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Objectives: We investigated whether pomegranate extract plays a protective antioxidant role against mesenteric ischemia–reperfusion injury (IR), which can lead to a systemic response and damage distant organs, such as the lung, liver, and kidney.

Methods: Forty female Wistar–Albino rats were separated into four groups: laparotomy, laparotomy + PG, mesenteric IR, and mesenteric IR and pomegranate (IR + PG). In the laparotomy + PG and IR + PG groups, pomegranate (225 mg/kg) was given by oral gavage at the beginning of the study. Ischemia was induced for 30 minutes, and reperfusion was subsequently allowed for 60 minutes in the IR and IR + PG groups. The malondialdehyde (MDA) and total antioxidant activity (AOA) levels were evaluated in blood samples. Additionally, all tissues were removed for the measurement of AOA and total oxidant status as well as for subsequent histopathological evaluation. The oxidative stress index was calculated.

Results: Histopathological changes in all organs were significantly higher in the IR group and significantly lower in the IR + PG group vs. the other groups. Serum MDA levels were significantly lower in the IR + PG group than in the IR group. No significant difference was found in AOA levels of the groups.

Discussion: These data may explain the positive protective effects of pomegranate based on the histopathologic findings in ischemic conditions in an intestinal IR injury model.

Keywords: Pomegranate, Intestinal ischemia, Reperfusion, Malondialdehyde, Total antioxidant activity, Total oxidant status

Introduction

Ischemia-associated diseases are among the most important causes of morbidity and mortality. Acute intestinal ischemic injury is caused by a decrease in blood flow (due to, e.g., intussusception, incarcerated hernia, necrotizing enterocolitis, transplantation, shock, and sepsis), leading to tissue damage. This tissue damage triggers mechanisms of cellular damage, including microvascular permeability, interstitial edema, damaged vasoregulation, and inflammatory cell infiltration.[1] Despite the known primary cellular damage, the pathophysiology of intestinal ischemic injury cannot be described without mentioning the mechanisms involved in reperfusion injury. Some reperfusion mechanisms are uncertain, while others are now well documented. For example,

oxidative stress mediators, such as reactive oxygen species (ROS), polymorphonuclear neutrophil activation, and nitric oxide production, are known to be major determinants of reperfusion injury. Many cellular models of the mechanisms of cellular dysfunction in ischemia–reperfusion (IR) injury are available.[2–4]

Defense against oxidative stress is mediated by antioxidant enzymes (e.g. superoxide dismutase, catalase, and glutathione peroxidase), which process and remove free radicals and other reactive products. Other mechanisms are non-enzymatic and include many natural or synthetic antioxidants (e.g. glutathione and alpha-tocopherol) that prevent oxidative damage by reacting with free radicals. A diet containing low-molecular-weight antioxidants benefits antioxidant defense.[5,6]

The rich phenolic contents of pomegranate include anthocyanins and hydrolyzable tannins.[7,8] Maternal dietary supplementation with pomegranate juice is

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neuroprotective against hypoxic–ischemic injury of the neonatal mouse brain.[9] Pomegranate juice also has protective effects against systemic oxidative stress in mice.[10] Investigations have shown that pomegranate extract has gastroprotective activity through antioxidant mechanisms.[11] Using pomegranate extract as a dietary supplement has been shown to ameliorate colonic inflammation. A review of these studies indicates that pomegranate exerts beneficial effects on oxidative stress and inflammation.[12]

Mesenteric IR injury and its effects on distant organs are closely linked to oxidative stress and inflammation. Thus, we sought to investigate the potential protective antioxidant effects of the pomegranate (*Punica granatum*) in a mesenteric IR model.

Materials and methods

Animal subjects

In this study, 40 female Wistar–Albino rats, weighing 200–250g, were obtained from the BLIND University School of Medicine Animal Care. Simple randomization method was used for selection of the animals in the groups. The Local Ethics Committee approved the study. All experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals.

Rats were housed in an air-conditioned room with a 12/12-hour light/dark cycle and a constant temperature ($22 \pm 2°C$). The rats were housed in cages and provided standard rat chow and water *ad libitum* before the experiments. The animals were fasted overnight before the experiments but were given free access to water. Rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar, Parke Davis, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer AG, Leverkusen, Germany) intramuscularly prior to the surgical procedures.

Study protocol

After the abdomen was shaved and disinfected, a midline incision was made and the rats underwent either laparotomy surgery or IR. Ischemia was induced by clamping the superior mesenteric artery and collateral vessels with an atraumatic vascular clamp at its origin for 30 minutes. Mesenteric ischemia was confirmed by the loss of mesenteric pulsations and the blanching of the intestine. During the surgical procedures, the peritoneal cavity was filled with warm Ringer's lactate to prevent hypovolemia and hypothermia during the ischemic episode, the abdominal incision was closed temporarily. Then, 30 minutes later, the ischemic intestine was reperfused by removing the clamp for 60 minutes. Reperfusion was confirmed by the restoration of pulsation and color prior to closing the incision. At the end of this

period, the animals were sacrificed by removing blood from the heart.

The animals were divided into four groups. In Group 1 (laparotomy), only laparotomy was performed. In Group 2 (laparotomy + PG), laparotomy was performed, and the animals were given 225 mg/kg pomegranate (transport maximum (T_{max}): 30 minutes; Pomella, Verdure Sciences, Noblesville, IN, USA)[13] by oral gavage at the beginning of the experiment. In Group 3 (IR), the superior mesenteric artery and collateral vessels were clamped. After 30 minutes, reperfusion was allowed for 60 minutes. In Group 4 (IR + PG), the superior mesenteric artery and collateral vessels were clamped. After 30 minutes, reperfusion was allowed for 60 minutes, and the rats were given 225 mg/kg pomegranate (T_{max}: 30 minutes; Pomella) by oral gavage at the beginning of the experiment.

At the end of the procedures, blood and tissue samples from the intestine, liver, lung, and kidney were obtained for biochemical and histopathological analysis. The intestine, liver, kidney, and lung tissues were divided for histopathological examination by removing adequate sample amounts and transferring the excess tissue to plastic clam-shell containers containing 10% formaldehyde solution for either further histological evaluation or storage at 80°C for the subsequent determination of the antioxidant activity (AOA), total oxidant status (TOA), and oxidative stress index (OSI) levels.

Biochemical analyses

Blood sampling

Venous blood samples were collected in tubes with a gel separator and centrifuged for 5 minutes at 1550g. The supernatant plasma was removed and placed in polypropylene plastic tubes. The tubes were properly labeled with the appropriate sample name and type. Samples were taken and stored at −80°C for the determination of the TOA, AOA, and malondialdehyde (MDA).

Tissue homogenization

To estimate the tissue oxidant and antioxidant levels, tissue samples were prepared at 4°C. The tissues were weighed, cut into small pieces, and homogenized in 10 volumes of ice-cold phosphate buffer (50 mM, pH 7.0) using a glass Teflon homogenizer (Ultra-Turrax T8, Staufen, Germany). The resulting homogenate was centrifuged (26 000g, 10 minutes, 4°C), and the supernatant was used immediately for the determination of the TOA and AOA.

Measurement of MDA

The MDA levels were estimated using the double-heating method of Draper *et al.*[14] This method is based on spectrophotometric measurements of the color generated by the reaction of thiobarbituric acid

(TBA) and MDA. For this purpose, 2.5 ml of trichloroacetic acid solution (10%) was added to 0.5 ml plasma in a centrifuge tube, and the tubes were placed in boiling water for 15 minutes. After cooling in tap water, the tubes were centrifuged (1000g, 10 minutes), and 2 ml of the supernatant was added to 1 ml of the TBA solution (6.7 g/l) in a test tube. The tube was then placed in boiling water for 15 minutes. The solution was cooled in tap water, and its absorbance at 532 nm was measured using a spectrophotometer (Shimadzu UV-1208, Japan). The MDA concentration was calculated from the absorbance coefficient of the MDA–TBA complex (an absorbance coefficient of 1.56×10^5/(cm M)) and expressed as μmol/l. All MDA analytical steps were performed in the Biochemistry Department Laboratory (BLIND University Medical Faculty).

Measurement of TOA

The TOA of the supernatant fractions was determined using a novel automated measurement method, developed by Erel.[15] Oxidants present in the sample oxidize the ferrous iron–*o*-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules, which are abundant in the reaction medium. The ferric ion produces a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide, and the results are expressed in the nmol H_2O_2 equiv./mg protein.

Measurement of AOA

The AOA of the supernatant fractions was determined using a novel automated measurement method developed by Erel.[16] In this method, the hydroxyl radical, the most potent biological radical, can be produced. In the assay, the ferrous ion solution present in Reagent 1 is mixed with the hydrogen peroxide in Reagent 2. The sequentially produced radicals, such as the brown-colored dianisidinyl radical cation produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidative effects of the sample against free radical reactions initiated by the produced hydroxyl radical can be measured. The assay has an excellent precision of less than 3%. The results are expressed as nmol Trolox equiv./mg protein.

Oxidative stress index

The OSI is the ratio of total peroxide to total antioxidant potential, an indicator of the degree of oxidative
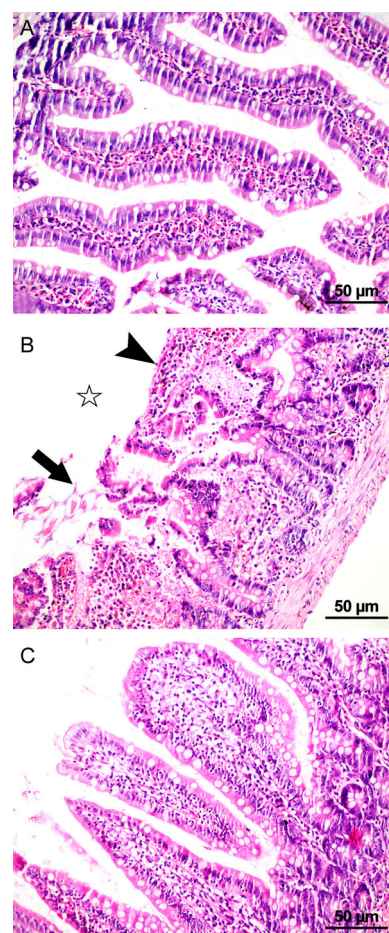
Figure 1 Microscopic images of the jejunum showing the histopathological samples of laparotomy (A), IR (B), and IR + PG (C) groups (H&E, 200×). (A) Mucosa with normal villous formation. (B) Disintegration of the villous (star), capillary dilatation, ulceration of lamina propria (arrow), capillary dilatation, infiltration, and hemorrhage (arrowhead) after IR injury. (C) Restitution of jejunal mucosa in the pomegranate-applied group.

stress. It was calculated as follows:[17]

$$OSI = \frac{TOA(\text{nmol } H_2O_2 \text{ equiv./mg})}{AOA(\text{nmol Trolox equiv./mg})}$$

Histopathological evaluation

Intestine, liver, kidney, and lung tissues were scored from mild to severe injury. Tissue specimens were fixed in 10% formalin for 24 hours, then embedded in paraffin wax, and cut into 4-μm sections. Slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope using a standard protocol. Each slide was evaluated by a pathologist who was blinded to the grouping and treatment of the specimen under analysis.

Intestinal injury was evaluated on a scale from 0 to 5 points. The tissue was assessed as having no diagnostic change, increased lamina propria cellularity, congestion and expansion of the subepithelial capillary, capillary dilatation, villus fragmentation, disintegration of

the lamina propria with ulceration, and hemorrhage[18] (Fig. 1).

Hepatic injury was evaluated according to its severity using the following ordinal scale: grade 0, minimal or no evidence of injury; grade 1, mild injury with cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate-to-severe injury with enlarged nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (Fig. 2).

Renal injury was graded as follows: grade 0, no visible change; grade 1, tubular cell swelling, brush border loss, and nuclear condensation with up to one-third of the tubular profile showing nuclear loss; grade 2, the same as grade 1 but with greater than one-third and less than two-thirds of the tubular profile showing nuclear loss; and grade 3, with greater than two-thirds of the tubular profile showing nuclear loss.
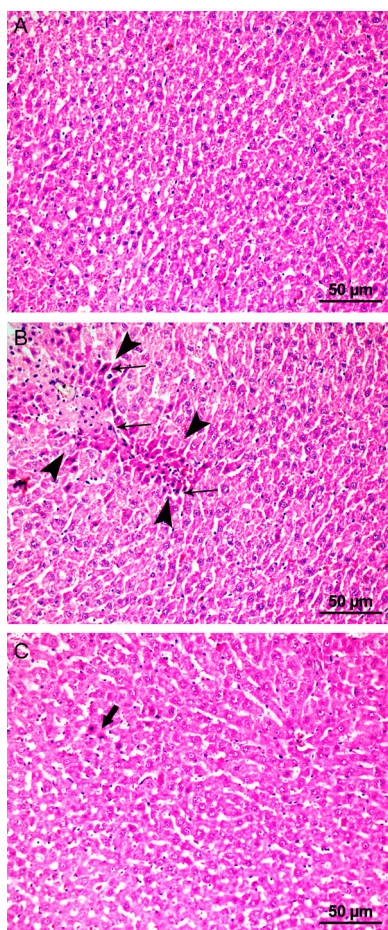
Figure 2 Microscopic images of the liver showing the histopathological samples of laparotomy (A), IR (B), and IR + PG (C) groups (H&E, 200×). (A) Normal hepatocyte formation. (B) Degenerative changes (area marked with arrowheads); necrosis and picnotic nucleus (arrows). (C) Hepatocytes in pomegranate applied group with few picnotic changes (arrows).

Lung injury was evaluated using the following ordinal scale: grade 0, no change; grade 1, mild neutrophil leukocyte infiltration and mild-to-moderate interstitial congestion; grade 2, moderate neutrophil leukocyte infiltration, perivascular edema formation, and disintegration of structure; and grade 3, dense neutrophil leukocyte infiltration and the destruction of pulmonary structures.[19]

Statistical analysis

SPSS software (ver. 11.5 for Windows; SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses. All values are given as the means ± standard error of mean (SEM). Differences between the groups were evaluated by Kruskal–Wallis variance analysis followed by a Mann–Whitney U test with a Bonferroni correction for binary comparisons. Spearman's test was used to determine the correlations between the groups. P-values <0.05 were considered to indicate statistical significance.

Results

The oxidative and antioxidative parameters of all groups are given in Table 1. The levels of MDA, a marker of oxidative stress, were significantly higher in the IR group than in the laparotomy group ($P = 0.003$). There were no significant differences between the MDA levels in the IR + PG and laparotomy groups. MDA levels were significantly higher in the IR group compared with the IR + PG group. AOA levels in the IR and IR + PG groups were significantly higher than in the laparotomy group ($P = 0.005$, $P = 0.019$, respectively). There was no significant difference in AOA levels between the IR and IR + PG groups.

No significant difference was found between the IR and IR + PG groups in terms of the AOA and TOA values in kidney and lung tissue. However, liver AOA values were significantly higher in the IR + PG group than in the IR group.

TOA levels did not differ significantly between the IR and IR + PG groups. Liver, lung, and kidney OSI levels were significantly higher in the IR group than in the IR + PG group. Furthermore, the lung and liver OSI levels were significantly higher in the laparotomy group than in the laparotomy + PG group (Fig. 3).

Histopathological scores in all organs were significantly higher in the IR group and significantly lower in the IR + PG group vs. the other groups (Fig. 4). The intestinal histopathological scores were well correlated with MDA levels ($P < 0.001$, $r = 0.566$), kidney histopathological score ($P < 0.001$, $r = 0.789$), liver histopathological score ($P < 0.001$, $r = 0.659$), and lung histopathological score ($P < 0.001$, $r = 0.749$).

Table 1 Oxidative and antioxidative parameters of all groups

	Sham (1)	Control (2)	IR (3)	IR + PG (4)	*P* values	Bonferroni
AOA (µmol Trolox equiv./l)	2.14 ± 0.32	2.35 ± 0.40	2.0 ± 0.54	2.67 ± 0.56	0.010	1 vs. 3,4
MDA (µmol/l)	0.51 ± 0.06	0.44 ± 0.10	0.77 ± 0.22	0.61 ± 0.15	0.001	1 vs. 3 and 3 vs. 4
Kidney-AOA (nmol Trolox equiv./mg)	2.15 ± 0.27	2.24 ± 0.11	2.29 ± 0.19	2.33 ± 0.11	NS	NS
Kidney-TOA (nmol H_2O_2 equiv./mg)	124.44 ± 11.03	119.32 ± 14.37	149.38 ± 30.56	132.63 ± 14.07	0.033	NS
Kidney-OSI	58.42 ± 5.49	53.13 ± 5.56	64.85 ± 9.40	57.16 ± 7.43	0.018	1 vs. 3 and 3 vs. 4
Liver-AOA (nmol Trolox equiv./mg)	3.46 ± 0.22	3.70 ± 0.29	4.06 ± 0.17	4.39 ± 0.32	<0.001	1 vs. 3,4 and 3 vs. 4
Liver-TOA (nmol H_2O_2 equiv./mg)	81.62 ± 11.83	72.36 ± 13.19	84.03 ± 7.56	78.06 ± 10.45	NS	NS
Liver-OSI	23.58 ± 3.12	19.82 ± 4.71	20.71 ± 2.04	17.96 ± 3.43	0.01	1 vs. 2,3,4 and 3 vs. 4
Lung-AOA (nmol Trolox equiv./mg)	3.77 ± 0.17	3.79 ± 0.23	4.07 ± 0.25	4.11 ± 0.13	<0.001	1 vs. 3,4
Lung-TOA (nmol H_2O_2 equiv./mg)	136.34 ± 15.44	118.95 ± 15.26	148.75 ± 18.74	127.57 ± 30.84	0.020	1 vs. 2,3
Lung-OSI	36.20 ± 4.36	31.57 ± 5.42	36.60 ± 4.44	31.03 ± 7.62	0.031	1 vs. 2 and 3 vs. 4

MDA, malondialdehyde; AOA, total antioxidant activity; TOA, total oxidant status; OSI, oxidative stress index.
Data are presented as mean ± SEM.
Statistically significant: *P* < 0.05. Comparisons between groups for Bonferroni test *P* < 0.008.

Discussion

IR injury caused by the temporary decrease in blood flow in an organ leads to tissue damage and loss of function. Our study showed that the IR-induced oxidative stress indices and cellular disintegration of local and distant organs, in particular, were decreased by pomegranate extract administration before IR protocol. The histopathological scores of the IR group were significantly higher than IR + PE group (*P* < 0.001) in local and all distant organs.

Hypoxia and reoxygenation are the major causes of IR injury. Many studies have shown that reoxygenation is more responsible for the mechanisms of IR injury than the hypoxia itself.[1,20] It is recognized that pathological process of IR injury principally stresses the systemic inflammatory response syndrome and may lead to the multiorgan dysfunction. In that manner, IR injury affects multiple organs in specific ways. Many pathophysiological mechanisms of ischemic tissue injury have been suggested. Recent studies of tissue damage have focused on leukocytes and leukocyte adhesion molecules as well as their effects of oxygen free radical production,[21] cytotoxic enzyme release,[22] and increased cytokine release.[23] Emerging hydroxyl radicals react with membrane structures, causing lipid peroxidation. Lipid peroxidation leads to cell damage, which can cause cell death.[24]

Defense against oxidative reactants is mediated by antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. Additionally, many native molecules, such as glutathione, vitamin E (alpha-tocopherol), and ascorbic acid, exert antioxidant effects.[25,26] Thus, studies of IR injury have focused on potential antioxidants that are natural
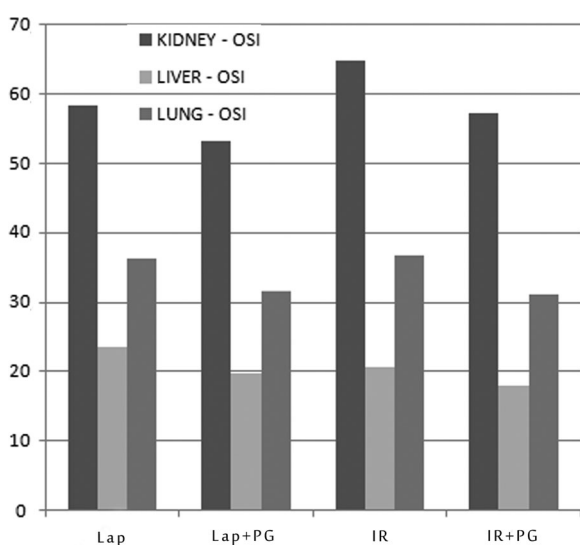
Figure 3 Comparison of OSI levels in the groups. Lap, laparotomy; PG, pomegranate; IR, ischemia–reperfusion.
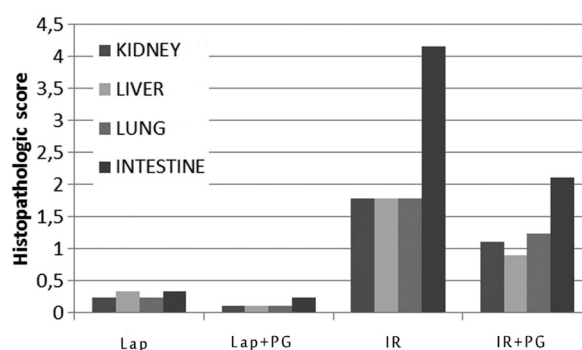
Figure 4 Comparison of the lung, kidney, intestinal, and liver histopathologic score levels in the groups. Lap, laparotomy; PG, pomegranate; IR, ischemia–reperfusion.

components of various food items which are in daily use.[27,28] Fruits and vegetables have high antioxidant activities, depending on their phenolic contents, which remove free radicals.[29] Among them, the pomegranate has a high phenolic content, containing anthocyanins, tannin, and ellagitannin, and is known to have marked antioxidant and anti-inflammatory effects.[30] A comparative study has shown that anthocyanin has a greater antioxidant effect than vitamin E (alpha-tocopherol), ascorbic acid, or beta-carotene.[31]

Oxidative stress causes lipid peroxidation. MDA is an end product of lipid peroxidation and is a well-known indicator of free radical formation in intestinal tissues.[32] In this study, not surprisingly, we found that the MDA levels were significantly higher in the IR group (Group 3) than in the laparotomy group (Group 4) ($P = 0.003$). Pomegranate is suggested to decrease MDA levels.[33] Similarly, when evaluating the preventative effects of pomegranate against lipid peroxidation, we found that MDA levels were significantly lower in the IR + PG group (Group 3) than in the IR group (Group 4) ($P = 0.031$).

Lung injury plays a key role in intestinal IR injury. Blood flow from damaged post-ischemic tissues drains predominantly into the pulmonary blood pool. Blood derived from ischemic tissue is rich in neutrophils and macrophages; these cell types produce cytokines and trigger an inflammatory response, causing alveolar capillary endothelial cell injury and pulmonary edema.[34,35] Pomegranate inhibits ROS-dependent luminol-amplified chemiluminescence in both resting and stimulated neutrophils and markedly inhibits myeloperoxidase activity, and it attenuates lipopolysaccharide-induced lung inflammation in mice.[36] Similarly, in the present study, lung TOA ($P = 0.049$) and lung OSI levels ($P = 0.034$), which are oxidative stress parameters, were found to be significantly higher in the laparotomy group than in the laparotomy + PG group However, irrespective of the absence of ischemia in the laparotomy group, oxidants could be generated by the laparotomy; therefore, pomegranate decreased the laparotomy-induced oxidative stress values in both the laparotomy + PG and laparotomy groups. Lung OSI levels and lung histopathological scores were higher in the IR group than

in the IR + PG group ($P = 0.049$) (Table 2). Pre-treatment with pomegranate reduced the lung tissue histopathological findings ($P = 0.019$).

Intestinal IR injury causes the release of cytokines and pro-inflammatory mediators (such as oxygen free radicals, platelet-activating factor, arachidonic acid metabolites, IL-6, IL-8, ICAM-1, and TNF-alpha) from damaged mesenteric tissue. These pro-inflammatory mediators pass into the blood through the lymphatic pathway and act on polymorphonuclear leukocytes in remote organs, such as the liver and kidney, causing cytotoxic cellular effects and increasing the levels of ROS in the affected organs.[37,38] In the present study, the intestinal histopathological scores were well correlated significantly with the liver ($r = 659$, $P < 0.001$) and kidney ($r = 789$, $P < 0.001$) histopathological scores (Table 2). Pomegranate juice consumption was found to reduce the hepatic oxidative stress scores in mice,[17] and pomegranate seed extract was found to exert protective effects against cisplatin hepatotoxicity in rabbits.[39] In our study, liver histopathological scores were not significantly different between the laparotomy and laparotomy + PG groups (Table 2). However, liver OSI levels were significantly higher in the laparotomy group ($P = 0.041$). Liver AOA levels were higher, and liver OSI and liver histopathological scores were significantly lower in the IR + PG group vs. the IR group. These results suggested that pomegranate decreases oxidative damage to the liver tissue.

Similar results in renal tissue have been reported in previous studies. In a recent study from Cekmen *et al.*,[33] pomegranate was reported to be preventive for oxidative stress in kidneys caused by gentamicin treatment. A different study demonstrated that pomegranate had a protective role against carbon tetrachloride-induced toxicity in the kidneys.[40] Another clinical study showed that pomegranate can attenuate hexachlorobutadiene-induced nephrotoxicity, with clinical findings of a dose-dependent decrease in serum creatinine and urea levels. In this study, as in previous reports, the kidney OSI levels and histopathological scores were significantly higher in the IR group compared with the IR + PG group (respectively, $P = 0.049$, $P = 0.017$) (Table 2). It should be noted that

Table 2 Histopathologic evaluation of intestine, kidney, liver, and lung for each group

	Sham (mean ± SEM)	Control (mean ± SEM)	IR (mean ± SEM)	IR + PG (mean ± SEM)	*P* values	Bonferroni *P* < 0.008
Intestine – HP	0.34 ± 0.47	0.23 ± 0.42	4.16 ± 1.00	2.11 ± 0.74	<0.001	1 vs. 3,4 and 3 vs. 4
Kidney – HP	0.23 ± 0.42	0.11 ± 0.31	1.78 ± 0.42	1.11 ± 0.57	<0.001	1 vs. 3,4 and 3 vs. 4
Liver – HP	0.34 ± 0.47	0.11 ± 0.31	1.78 ± 0.63	0.89 ± 0.74	<0.001	1 vs. 3 and 3 vs. 4
Lung – HP	0.23 ± 0.42	0.11 ± 0.31	1.78 ± 0.42	1.23 ± 0.42	<0.001	1 vs. 3,4 and 3 vs. 4

HP, histopathologic score. Data are presented as mean ± SEM.
Statistically significant: *P* < 0.05.

our results on the kidneys were achieved using a distant IR protocol.[41]

Unlike previous reports, this study found that the AOA levels were lower in the IR + PG group compared with the IR group. AOA levels increased in response to oxidative stress. If the oxidative effects were reduced, AOA levels would be expected to be lower than in the IR + PG group. In contrast, the bioavailability of pomegranate is low in animals following oral consumption. Furthermore, the ellagitannins, which have an antioxidant effect, are connected to cellular DNA and proteins, thus limiting the oral absorption of pomegranate.[42,43]

There were some limitations in our study. We reported only a single point of reperfusion because we had rat deaths; blood samples could not be supplied for more than 60 minutes in all groups. Additional groups for determining the time variation of pomegranate consumption and dose titration curve may be more helpful. Cytokine levels and quantitation of neutrophils in tissues were not studied. Female-only option might influence on the inflammatory markers because of hormonal changes.

In conclusion, the consumption of pomegranate extract can ameliorate the oxidative stress effect, particularly in terms of the degenerative histopathological changes in tissues damaged by IR injury. Pomegranate was also found to exert beneficial effects by preventing distant organ damage after IR injury. However, the protective effects of pomegranate must be supported by further studies.

Disclaimer statements

Contributors None.

Conflicts of interest I (we) certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Ethics approval Dicle University School of Medicine Animal Care. The Local Ethics Committee approved the study.

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