The Effect of Pomegranate Juice on Diabetes-Related Oxidative Stress in Rat Lung

Nar Suyunun Sıçan Akciğerinde Diyabete Bağlı Oksidatif Stres Üzerine Etkileri

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ABSTRACT Objective: It is known that diabetes leads to depletion of the cellular antioxidant defense system and this causes the organ injuries related to the diabetic complications. Antioxidants have been shown to reduce indices of oxidative stress measures in experimental disease models and in humans. The present study was designed to evaluate the oxidative stress in lungs as well as the therapeutic effect of pomegranate juice (PJ), as an antioxidant, in rats with streptozotocin-induced diabetes. Material and Methods: Twenty seven rats were enrolled in the study where diabetes was induced by streptozotocin injection. Rats were treated with either PJ or saline for 10 weeks, and their lungs were harvested for histologic and immunohistochemical evaluation at the end. Protein carbonyl content (PCC), sialic acid (SA), superoxide dismutase (SOD) and reduced glutathione (GSH) activities were measured in the pulmonary tissue as well as the presence of endothelial nitric oxide synthase (eNOS), through immunohistochemistry. Results: The pulmonary tissue in diabetic rats showed oxidative alterations related to the streptozotocin treatment. There was increased eNOS expressions in diabetic lungs, but PJ treatment diminished both eNOS expressions and inflammatory changes in pulmonary tissue. PCC and SA levels were higher in diabetic lungs (p<0.01) whereas SOD was lower (p=0.021) and GSH did not change significantly in comparison to PJ-treated diabetic group (p=0.374). Conclusion: The PJ treatment effectively reduced the oxidative stress and contributed to tissue recovery in experimental diabetic lungs. These findings would be of clinical relevance.

Key Words: Oxidative stress; diabetes complications; lung; models, animal; punicaceae

ÖZET Amaç: Diyabetin hücresel antioksidan savunma sisteminde azalma yol açtığı bilinmektedir ve bu da diabetik kompleksiyonlara bağlı organ hasarına neden olur. Antioxidanların oksidatif stres ölçütleri göstergelerini azalttığı deneylerde farklı modellerde ve insanlarda gösterilmiştir. Bu çalışma akciğerlerdeki oksidatif stresi ve streptozotocinle indüklenmiş diyabet olan sıçanlarda, bu antioksidan olan nar suyu tedavisini edici etkisini değerlendirerek bu için tasarlandı. Gerer ve Yöntemler: Streptozotocin enjeksiyonu ile diyabetin indüklençisi 27 sıçan çalışmayı alındı. Sıçanlar 10 hafta boyunca nar suyu veya serum fizyozikikles tedavi edildi ve bitiminde histologik ve immunohistokimyasal değerlendirme için akciğerleri toplandı. Akciğer dokusunda protein karbonil içeriği (PCC), sialik asit (SA), süperoksit dızmutaz (SOD) ve redüktre glutatyon (GSH) aktiviteleri ve endotelial nitrık oksit sentaz (eNOS) varlığı immunoistokimyasal olarak ölçüldü.

Bulgular: Diyabetin sıçanlarında akciğer dokusu streptozotosin uygulamasına bağlı oksidatif değişiklikler gösterdi. Diyabetik akciğerlerde artmış eNOS enzim sinyalini vardi fakat nar suyu tedavisi akciğer dokusunda hem eNOS enzim sinyalini hem de inflamatur değişiklikleri azalttı. Diyabetik akciğerlerde PCC ve SA düzeyleri daha yüksekti (p<0.01) ve SOD daha düşültüktü (p=0.021), oysa GSH nar suyu tedavisi edilen diyabetik grupla karşılaştırıldığında önemli ölçüde değişmedi (p=0.374). Sonuç: Nar suyu tedavisinin oksidatif stres etkisi görülmedi ve deneyel diyabetik akciğerlerde doku iyileşmesi katkında bulunduğunu göstermiştir. Bu bulgular klinik çalışmalarla desteklenmeliidir.

Anahtar Kelimeler: Oksidatif stres; diyabet kompleksiyonları; akciğer; modeller, hayvan; nargiller

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there is clear evidence that diabetes leads to depletion of the cellular antioxidant defense system and increases levels of reactive oxygen species (ROS). This known concept of oxidation stress, being an important trigger in the onset and progression of diabetes and its complications, may offer a unique therapeutic option for the treatment of diabetes and its complications by using antioxidants or nutrients with high antioxidant capacity. Antioxidants have been shown to reduce indices of oxidative stress measures in experimental disease models and in humans.

The increase of nitric oxide (NO), formed by the action of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) is one of the factors responsible for both the pathogenesis and the complications resulting from diabetes mellitus (DM). In our previous experimental study, we demonstrated the lung injury caused by streptozocin-induced diabetes through nuclear factor kappa B (NF-κB) and eNOS activity.

The use of exogenous antioxidants may represent a great therapeutic potential for the treatment of DM. Pomegranate (Punica granatum), is a rich source of potent polyphenolic, flavonoid antioxidants which include tannins and anthocyanins. In general, epidemiological studies suggest that intake of flavonoids, a group of polyphenolic compounds found in vegetables and fruits, is beneficial for prevention of cardiovascular, inflammatory and other diseases. It has been suggested that free radical scavenging and antioxidant activities play important roles in the prevention of free radical-related diseases. Meanwhile, anthocyanins, which differ structurally from other flavonoids, prevented lipid peroxidation of cell or liposome membranes. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10 and α-lipoic acid. The antioxidant level in pomegranate juice (PJ) was found to be higher than that in other natural juices, and red wine. It has also been shown that PJ can suppress NF-κB activation through a novel mechanism in vascular endothelial cells. Pomegranate juice showed potent anti-atherogenic effects in vivo. PJ consumption by diabetic patients resulted in anti-atherogenic effects with a significant reduction in oxidative stress in the patients’ serum and macrophages.

Decreases in the pulmonary function over the years, related to the decreased measures of pulmonary volumes and capacity, were evidenced in diabetic patients with impaired metabolic control. An increasing number of studies indicate physiological and structural abnormalities in the lung of diabetics. Structural alterations to the basal membrane of the pulmonary capillary endothelium are also present in DM, with a thickening of the alveolus-capillary membrane and reduction in the diffusional capability. Decreased capacity of the antioxidative defense system and increased oxidative stress were seen in the lung of diabetic animal models. Herein, we studied the potential antioxidative and preventive effect of pomegranate juice on diabetic lung injury.

**MATERIALS AND METHODS**

**POMEGRANATE PROCESSING**

Fresh pomegranates were washed, crushed, then squeezed, and treated enzymatically with pectinase to yield PJ and by-products, which included the inner and outer peels and seeds. Pectinase hydrolyses α-1.4-galacturonide bonds in pectin and thus improves extraction and filtration and prevents the formation of pectin gels. The juice was filtered, pasteurized, concentrated, and stored at -18°C, as described previously. Concentrated PJ was diluted in water (20 mL of concentrated juice in 500 mL of distilled water). The average of 2.5 mL diluted PJ contains 100 µL PJ, which is equivalent to 2.8 µmol total polyphenols per day.

**ANIMALS**

This study was approved by the Animal Care and Use Committee of Istanbul University. Twenty-eight adult male Sprague-Dawley rats (230–250 g) were acquired from the Experimental Animal Laboratory of Istanbul University, Cerrahpasa Medical Faculty vivarium sources and maintained in a 14-hour light/10-hour dark cycle with free access to food and water.

DM was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ, Sigma Chem-
ical Company, Deisenhofen, Germany) at a dose of 70 mg/kg of bodyweight. STZ was dissolved in sodium citrate buffer (0.1 M, pH 4.5) and administered in the left abdominal region of the animal about 10 minutes after dissolution in the buffer solution. The animals in the control groups received only NaCl 0.9% IP at the same volume of the buffer used to dissolve STZ. Two days after the STZ injection, blood samples were collected from the tail vein, and blood glucose levels were determined by a glucometer. Rats with blood glucose levels higher than 250 mg dl<sup>-1</sup> were accepted as diabetic.

The animals were randomized in four different groups: control (CO), control treated with pomegranate juice (CO+PJ), diabetic treated with NaCl (DM), and diabetic treated with pomegranate juice (DM+PJ). The control and diabetic rats in the PJ- treated groups (n=5, n=9, respectively) received 100 µL/day PJ via gastric gavage for a total of 10 weeks, whereas the rats in the control and diabetes group (n=5, n=9, respectively) were given 2.5 ml of saline instead. One of the diabetic rats died before the termination of the trial, so the total number of rats evaluated at the end was 27.

At the end of the 10 weeks of trial, the animals were sacrificed under anesthesia with xylazine and ketamine. Blood was sampled from the retro-orbital plexus, and the right lung was dissected out and kept in 4% formaldehyde for histological analysis. The left lung was removed and frozen at -80°C for additional analyses.

**HISTOPATHOLOGICAL EXAMINATION**

For the histopathological examination, right lung tissue samples were fixed in 10% neutral buffered formaldehyde solution for 24 h. After dehydration procedures the samples were embedded in paraffin twice. Using a microtome, the paraffin blocks were cut into sections of 4 µm. In staining phase, the slides were immersed in hematoxylin and eosin (HE), and for the evaluation of basal membrane thickening, they were also stained with periodic acid Schiff (PAS) staining. Mounted slides were examined under a light microscope (Nikon microscope ECLIPSE E600W, Tokyo, Japan) by two pathologists blinded to the study protocol.

**IMMUNOHISTOCHEMICAL EVALUATION**

Specimens were processed for light microscopy and sections were incubated at 60 °C overnight and then dewaxed in xylene for 30 min. After being soaked in a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin in 50 m M Tris buffer (pH 7.5) at 37 °C for 15 min washed with PBS. This was followed by their delineation with a Dako pen (Dako, Glostrup, Denmark) and incubation in a solution of 3% H<sub>2</sub>O<sub>2</sub> for 15 min to inhibit endogenous peroxidase activity. Then, the sections were incubated for 1 h at room temperature with endothelial nitric oxide synthase (eNOS) (eNOS rabbit Pab Neomarker, RB-1711-P) antibodies at a 1: 50 dilution. Ultra vision HRP-AEC (3-amino-9-ethyl carbazole) staining protocol was used in this stage. The colored product was developed following an incubation period of 5 min with AEC substrate kit (TA-004-HAC; Lab Vision). Having been washed in distilled water, the slides were counterstained with hematoxylin and mounted in glycerol gelatin. The staining of cytoplasmic eNOS in the interstitial and alveolar endothelial cells and epithelial cells was evaluated, and the results were expressed as the percentage of interstitial and alveolar endothelial cells and epithelial cells cytoplasmically stained positive for eNOS in 1 000 cells counted in the same section. The cases were evaluated for diffuseness and intensity of staining. According to staining diffusion, sections were graded as follows: 0=no staining; 1=less than 25% staining; 2=staining between 25 and 50 %; 3=staining between 50 and 75%; 4=more than 75% staining. According to staining intensity, sections were graded as follows: 0=no staining; 1=weak but detectable above control; 2=distinct; 3=intense staining. Immunohistochemical scores were obtained by adding diffuseness and intensity subscores.

**BIOCHEMICAL ANALYSES OF OXIDATIVE STRESS AND ANTIOXIDANT ASSAY**

All tissues were washed twice with cold saline solution and immediately stored at -80°C for the measurement of superoxide dismutase (SOD), re-
duced glutathione (GSH), protein carbonyl content (PCC) and sialic acid (SA) levels. Tissues were homogenized in a 4 volumes of ice-cold buffer containing 20 mM Tris, 10 mM EDTA (pH 7.4).

GSH level was determined by the spectrophotometric method of Elman based on the development of a yellow color with the addition of 5,5’-dithiobis-2-nitrobenzoic acid to compounds containing sulphydryl groups.20 The results are expressed as nmol/g wet tissue.

SOD activity was measured with a modified method of Sun et al. This assay involved the inhibition of nitroblue tetrazolium reduction, with xanthine oxidase used as a superoxide generator. The reaction mixture consisted of 40 ml of 0.3 mmol/l xanthine solution, 20 ml of 0.6 mmol/l EDTA solution, 12 ml of 400 mmol/l Na₂CO₃, and 6 ml of bovine serum albumin.21 The final concentration of xanthine oxidase was 167 U/l. Formazan production was measured spectrophotometrically at 560 nm.

Tissue protein levels were determined by the method of Lowry et al. PCC levels were measured according to the method based on spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine with protein carbonyl to form protein hydrazones.22

Serum sialic acid level was assayed using Warren’s thiobarbituric acid assay.23 Samples were in-

STATISTICAL ANALYSIS

The data are presented as median and interquartile range (IQR), and were analyzed through statistical software NCSS 2007. Kruskal Wallis test was used for nonparametric variables and subgroup comparisons were done with Dunn’s post test. The level of significance used was p<0.05.

RESULTS

There was no significant difference in terms of body weight between the control and experimental groups, and the two control groups revealed almost similar results in all aspects (p>0.05).

The measurement of protein carbonyl content showed that it was significantly increased in the lungs of the diabetic group compared to the controls whereas it was reduced in PJ-treated diabets group (p=0.004). Enzyme GSH activity did not show any differences between the groups (p=0.374). However, activity of antioxidant enzyme SOD was significantly reduced in the diabetic group and increased in the PJ-treated group (p=0.021). SA measurement showed that it was significantly increased in diabetes group (p=0.003), whereas PJ treatment decreased SA levels significantly (p=0.03) (Tables 1 and 2).

<table>
<thead>
<tr>
<th>TABLE 1: Comparisons of antioxidant assay (GSH, SOD, PCC and SA) between groups.</th>
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<tr>
<td>CO</td>
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<tr>
<td><strong>GSH</strong></td>
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<tr>
<td>nmol/mg protein</td>
</tr>
<tr>
<td><strong>SOD</strong></td>
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<tr>
<td>U/mg protein</td>
</tr>
<tr>
<td><strong>PCC</strong></td>
</tr>
<tr>
<td>nmol/mg protein</td>
</tr>
<tr>
<td><strong>SA</strong></td>
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<tr>
<td>µg/mg protein</td>
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KW: Kruskal Wallis, p<0.05 statistically significant
CO: control, CO+PJ: control group treated with pomegranate juice, DM: diabetic group, DM+PJ: diabetic group treated with pomegranate juice
GSH: reduced glutathione, SOD: superoxide dismutase, PCC: protein carbonyl content, SA: sialic acid
Light microscopy revealed that alveolar basal membranes were thickened in diabetic lungs. Furthermore, there were mononuclear inflammatory cell infiltration in the interstitium of STZ-induced diabetic lungs. However, these histopathological changes were less intense in the PJ treated group (Figure 1).

Figure 2 shows eNOS distribution in the lung tissue as detected through immunohistochemistry. The positive brown staining seen in the pulmonary bronchial epithelium and capillary endothelium in the DM group indicated eNOS positivity. eNOS staining was less apparent in the PJ treated group and almost absent in the CO group (Figure 2, Table 3).

**DISCUSSION**

The main finding of this study was the reduction of pulmonary lipoperoxidation in rats with streptozotocin-induced diabetes after treatment with pomegranate juice. PJ apparently diminished diabetes-caused alterations in antioxidant capacity and structural derangements in the rat lungs.

In our experimental model, some histological alterations were observed in the pulmonary tissue. These alterations are in agreement with those reported in the literature, especially as regards the increase in the conjunctive tissue and thickening of the basal lamina. After treatment with PJ, such alterations became less evident. The formation of intra-and intermolecular binding with collagen, resulting from the process of glycosylation, leads to structural alterations in tissue proteins. In our study, the main factor for the reversion of this process after treatment with PJ can be explained by the reduction of damage resulting from the oxidative stress demonstrated by the reduction of pulmonary protein oxidation and lipoperoxidation.

Reactive species can be eliminated by a number of enzymatic and nonenzymatic antioxidant mechanisms. Maritim and colleagues have recently reviewed in detail that diabetes has multiple effects on the protein levels, and activity of these enzymes further augment oxidative stress by causing a suppressed defense response. Several antioxidants are tried to restore this suppressed response. The effects of antioxidants on oxidative stress are measured through certain observable biomarkers. These markers include the enzymatic catalase, SOD, GSH, as well as thiobarbituric acid reactant levels (TBARS), an indirect measurement of free-radical production that has been shown to be consistently elevated in diabetes. Gumieniczek et al. demonstrated that pulmonary oxidative stress is present in experimental DM because of the reduction of antioxidant enzyme activity and increased lipoperoxidation where there was a decrease of Cu, Zn-SOD activity and an increase of catalase activity. In another study by Hurdag et al., SOD levels were low in the diabetic lung and raised by α-lipoic acid treatment. These findings were in agreement with our findings. We demonstrated increased SOD levels in the lungs of the PJ-treated diabetic group.

The best marker for intracellular oxidative stress-dependent cellular damage is the PC content. The unique advantage of the carbonyl measurement as a good marker of oxidative stress is that it covers a much wider range of oxidative damage than do the other markers. We found increased protein carbonyl levels in diabetic lung, and our data are in agreement with other investigators and confirm the presence of increased oxidative protein damage in DM.

SA is a fragment of some glycoproteins and glycopeptides found in the structure of hormones and enzymes and it is reported to be a marker of

<table>
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<tr>
<th>Dunn’s Multiple Comparisons</th>
<th>SOD U/mg protein</th>
<th>PCC nmol/mg protein</th>
<th>SA µg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO / DM</td>
<td>0.558</td>
<td>0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>CO / CO +PJ</td>
<td>0.602</td>
<td>0.075</td>
<td>0.917</td>
</tr>
<tr>
<td>CO / DM + PJ</td>
<td>0.071</td>
<td>0.053</td>
<td>0.039</td>
</tr>
<tr>
<td>DM / CO + PJ</td>
<td>0.305</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>DM / DM + PJ</td>
<td>0.021</td>
<td>0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>CO + PJ / DM + PJ</td>
<td>0.063</td>
<td>0.095</td>
<td>0.02</td>
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microvascular complications in diabetic patients.\textsuperscript{31} In our experimental study, SA levels were significantly increased in the diabetic lung and reduced after PJ treatment. However GSH, which is a major intracellular redox tampon system, was not changed in any of the four groups.

Unlike eNOS expressions in other organs, eNOS levels in lungs in diabetes may increase as shown in a research carried out by Sridulyakul et al., who have concluded that high, not low, flow-mediated eNOS expression is a good measure of hyperglycemic-induced endothelial dysfunction.\textsuperscript{32}

\textbf{FIGURE 1:} Histology of the lung tissue (stained by HE in a, b, c and e; and by PAS in d and f. Magnification 100x in a, c and d; and 40x in b, e and f.) \textsuperscript{[a] and (b) Control and Control+pomegranate juice (PJ), (c) and (d) Diabetes, (e) and (f) Diabetes treated with PJ.] Thickened basal membranes and intense mononuclear cell infiltration (arrows) are seen in the diabetic lung (c and d). But inflammatory reaction is less in the PJ-treated diabetic rat lung tissue (e and f). (See for colored form http://tipbilimleri.turkiyeklinikleri.com/)
Similarly, in the immunohistochemical analysis of our study, eNOS was significantly increased in the pulmonary tissue of the diabetic rats and significantly decreased when the animals were treated with PJ. Likewise, in a recently published study, lipoperoxidation, superoxide dismutase activity, and the distribution of iNOS and eNOS isoforms were evaluated in the lung tissue of diabetic rats. An increase in the oxidative stress concomitant with the increased eNOS was observed in the lung tissue of diabetic rats, which was reversed in the group treated with antioxidant α-lipoic acid.28 These findings are in agreement with those reported in our present work, such as the observed increased oxidative stress, the histological pulmonary alterations, and the effect of antioxidant (PJ) therapy in this DM model.

The present study demonstrated, for the first time, that pomegranate treatment to the diabetic rats attenuated the lung injury induced by diabetes. There are several possible explanations for this phenomenon. Extract of pomegranate fruit was shown to have lasting hypoglycemic effects in diabetic rats.33 Pomegranate consumption decreases
the high oxidative stress in diabetics.\textsuperscript{34} In pomegranate, the sugars are attached to their unique polyphenols, forming a complex with beneficial properties against diabetic complications.\textsuperscript{35} Increased serum oxidative stress in DM could be the result of glycation and glycol-oxidation of lipids by glucose.\textsuperscript{36} The antioxidative properties of pomegranate are related to the high capability to scavenge free radicals and to inhibit LDL oxidation. Furthermore it has also been shown that PJ can suppress NF-κB activation through a mechanism in vascular endothelial cells.\textsuperscript{37} The blockade of NF-κB activation by antioxidants has been suggested to be an effective strategy for prevention of late diabetic complications.\textsuperscript{1,38} In our previous study, we found that pyrrolidine dithiocarbamate, which is a well-known NF-κB inhibitor, prevented diabetic pulmonary injury.\textsuperscript{6} It has been demonstrated that shear stress-induced upregulation of eNOS is mediated by NF-κB. A study by Zhen et al. has suggested that ROS-stimulated upregulation of eNOS expression is mediated by diminished NO availability and consequent reduction in the negative feedback regulatory action of NO on eNOS expression, which is possibly mediated by NF-κB.\textsuperscript{39} In our present study, PJ treatment diminished the increased eNOS activity in diabetic lung most probably through NF-κB inactivation.

**CONCLUSION**

Therapeutic intervention with antioxidant polyphenols contained in pomegranate may promote a sustained correction of the oxidative stress related to diabetes in vitro. These findings may have important implications for the prevention of clinical sequelae of diabetes over lungs.

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