such as punicalagin (Newman and Lansky, 2007). Compounds with this antioxidant property are generally found in the pericarp and mesocarp layers of the pomegranate peel (Fischer et al., 2011) and have strong antioxidant activity, anti-mutagenic, anticancer, anti-inflammatory and anti-diabetic effects (Akhtar et al., 2015).

In the light of all these data, in this study, it was aimed to determine the effect of ethanol extract of Punica granatum L. bark on the pathological and biochemical changes in experimental diabetic rats.

MATERIAL and METHODS

Plant Material and Extraction Procedure

The pomegranate peel extract was made with the modification of the method described by Tayel et al.(Tayel and El Tras, 2010). Commercially available pomegranates were separated

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from the seeds and the peels were dried in the shade and were crushed. 100 grams of pomegranate peel was taken and placed in a 1 liter flask, 500 ml of ethanol was added to it and left for 48 hours. The resulting mixture was filtered and coarse particles were separated. Then, this mixture was separated from the organic solvent part with the help of evaporator and the extract was obtained. The extract was stored at +4ºC until the experimental stage to be used in the study.

Experimental Design

In this study, 28 male Sprague-Dawley rats weighing 200-250 grams, 5-6 weeks old, were used as the control and experimental groups. Rats were fed with standard water and pellet feeds. It was kept at 21 °C (±2) in the 12 hour light / 12 hour dark cycle.

Group I (n:7); Physiological saline via oral gavage to the rat.

Group II (n:7); STZ (60 mg / kg) via intraperitoneal injection(IP).

Group III (n:7); Punica granatum L. Extract(PGE) was administered to rats (10 mg/ kg) peros.

Group IV (n:7); STZ (60 mg / kg) via IP+PGE (10mg/ kg) peros.

STZ was dissolved in cold citrate buffer and administered IP after 18 hours of fasting to induce diabetes. The blood glucose level was measured with a glucometer in the rats in which diabetes was desired. Rats with a glucose level of 250 mg/dl and above were evaluated as diabetic. PGE was given to the rats in the experimental groups by oral gavage for 20 days. At the end of the 20th day, blood and tissue samples were taken by performing decapitation under anesthesia. Blood samples taken into anticoagulant tubes were centrifuged at 3000 rpm at +4°C for 10 minutes, the plasma part was removed and stored at -20°C until analysis. Testicles of rats were removed and cleaned from surrounding tissues. The testicular tissues obtained were washed with 0.15 M potassium chloride (KCl) at +4 °C and dried. Then, the tissues were homogenized with a homogenizer in 0.15 M KCI solution at 16000 rpm for 3 minutes. Homogenization was carried out in an ice bucket. The homogenate was centrifuged at 5000 g for 1 hour (at + 4 ° C) then GSH, MDA, CAT, GPx and SOD levels were measured from the supernatant with the help of Biotek ELISA Reader.

Measurement of Oxidative Stress

Testicular tissue CAT activity was measured by the method described by Goth (1991). Testicular tissue MDA level was measured by the method specified by Placer et al. (1966), SOD activity by Sun et al. (1988), and GPx level by Matkovics et al. (1988).Tissue extraction and analysis of GSH was done according to the method of Ball (1966), Fernandez and Videla(1981).

Plasma MDA levels were measure by the method of Yoshioka et al.(1979) and GSH, CAT, SOD, GPx levels were determined according to the method of Tietze(1969), Goth(1991), Sun et al.(1988), Matkovics et al.(1988) respectively, using Biotek ELISA Reader.

Immunohistocemical and in situ Hybridisatiaon

Testis tissues were taken into 10% buffered formalin solution. The samples were embedded in paraffin blocks and 4μm thick sections were stained with hematoxylin-eosin using a microtome and examined with a light microscope for immunohistochemical examination. Sections were classified as absent (-), mild (+), moderate (++), and severe (+++) according to the degree of lesion.

For immunoperoxidase analysis, all sections taken from adhesive (poly-L-Lysine) wares were passed through xylol and alcohol series, deparaffinized and dehydrated. Samples were washed in distilled water for 5 minutes. Sections were heated in citrate buffer (pH 6.1) 4 times in a microwave device for 5 minutes to ensure antigen recovery in the nucleus. Samples removed from the microwave were incubated for 30 minutes at room temperature. Then the samples were washed with distilled water, the sections were dried and drawn with a special glass pen. Endogenous peroxidase was washed with phosphate buffer solution (PBS) for 5 minutes and inactivated by holding in 3% H_2O_2 for 10 min. After washing the sections in PBS samples were left to incubate for 5 minutes with a Protein block compatible with all primary and secondary antibodies to prevent nonspecific background staining. Primary antibodies (caspase 3) were dropped without washing after the excess block solution remaining on the tissue sections at the end of the incubation was poured. It was kept at room temperature for 1 hour or at +4 ^{0}C for 1 night in accordance with the primary antibody. It was washed with PBS 2 times for 5 minutes and incubated with biotinized secondary antibody about 30 minutes at room temperature. The sections washed again with PBS were kept in streptavidinperoxidase for 30 minutes and then washed in the same way with PBS. After washing, AEC (3-amino-9-ethyl carbazole) chromogen was dropped to the sections and kept for 5-10 minutes depending on the chromogen retrieval. For the floor staining, it was kept in Mayer's hematoxylin for 1-2 minutes and then washed in tap water. It was coverslipped using a water-based adhesive and examined with a light microscope (Leica DM 1000). Incubation time in the other stages with primary antibodies, whether antigen retrival or enzyme will be applied, and dilution rates of primary antibodies may vary depending on the commercial kit used. Sections were evaluated as none (-), mild (+), moderate (++) and severe (+++) according to immune positives.

Statical Analysis

The data obtained in the study were analyzed in the SPSS (V13) program. Kruskal-Wallis test was used to determine the difference between groups. The difference between the two groups was determined using the Mann-Whitney U test.

RESULTS

When testicular and plasma biochemical parameters are evaluated (Table 1, 2), The MDA levels in the Group2 were found to be higher compared to the other experimental groups. The levels of GSH, CAT and SOD were lower in the group 2 in comparison the other groups. While a clearly positive effect was observed in the group 3 in terms of the evaluated parame-

ters, the parameters detected in the group 4 confirm the protective efficacy of *Punica granatum L. peel extract* against STZ.

Table 1. Testicular biochemical parameters (Mean ± SEM)

Groups	GSH (mmol/g)	MDA (nmol/g)	CAT (kU/g)	GPx (U/mg)	SOD (EU/mg)
Group I	0.62±0.00^{c}	36.01±0.26^{a}	239.90±1.14^{b}	0.05±0.00^{b}	5.12±0.08^{c}
Group II	0.33±0.00^{a}	56.63±0.44^{c}	213.07±0.86^{a}	0.03±0.00^{a}	3.41±0.08^{a}
Group III	0.64±0.00^{c}	34.42±0.69^{a}	248.34±2.05^{c}	0.06±0.00^{c}	7.36±0.09^{d}
Group IV	0.54±0.01^{b}	38.53±0.63^{b}	239.31±1.46^{b}	0.04±0.00^{a}	4.69±0.06^{b}

a-c The values represented by different letters within the same row are significantly different from each other, **P<0.05

Table 2. Plasma biochemical parameters (Mean ± SEM)

Groups	GSH (mmol/g)	MDA (nmol/g)	CAT (kU/g)	GPx (U/mg)	SOD (EU/mg)
Group I	3.13±0.00^{b}	27.07±0.14^{b}	256.79±0.50^{c}	0.23±0.01^{a}	13.90±0.08b
Group II	2.66±0.03^{a}	37.10±0.18^{c}	138.48±0.42^{a}	0.21±0.00^{a}	11.79±0.16a
Group III	3.18±0.06^{b}	25.72±0.05^{a}	259.15±1.95^{c}	0.25±0.00^{b}	15.07±0.13c
Group IV	3.07±0.06^{b}	27.17±0.39^{b}	243.91±1.78^{b}	0.22±0.00^{a}	13.72±0.11b

a-c The values represented by different letters within the same row are significantly different from each other, **P<0.05

Group 1: Normal testicular histological structure is seen in rats. (Figure1- A).

Group 2: Edema in intertubular spaces and dilated and hyperemic vessels were observed in testicular tissues of rats. It was observed that the walls of the tubules became thinner due to the reduction of spermatocytes in the walls of the seminiferous tubules. In addition, severe degenerative and necrotic changes were detected in spermatocytes. Very few spermatozoa were found in the lumen of the tubules, and some were absent. (Figure1- B).

Group 3: It was observed that both the wall and interstitial spaces of the seminiferous tubules were in normal histological structure in the testicular tissues of the rats. (Figure1- C).

Group 4: Mildly degenerative spermatocytes were found in the tubules of the testicular tissues of the rats in this group, but no necrotic spermatocytes were found. It was determined that there were moderate levels of spermatozoon in the tubulus lumens (Figure1- D).

In addition, ın rats with diabetes, thinning of the tubular wall, degeneration of spermatocytes, necrotic spermatocytes and edema in intertubular spaces were observed. however, it was determined microscopically that orally administered PGE caused a decrease in these changes (Table 3).

Table 3: Histopathological and immunohistochemical scores of testicular tissues.

Groups	Thinning of tubulus wall	Degenerative spermatocytes	Necrotic spermatocytes	Edema in intertubular spaces	Caspase 3
Group I	-	-	-	-	-
Group II	+++	+++	++	+++	+++
Group III	-	-	-	-	-
Group IV	-	+	-	-	++

It was observed that Caspase 3, which was used to determine apoptosis, was expressed at a very low level in the testes of rats in the control and groups 3. This situation was evaluated as negative (-) (Figure 2, A-C). In the group 2, a large number of spermatocytes were found to be positive (+++) (Figure 2, B). Mild positive (+) cells were found in the testicles of rats in the group 4 (Figure 2, D). When the groups 2 and 4 were compared in terms of the number of caspase positive cells, a statistically significant difference was found (p <0.05).

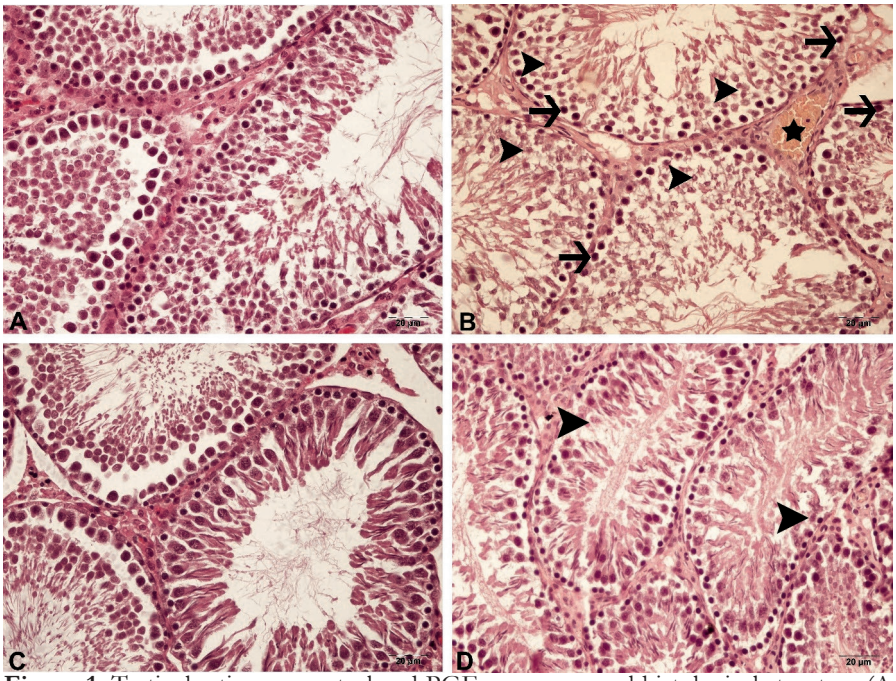


Figure 1. Testicular tissues, control and PGE group, normal histological structure (A and C). Severe necrotic-degenerative spermatocytes in tubuli (arrows-arrowheads), thinning of tubulus wall, dilated and hyperemic vessels in intertubular spaces (star), edema in intertubular spaces (B). Mild degenerative spermatocytes in tubuli (arrowheads), moderate spermatozoon in tubulus lumens (D), H&E, Bar: 20µm.

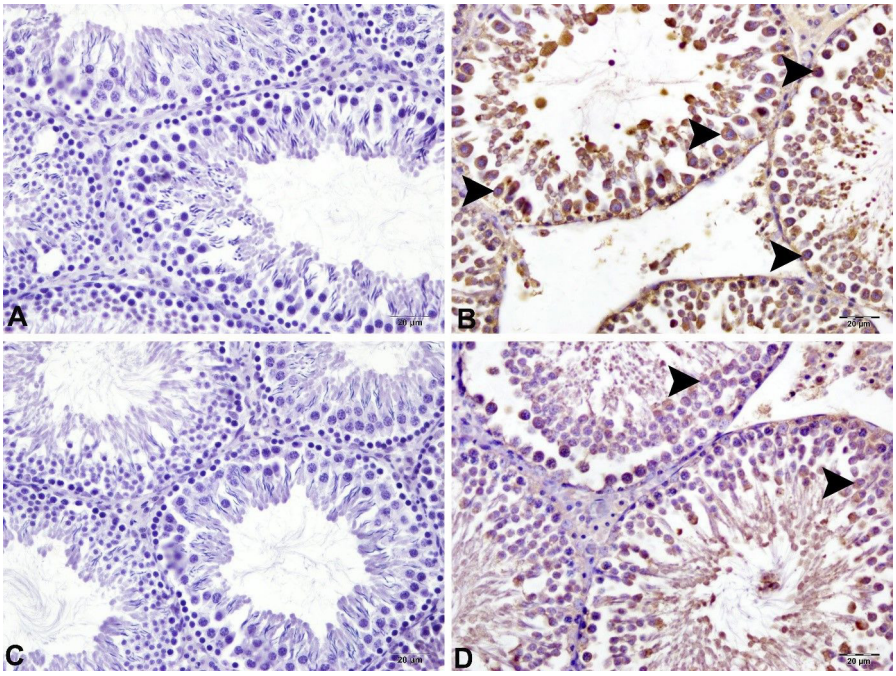


Figure 2. Testicular tissues, control and PGE group, caspase 3 negative (A and C). Severe caspase 3 positive in spermatocytes (arrowheads) (B). Testicular tissues, mild caspase 3 positive in very few spermatocytes (arrowheads) (D), IHC-P, Bar: 20µm.

DISCUSSION

Diabetes Mellitus is a chronic disease characterized by hyperglycemia and significantly affects many functions of the body. It is also important in that it causes a decrease in fertility(Khaneshi et al., 2013). STZ and alloxane are the agents most commonly used to induce experimental diabetes. Diabetes can be caused by intraperitoneal, subcutaneous, intravenous, parenteral administration of these agents. These agents cause hypoinsulinemic and hyperglycemic state by destroying beta cells in Langerhans islets in the pancreas(Erbaş, 2015).

In the present study, it was determined that oxidative stress products increased and antioxidant enzymes decreased in STZ applied groups. In histopathological examination of testicular tissues, edema in the intertubular spaces, thinning of the tubular walls due to the decrease of spermatocytes in the walls of the seminiferous tubules, severe degenerative and necrotic changes in the spermatocytes were detected. Ibrahim (2018) stated that diabetes related to STZ, similar to our study, causes a disorder in the reproductive system in male rats. This situation is thought to be related to the fact that STZ increases oxidative stress and consequently leads to a decrease in sperm quality and causes histopathological changes in the testis.

It has been reported that a new ellagitannin and punigluconin containing a gluconic acid were found in the fresh body peels of P. Granatum (Tanaka et al., 1986). So, when evaluated in terms of antioxidative properties, because of the content of P. Granatum, it was observed in the current study that Punica granatum L peel extract increased antioxidant activity and decreased malondialdehyde levels in testicular tissue and plasma of diabetic rats.

Studies have reported that diabetes increases oxidative stress due to hyperglycemia, causes damage to the seminiferous tubules and drives spermatocytes to apoptosis(Khaneshi et al., 2013). In our study which supports the findings; thinning of tubule walls, severe degenerative and necrotic changes in spermatocytes were found to be very mild in the group 4. According to the immunohistochemical findings, caspase 3 expression, which was performed to detect apoptosis, was severely expressed in spermatocytes. On the other hand, it was observed to be very mildly expressed in the group 4.

In a study by Mahmoud and Mahmoud(2017), it was determined that pomegranate peel extract provided protection against damage to the tissues of rats, which were evaluated differently from our study in the diabetes table created with STZ. In addition, in another study, it was determined that pomegranate peel extract was effective against oxidative stress in rats with oxidative stress(Doostan et al., 2017).

In addition, according to the findings of the study by Dkhil et al. (2013), it was observed that the application of Methanolic Bark Extract and Pomegranate (Punica granatum L.) juice decreased oxidative stress in testicular tissues of rats and increased antioxidant activity. Also, in view of the application of Pomegranate peel extract, results showed an improvement of morphological condition of seminiferous tubules in adult wistar rats(Boroujeni et al., 2017). And, in another study, Minisy et al.(2020) have shown pomegranate seeds extract to be effective against testicular toxicity in experimental rats. Again, in this sense, a study on rabbits is also remarkab-le. Bakeer et al.(1988) found that pomegranate peels stimulate spermatogenesis and increase fertility in rabbits. These situations were consistent with the results we obtained.

The mechanism of action of free radicals resulting in lipid peroxidation (LPO) is as follows: Free radicals are atoms or molecules that contain one or more unpaired electrons, and they react rapidly with other molecules to share these electrons. Since one electron of molecules that react with radicals decreases, they become reactive and this reaction continues in a chain. Abundant free radicals are produced in mitochondrial, endoplasmic and nuclear electron transport systems (cytochrome P-450), peroxisomes, and during normal metabolic events such as phagocytosis of monocytes and neutrophils. Many defense mechanisms have been developed in the body to prevent the formation of these radicals and the damage they may cause. If these radicals exceed the capacity of the defense mechanisms, they cause damage to important components of cells such as lipids, proteins, DNA, carbohydrates and enzymes. Lipids are the structures most susceptible to free radical damage. Free radicals easily react with unsaturated bonds in fatty acids and cause peroxidation of lipids(Aydilek and Aksakal, 2003).

In general, antioxidant substances prevent the stealing hydrogen by free radicals from tissues. They provide this situation by directly supplying the hydrogen needed by free radicals. In other words, antioxidant substances saturate free radicals at the beginning and prevent their continuous activities. However, if the formation of free radicals continues due to light and metals, the antioxidant substance is consumed. If necessary precautions are taken and sufficient amount of antioxidant material is present, free radicals will be stopped before and antioxidant effect will continue for a long time(Çakmak, 2003). The effects of antioxidants against free oxygen radicals are as follows; stopping the initiation of the chain reaction, breaking the radical chain reaction that started, preventing the radical formation to start. In addition, they disrupt the structure of peroxides and reduce the local oxygen density(Cheeseman and Slater, 1993). By the way, the most important antioxidant enzymes; SOD, which converts superoxide anion to H_2O_2, GPx, which detoxifies organic peroxides and catalase that H_2O_2, reduces to H_2O(Marti et al., 2008). When the effectiveness of antioxidant enzymes mentioned in the findings section of our study is interpreted in terms of literature information, the results are significant.

In the light of the literature, the results of many different studies related to the positive effect of pomegranate on reproductive parameters in lab animals support our findings(Lydia, 2019).

CONCLUSION

In conclusion, it was concluded that lipid peroxidation of testicular damage occurs in the case of diabetes induced by STZ and pomegranate peel extract (PGE 10 mg/kg/20days/po) prevents this damage.

DECLARATIONS

Ethics Approval

The rats used in the study were obtained from Atatürk University Experimental Application and Research Center (ATADEM). Permission was obtained from the Local Ethics Committee of Ataturk University (75296309-050.01.04-E.2000065644/4).

Conflict of Interest

The authors declare that they have no known competing fi-

nancial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for Publication

The authors have given the consent for publication to the journal.

Author contribution

Idea, concept and design: ADO, BAY.

Data collection and formal analysis: ADO, BAY, SY.

Drafting of the manuscript: ADO, SAA.

Critical review, writing and editing SAA.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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