

# Protective effect of pomegranate juice on retinal oxidative stress in streptozotocin-induced diabetic rats

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## Abstract

• **AIM:** To investigate the effect of pomegranate juice (PJ) intake on overall oxidation status in retinas of diabetic rats.

• **METHODS:** Twenty-seven rats were divided into four groups as control (CO), diabetic (DM), control treated with PJ (CO-PJ), and diabetic treated with PJ (DM-PJ). The retina tissues were used to determine 8-hydroxy-2'-deoxyguanosine (8OHdG), malondialdehyde (MDA), reduced glutathione (GSH) levels, and the enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

• **RESULTS:** The levels of 8OHdG and MDA were significantly increased in the retina of DM group compared to CO group ( $P=0.001$ ,  $P<0.001$  respectively). Both 8OHdG and MDA levels were decreased in PJ-DM group compared to DM group ( $P=0.004$ ,  $P<0.001$  respectively). The activities of antioxidant enzymes GSH, SOD, and GDH-Px were significantly decreased in the retina of DM group compared to CO group ( $P\leq 0.01$ ). GSH and GSH-Px activities were higher in PJ-DM group compared with DM group ( $P=0.010$ ,  $P=0.042$ , respectively) but SOD activity was not statistically different ( $P=0.938$ ).

• **CONCLUSION:** PJ intake is found to be effective in decreasing oxidative end products, and in increasing the activities of antioxidant enzymes in diabetic retinas of rats, which suggests it may be effective against oxidative stress in diabetic retinas.

• **KEYWORDS:** antioxidant enzymes; diabetic retinopathy; immunohistochemical staining; oxidative stress; pomegranate juice

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## INTRODUCTION

Diabetes mellitus (DM) causes alterations in antioxidant activities, and there is emerging interest in oxidative stress causing a complex dysregulation of cell metabolism in the pathogenesis of diabetic vascular complications. Increased oxidative stress and decreased capacity of the antioxidative defense system have been demonstrated in the retina of diabetic animals<sup>[1-2]</sup>. Likewise, inflammatory responses in retinas of diabetic rodents have been shown by microarray analyses<sup>[3]</sup>. Additionally, it has been demonstrated that DM has led activation of transcriptional nuclear factor-kappa-B (NF- $\kappa$ B) in rodent retinas<sup>[4-5]</sup> and also caused the migration of the P65 subunit into the nuclei of retinal endothelial cells, pericytes, ganglion cells, or cells of the inner nuclear layer in rodent retina<sup>[6-8]</sup>. Besides, there is clear evidence that DM causes an increase of nitric oxide (NO), formed by the activity of endothelial nitric oxide synthase (eNOS), and it is one of the factors responsible for the pathogenesis of diabetic complications<sup>[9]</sup>. Laboratory measurement of NO is extremely difficult, hence other tissue indicators 8-hydroxy-2'-deoxyguanosine (8OHdG), malondialdehyde (MDA) for determining the cellular oxidant status have been studied biochemically. A deterioration in antioxidant enzyme activity [glutathione (GSH) redox system, superoxide dismutases (SOD), glutathione peroxidase (GSH-Px)] increases the susceptibility to oxidative stress; therefore, measurement of these enzymes have also been evaluated in the recent literature<sup>[10]</sup>. Accordingly, exogenous antioxidants have been used to decrease oxidative stress-dependent cellular alterations related with diabetes. Previous studies have demonstrated that certain fruits and vegetables having high levels of phenolic compounds are ideal sources of natural antioxidants<sup>[11-13]</sup>. Consumption of pomegranate juice (PJ) with a high content of

polyphenols decreases oxidative stress by stimulating the catalytic enzyme activities<sup>[11-15]</sup>. Pomegranate, in particular, condensed tannins and anthocyanins, are potent antioxidants which suppress NF- $\kappa$ B activating pro-inflammatory cytokines such as TNF, IL-1 and IL-12, and oxidative enzymes. Apart from its antioxidant capacity, anti-proliferative, anti-atherosclerotic and anti-inflammatory effect of PJ have been proposed<sup>[16-20]</sup>. The present experimental study was designed to evaluate the potential antioxidant effect of PJ in retinas of diabetic rats.

## MATERIALS AND METHODS

**Pomegranate Processing** Fresh pomegranates were washed, crushed, and then squeezed. It was treated enzymatically with pectinase to produce PJ and by-products. For improvement of extraction and filtration,  $\alpha$ -1,4-galacturonide bonds were hydrolysed by pectinase. The juice was filtered, pasteurized, concentrated, and stored at  $-18^{\circ}\text{C}$ <sup>[21-23]</sup>. Then dilution of 20 mL of concentrated juice in 500 mL of distilled water was acquired. The average of 2.5 mL diluted PJ includes 100  $\mu\text{L}$  PJ, which is equivalent to 2.8  $\mu\text{mol}$  total polyphenols per day.

**Study Design** This study was approved by the Scientific and Ethics Committee of animal care of Istanbul University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Twenty-eight adult Sprague-Dawley rats (230-250 g) were housed in cages under standard laboratory conditions with dark and light cycle. They were permitted free access to standard diet and water *ad libitum*. A single dose of intraperitoneally (i.p.) streptozotocin (STZ) (70 mg/kg STZ; Sigma, Deisenhofen, Germany) dissolved in 0.1 mol/L sodium citrate buffer with pH 4.5 was injected intraperitoneally<sup>[24]</sup>. NaCl 0.9% was injected i.p. only in the control group. Glucose levels were measured by glucometer, in blood samples obtained from the tail vein, 48h after the STZ injection. Blood glucose levels higher than 250 mg/dL were accepted as diabetic.

The animals were divided into four groups randomly: 1) control (CO); 2) control rats treated with pomegranate juice (PJ-CO); 3) diabetic rats (DM); 4) diabetic rats treated with pomegranate juice (PJ-DM). PJ-treated groups [PJ-CO ( $n=5$ ), PJ-DM ( $n=9$ ), respectively] were given 100  $\mu\text{L}/\text{day}$  PJ *via* gastric gavage for a total of ten weeks, whereas the control and diabetes groups ( $n=5$ ,  $n=9$ , respectively) received only 2.5 mL of saline. The total number of rats evaluated at the end was 27, because one of the diabetic rats died during the study.

The animals were anaesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg) and sacrificed by cervical dislocation after 10wk. The globes were enucleated, then the retinas were detached and used for DNA extraction or colorimetric analysis; these tissues were stored at  $-80^{\circ}\text{C}$  until the biochemical analyses. The other globes were immersed in 10% formaldehyde for histological analysis.

**Biochemical Analyses** The retina tissue was evaluated for oxidative DNA damage, lipid peroxidation products and antioxidant enzymes. 8OHdG in retina tissue was determined using NWLSS 8OHdG ELISA Kit, a competitive enzyme-linked immunosorbent assay purchased from Northwest (Vancouver, WA, Canada) following the manufacturer's instructions. It was necessary to extract and digest sample DNA prior to assay. Retina DNA was extracted using DNasy Blood and Tissue Kit (Qiagen), Spin-Column Protocol. This protocol is designed for purification of total DNA from animal tissues. Samples were assayed in the same day of enzymatic digestion. 8OHdG levels were expressed as 8OHdG pg/mg DNA.

All tissues were weighed and homogenized with 0.15M KCl solution and, 10% homogenates (w/v) of these tissues were prepared. First, tissue homogenates were centrifuged for 10min at  $4^{\circ}\text{C}$  cold centrifuge 600 g. Then, it was centrifuged for 20min at 10 000 g and post-mitochondrial fraction was obtained. MDA and GSH levels were determined in tissue homogenates; SOD, GSH-Px activities were examined in the post-mitochondrial fraction of these homogenates. The bicinchoninic acid method was used for determination of the amount of protein in samples<sup>[25]</sup>.

MDA, as an endpoint of lipid peroxidation, was calculated by detecting the absorbance of thiobarbituric acid reactive substances at 532 nm<sup>[26]</sup>. MDA levels were expressed as MDA nmol/mg protein. GSH levels were determined by using Elman reagent (5,5'-dithiobis-2-nitro benzoic acid)<sup>[27]</sup>, and the results were expressed as GSH nmol/mg protein. SOD activity was measured by principle of increasing the ability of photo-oxidation rate in o-dianisidin sensitized with riboflavin<sup>[28]</sup>. Colored product was measured spectrophotometrically at 460 nm, the results were expressed as U/mg protein, as specified. GSH-Px activity was measured according to the protocol of Lawrence *et al*<sup>[29]</sup>. The results were calculated using NADPH extinction coefficient and were expressed as nmol NADPH /mg protein /min.

**Immunohistochemical Evaluation** Eyesballs of sacrificed rats were fixed in 10% buffered formaldehyde for 12h. Dehydration, clearing, paraffin infiltration and embedding were alternated to all tissue samples. The paraffin blocks were cut into 3 microns sections by a microtome. The slides were stained with hematoxylin and eosin and examined under light microscopy. Briefly, sections were deparaffinized in xylene ( $2\times 5\text{min}$ ) and rehydrated with successive 1min washes in 100%, 96%, 80%, and 70% ethanol. They were then stained with hematoxylin (2min), rinsed with distilled water, rinsed with 0.1% hydrochloric acid in 50% ethanol, rinsed with tap water for 15min, stained with eosin for 1min, and rinsed again with distilled water. The slides were then dehydrated with 95%

**Table 1 The levels of 8OHdG, MDA, and activities of antioxidant enzymes GSH, SOD and GSH-Px of the four groups** Mean±SD

Groups	8 OH dG pg/μg DNA	MDA nmol/mg protein	GSH nmol/mg protein	SOD U/mg protein	GSH-Px nmol NADPH/mg protein/min
CO	1.32±0.20	0.67±0.14	25.01±4.99	29.53±4.63	216.92±32.87
PJ-CO	1.31±0.11	0.70±0.11	26.12±3.85	33.47±4.58	228.88±31.87
DM	4.11±1.19	1.28±0.11	16.15±2.63	20.58±5.34	148.09±26.61
PJ-DM	1.98±0.18	0.92±0.09	21.58±1.65	21.87±3.54	187.95±26.62

CO: Control group; PJ-CO: Pomegranate juice treated control group; DM: Diabetes mellitus group; PJ-DM: Pomegranate juice treated DM group.

and 100% ethanol successively followed by xylene (2×5min) and mounted with coverslips<sup>[30]</sup>.

Immunohistochemical study was performed with NOS2/ iNOS polyclonal antibody (Mybiosource) after antigen retrieval of paraffin-embedded tissue sections. Citrate buffered, pH 6.0 Avidin-Biotin-Peroxidase Complex (ABP) method and aminoethyl carbazole (AEC) chromogen were applied for immunohistochemical staining. The microscopic examination were performed by a single pathologist blinded to the study.

The staining of cytoplasmic eNOS (a surrogate for nitric oxide) and P65 (showing the activity of NF-κB) in the sensorial retina and choroid was evaluated, and the results were expressed as the percentage of retina and choroid cytoplasmically stained positive for eNOS and P65 in 1000 cells counted in the same section. The cases were evaluated for diffuseness and intensity of staining. According to staining diffuseness, 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<sup>[31]</sup>.

**Statistical Analysis** Analyses were performed using IBM SPSS Statistics v.19. Biochemical parameters were compared by oneway-ANOVA and multiple comparisons were done with Tukey's Post-hoc tests. The results of the histopathological and immunohistochemical evaluation of the groups were carried out by Pearson  $\chi^2$  test.  $P < 0.05$  was accepted as statistically significant.

**RESULTS**

There was no significant difference in terms of body weight among the control and experimental groups ( $P=0.08$ ). The levels of 8OHdG and MDA were significantly increased in the retina of the DM group compared to the CO group ( $P=0.001$ ,  $P < 0.001$  respectively). Both 8OHdG and MDA levels were decreased in PJ-DM group compared to DM group ( $P=0.004$ ,  $P < 0.001$  respectively). However, the levels of 8OHdG and MDA in PJ-DM group were still higher than the CO group ( $P=0.002$ ,  $P=0.003$  respectively) (Tables 1, 2).

The activities of antioxidant enzymes of SOD and GDH-Px were significantly decreased in the retina of the DM group

**Table 2 Comparison of 8OHdG, MDA, GSH, SOD and GSH-Px between groups**  $P^1$

Groups	8OHdG	MDA	GSH	SOD	GSH-Px
CO/PJ-CO	1.000	0.976	0.999	0.539	0.912
CO/DM	0.001	<0.001	0.075	0.010	0.001
CO/PJ-DM	0.002	0.003	0.744	0.036	0.314
DM/PJ-CO	0.001	<0.001	0.011	<0.001	<0.001
DM/PJ-DM	0.004	<0.001	0.001	0.938	0.042
PJ-CO/PJ-DM	<0.001	0.010	0.278	0.001	0.087

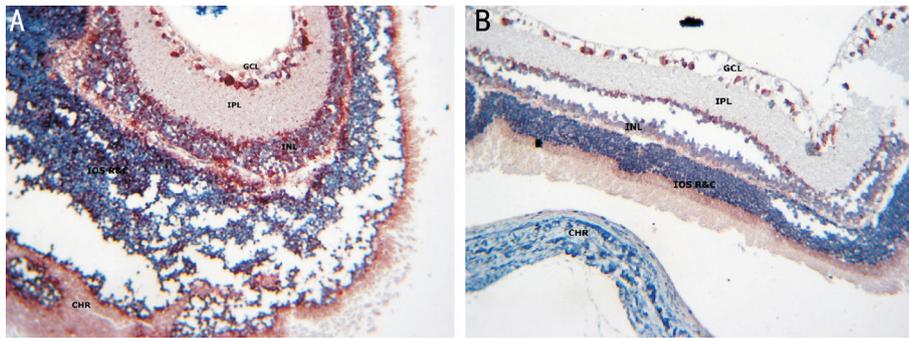
CO: Control group; PJ-CO: Pomegranate juice treated control group; DM: Diabetes mellitus group; PJ-DM: Pomegranate juice treated DM group. <sup>1</sup>Oneway-ANOVA, multiple comparisons were done with Post-hoc tests.



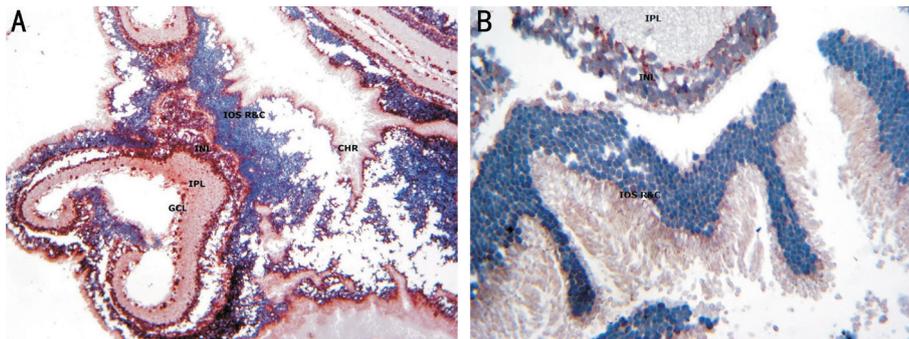
**Figure 1 Normal control retina tissue.**

compared to the CO group ( $P \leq 0.01$ ; Tables 1, 2). The activity of antioxidant enzyme of GSH was also decreased, albeit this difference was not statistically significant ( $P=0.075$ ; Tables 1, 2). The activities of all three antioxidant enzymes increased with PJ intake both in the control, and the DM groups (Table 1). This increase was not statistically significant in the control group ( $P > 0.05$ ; Table 2). In the DM group, there was a statistically significant increase in the activity of GSH-Px ( $P=0.042$ ; Table 2); whereas the increase in the activity levels of GSH, and SOD was not statistically significant ( $P=1$ , and  $P=0.938$ , respectively; Table 2). However, in PJ-DM group, the activities of both GSH and GSH-Px reached similar levels compared to the control group ( $P=0.744$ ,  $P=0.314$ , respectively; Table 2), whereas the activity of SOD was still lower compared to the CO group ( $P=0.036$ ; Table 2).

Immunohistochemical staining of the retina and the choroid was evaluated (Figure 1), and it was found that the staining



**Figure 2 P65 levels** Intense staining by P65 immunohistochemistry of the diabetic retina tissue (A) and reduction of staining by P65 (B) immunohistochemistry of the diabetic retina tissue with pomegranate juice treatment. Magnification 100×, GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; IOS R&C: Inner and outer segments of rod sand cones; CHR: Choroid.



**Figure 3 eNos levels** Intense staining by eNOS immunohistochemistry of the diabetic retina tissue (magnification 40×) (A); Reduction of staining eNOS (B) immunohistochemistry of the diabetic retina tissue with pomegranate juice treatment (PJ-DM group, magnification 100×, GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; IOS R&C: Inner and outer segments of rods and cones; CHR: Choroid).

**Table 3 Immunohistochemical staining scores**

Staining scores <sup>1</sup> eNOS/P65	0	1	2	3	4	5	6	7
CO	7/7	1/1						
DM					1/-	2/3	5/5	2/2
PJ-DM	3/3	3/4	4/2	-/1				

<sup>1</sup>Immunohistochemical staining scores were obtained by adding diffuseness and intensity subscores.

of eNOS and P65 was more intense in diabetic retinas than in those of the PJ-DM groups ( $P < 0.001$ ; Figures 2, 3). The intensity of staining of DM group diminished with PJ-treatment and approximated to the CO group (Table 3; Figures 2, 3).

## DISCUSSION

The present study represented that lipid peroxidation measured as MDA and DNA oxidative damage evaluated by estimation of 8OHdG were significantly increased in the retinas of diabetic rats compared to CO group. Oxidative damage of cellular components mainly lipids, proteins and nucleic acids results in metabolic disturbances. In the literature, 8OHdG has been used as a key indicator of DNA oxidative damage and MDA as an indicator of lipid peroxidation<sup>[32-34]</sup>. There are a few reports showing the protective effect of PJ on DNA damage in the literature as well. In one of these, Faria *et al*<sup>[35]</sup> have demonstrated lower levels of pg 8OHdG/ $\mu\text{g}$  indicating

oxidative damage of DNA in the liver of PJ treated mice. Similarly, we also found a reduction in the level of 8OHdG in retinas of PJ treated diabetic rats in our study.

Phenolic compounds of PJ are well known in inhibiting lipid oxidation and scavenging free radicals *in vitro*<sup>[36-37]</sup>. In this study, lower level of MDA was detected as an indicator of lipid peroxidation in PJ-DM group compared to DM group, consistent with the results of 8OHdG. Sohrab *et al*<sup>[38]</sup> recently reported in their randomized double-blind clinical trial that after 12wk of PC intake MDA decreased, total antioxidant capacity increased with a decrease in lipid peroxidation in adults with type 2 diabetes, concluding that PJ consumption may delay onset of diabetic complications related to oxidative stress. Besides, Shema-Didi *et al*<sup>[37]</sup> have found significantly lower levels of MDA in hemodialysis patients with one year of PJ intake in a randomized placebo-controlled trial. As a secondary endpoint, they concluded that prolonged PJ

intake improves nontraditional cardiovascular risk factors, attenuates the progression of the atherosclerotic process, strengthens the innate immunity, and thus reduces morbidity among hemodialysis patients<sup>[37]</sup>. Yilmaz *et al*<sup>[39]</sup> reported that pomegranate extract decreased MDA, increased total antioxidant capacity, and decreased total oxidative stress in liver tissue, and remote organs of rats with experimental jaundice. In our experimental study we also found that there was a significant decrease in 8OHdG and MDA levels in PJ-DM group compared to DM group. Although the decreased levels of both of these parameters in PJ-DM group were still higher than the average level in control group, PJ intake decreased the oxidative stress biomarkers in diabetic rat retina tissues.

GSH and GSH-Px play important roles in preventing cellular damage caused by reactive oxygen species (ROS) such as free radicals and peroxides. SOD is specific for catalytic removal of superoxide and converts superoxide to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Correspondingly, Guo *et al*<sup>[32]</sup> have obtained an increase in GSH-Px levels and a decrease in SOD levels at final measurement in elderly subjects who were treated with PJ. In another experimental study by Çukurova *et al*<sup>[22]</sup>, SOD levels were low in the diabetic lung and improved by PJ treatment. In a recent randomized, controlled study in patients with rheumatoid arthritis, pomegranate extract has shown to alleviate disease activity, and erythrocyte sedimentation rate as a biomarker of inflammation, and to increase GSH-Px concentration<sup>[40]</sup>. Ekhlas *et al*<sup>[41]</sup> also reported that total antioxidant capacity increased in patients with non-alcoholic fatty liver disease who received PJ for 12wk in a randomized clinical trial. Consistent with these reports, we also noticed that GSH, SOD and, GSH-Px levels increased with PJ treatment both in the control and the diabetic groups, although this difference was statistically significant only with GSH-Px levels in diabetic rats. Moreover, the activities of both GSH and GSH-Px in PJ treated diabetic group increased to similar levels of the control group. These findings are compatible with the findings in the literature. Thus, recently, six weeks supplementation of PJ has been reported to increase total antioxidant capacity in patients with type 2 diabetes in a single-blind, randomized clinical trial<sup>[42]</sup>. Likewise, Shishehbor *et al*<sup>[43]</sup> found similar results with 4wk of concentrated PJ intake.

In literature, both NF-κB and eNOS are considered to play an important role in the pathogenesis of diabetic complications. Infact, these are two different pathways and the relationship between these pathways remains poorly understood. While some studies show that eNOS leads to the activation of NF-κB pathways<sup>[44-46]</sup>, conversely in others eNOS inhibits NF-κB activation<sup>[47]</sup>. Besides, it has been demonstrated that NF-κB responsive miRNAs plays an important role in eNOS

expression under inflammatory conditions<sup>[48-50]</sup>. For this reason, inhibition of NF-κB has been suggested as a new therapeutic intervention to improve endothelial dysfunction, by functional restoration of the eNOS/NO/cGMP pathway.

Inhibition of NF-κB has been suggested as a new therapeutic intervention to improve endothelial dysfunction underlying diabetic complications. In an experimental study, Eren *et al*<sup>[31]</sup> established the lung injury caused by NF-κB and eNOS activity in diabetic rats. Schubert *et al*<sup>[51]</sup> have shown that natural antioxidants like pomegranate wine can suppress NF-κB activation through a novel mechanism in vascular endothelial cells. Likewise, in our study staining of eNOS and P65 was found to be less intense in PJ treated diabetic retinas than those of the diabetes groups. Also, phosphorylated eNOS can be checked to ascertain decreased activity of eNOS in diabetic retina upon PJ treatment. Phosphorylated eNOS could not be studied in our study. However benefit of this decreased oxidative stress on the function of diabetic retinas still yet to be elucidated. Electrophysiological testing may be helpful in detecting these differences in diabetic, PJ treated, and control groups with further studies.

To the best of our knowledge, this is the first report about the evaluating the potential protective effect of PJ on retinal oxidative stress in diabetic rats. As a conclusion, in this study, assessment of biochemical oxidative markers revealed that prolonged PJ intake was found to decrease the oxidative stress *via* inhibition of lipid peroxidation and DNA oxidation. In addition, consistent with these results, immunohistochemical evaluation of the retina demonstrated less oxidative activity in PJ-DM group compared to that of the diabetic group. Further comprehensive studies are warranted to verify these experimental results.

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