

Pomegranate (*Punica granatum* L.) Juice Supplementation Attenuates Isoproterenol-Induced Cardiac Necrosis in Rats

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Abstract The aim of the present study was to evaluate the efficacy of pre-supplementation with pomegranate (*Punica granatum* L.) juice (PJ) on heart weight, infarct size, plasma marker enzymes of cardiac damage, lipid peroxidation, endogenous enzymatic and non-enzymatic antioxidants, cardiac ATPases and histopathology of isoproterenol (IP)-induced cardiac necrosis (CN) in rats. Rats treated with IP (85 mg/kg, s.c.) for 2 days at an interval of 24 h caused significant ($P < 0.05$) infarct in myocardium and increase in heart weight, lipid peroxidation (LPO), activity levels of Ca^{+2} ATPase and plasma marker enzymes, while there was significant ($P < 0.05$) decrease in endogenous enzymatic and non-enzymatic antioxidants and $\text{Na}^{+}\text{-K}^{+}$ and Mg^{+2} ATPases. Pre-supplementation with PJ for 30 consecutive days and treated with IP on days 29th and 30th showed significantly ($P < 0.05$) lesser increase in heart weight, infarct size, plasma marker enzymes, lipid peroxidation, Ca^{+2} ATPase and a significant protective effect in endogenous enzymatic and non-enzymatic antioxidants, $\text{Na}^{+}\text{-K}^{+}$ and Mg^{+2} ATPases compared to IP alone treated group. Present study provides first scientific report on protective effect of supplementation of Pomegranate juice against IP-induced CN in rats.

Keywords *Punica granatum* L · Isoproterenol · Cardiac necrosis · Free radicals · Oxidative stress

Introduction

Isoproterenol (IP), a synthetic catecholamine β -adrenergic agonist, has been found to cause severe stress in the myocardium resulting in infarct like necrosis of the heart muscles [1]. The effects of IP on heart are mediated through β_1 and β_2 adrenoreceptors, as both mediate the positive inotropic and chronotropic effects of β -adreno-receptor agonists [2]. Isoproterenol thereby causes relative ischaemia or hypoxia due to myocardial hyperactivity and coronary hypotension [3], and induces myocardial ischaemia due to cytosolic Ca^{2+} overload [4]. Additionally, generation of highly cytotoxic free radicals through the auto-oxidation of catecholamine has also been implicated as one of the important causative factors [5]. Increased load of tissue reactive oxygen species (ROS) can affect a variety of cellular functions such as enzyme kinetics, ion transport, DNA repair and even transcription. As IP-induced cardiac necrosis (CN) resembles histological deletions seen in human CN [6], study of appropriate markers is likely to provide mechanistic explanation to possible biochemical alterations occurring during CN.

The juice of pomegranate (*Punica granatum* L.) fruit (PJ) has gained recognition for its varied medicinal properties. The potent antiatherogenic effect of PJ has been recently reported in healthy humans as well as in atherosclerotic mice [7, 8]. Clinical trials have reported its hypocholesterolemic [9], hypotensive [10] and antioxidant [11] properties. Chronic supplementation with PJ results in significant reduction in intima-media thickness, systolic blood pressure and serum lipid peroxidation in human subjects [12]. Pomegranate juice has been rated to contain the highest antioxidant capacity compared to other commonly consumed polyphenol-rich beverages and other fruit

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juices [11, 13] and in fact, reportedly the antioxidant potential of PJ is three times higher than that of red wine and green tea [14].

Dietary factors do play a key role in the control of various human diseases, including cardiovascular. Epidemiological studies have shown that people who consume diets rich in antioxidants (fruits, herbs and spices) are at a lower risk of developing cardiovascular disorders [15]. Based on this background, it is hypothesized that PJ would be effective in protecting against CN, and the same has been tested in the present inventory on the effects of PJ supplementation on biochemical and histopathological changes in isoproterenol-induced CN.

Materials and Methods

Pomegranate Processing and Dose Preparation

Pomegranates were handpicked, washed, and the entire fruit was then squeezed in a manual juicer and the juice was treated enzymatically with pectinase to yield a clear PJ. Regular or high doses of PJ (6.25 or 12.5 ml PJ made up to 100 ml with drinking water) were prepared as per previous reports [7, 8].

Experimental Animals

Male *Wistar* rats (170–190 g), procured from Zydus Research Centre, Ahmedabad, India, were maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd Baroda, India) food and water were provided ad libitum. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of Department of Zoology, The M.S University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Experimental Design

Rats were randomly divided into five groups ($n = 6$ each). Groups I and III were given pure drinking water, whereas groups II, IV and V were given PJ in drinking water for 30 days. Mean fluid intake per day of rats from all the groups was 20 ml/day. Total polyphenol intake per day calculated [7, 8] in groups II, IV and V was found to be 16, 8 and 16 µmoles/day, respectively. Groups III, IV and V were treated with isoproterenol (85 mg/kg, s.c.) on 29th and 30th days [16], whereas Groups I and II received equal volume of the vehicle (0.9% NaCl). On the 31st day (24 h after the last injection of IP), animals were fasted over

night (12 h) and blood samples were collected from retro-orbital sinus under mild ether anaesthesia. Animals were subjected to cervical dislocation under mild ether anaesthesia (as per the CPCSEA guidelines) and hearts of control and experimental rats were excised.

Plasma Markers of Cardiac Injury

Activity levels of creatine phosphokinase-MB (CK-MB) and lactate dehydrogenase (LDH) were estimated in plasma by using commercially available kits (Eve's Diagnostics, Baroda, India).

Cardiac Antioxidants and Lipid Peroxidation

Cardiac tissue pieces from control to treated groups were weighed and homogenized (10% w/v) in chilled tris buffer (10 mM, pH 7.4) and centrifuged at 10,000×g for 20 min in high speed cooling centrifuge (0°C). Clear supernatant was used for assaying MDA levels, an index of lipid peroxidation (LPO) [17]. Activity levels of superoxide dismutase (SOD) [18] and catalase (CAT) [19], and the contents of reduced glutathione (GSH) [20] and ascorbic acid [21] were assayed.

Cardiac ATPase Activity

Pellet obtained after centrifugation of homogenate was re-suspended in ice-cold tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of levels of activity of $\text{Na}^+ \text{-K}^+$ ATPase [22], Ca^{2+} ATPase [23] and Mg^{2+} ATPase [24]. Total protein was estimated by the method of Lowry et al. [25].

Measurement of Cardiac Infarct Size by Nitro Blue Tetrazolium Staining

Cardiac tissue samples were washed in phosphate buffer saline (PBS) thrice and frozen at –20°C for 24 h. The ventricle was sliced (1 cm thick) perpendicular to the apex-base axis, and the slices were then incubated at 37°C for 20 min in 5% nitro blue tetrazolium (NBT). The dehydrogenases present in the viable myocardium reduce NBT to a visible blue colour, while the infarcted areas remain unstained. The infarcted and non-infarcted areas were separated out under a stereo zoom microscope (Leica, MZ 16 A) using micro forceps. The ventricular slices were pre-weighed to obtain the total weight. Later, the infarcted areas were weighed on a digital ultra sensitive weighing balance (Sartorius, BP 61). Infarct size was calculated as per Wei and Zang [26] and expressed as percentage of total left ventricular weight.

Microscopic Evaluation of Cardiac Tissue

Heart samples were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five micrometre sections were cut (on a Leica RM 2155 Microtome), stained with haematoxylin and eosin and examined under a Leica DMRB microscope (100 \times). The section was photographed with Canon power shot S72 digital camera. Lesions were graded as (0) nil, (1) minimum (focal myocytes damage), (2) mild (small multifocal degeneration with slight degree of inflammatory process), (3) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process) and (4) severe (necrosis with diffuse inflammatory process) as per Joukar et al. [27].

Statistical Analysis

Statistical evaluation of the data was done by student's *t* test and one-way ANOVA. The results are expressed as mean \pm S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA. Differences in the histopathological scores were determined by chi square and Fisher's exact tests.

Results

Plasma Markers of Cardiac Injury

Isoproterenol-treated rats registered a significant increment ($P < 0.05$) in activity levels of plasma CK-MB (45.82%) and LDH (55.15%) compared to CON. However, PJ supplemented rats showed a lesser increment of 29.68 & 21.88% for CK-MB and 44.41 & 20.71% for LDH. Control rats treated with PJ alone did not register any significant alterations in the plasma levels of CK-MB and LDH compared to CON rats (Table 1).

Cardiac Antioxidants and Lipid Peroxidation

Isoproterenol-treated rats recorded significant decrement in activity levels of SOD (53.63%), CAT (50.00%), contents of GSH (60.15%), and AA (60.13%) with concurrent

significant increment ($P < 0.05$) in cardiac LPO (41.31%) compared to CON rats. IP+PJ rats showed only 22.45 & 8.40% decrement in SOD and 20.37 & 4.00% decrement in CAT activity levels. GSH and AA contents showed a significantly lower decrement of 30.87 & 14.54% and 33.55 & 5.64% while LPO registered a minimal increment of only 22.00 & 2.25%. Control rats treated with PJ alone did not register any significant alterations in levels of lipid peroxidation and cardiac antioxidants compared to CON rats (Table 2).

Cardiac ATPases Activity

Isoproterenol-treated rats recorded significant decrement in activities of Na⁺-K⁺ ATPase (40.58%) and Mg²⁺ ATPase (44.14%) but a significant increment ($P < 0.05$) in cardiac Ca²⁺ ATPase activity (52.86%) compared to CON rats. However, PJ-supplemented rats showed only 19.41 & 14.50% decrease in Na⁺-K⁺ ATPase and 25.41&12.70% decrease in Mg²⁺ ATPase activities and only 35.61 & 17.75% increase in Ca²⁺ ATPase activity. Control rats treated with PJ alone did not register any significant alterations in activity levels of cardiac ATPases compared to CON rats (Table 3).

Heart Weight and Infarct Size

Mean relative heart weight was significantly increased (35%) in IP-treated rats compared to CON. Pre-supplementation with regular and high doses of PJ restricted the IP-induced increase in heart weight to only 22.00 and 8.00%, respectively, compared to CON rats (Fig. 1). Heart from IP-treated rats registered 37.54% infarct size, while the infarct size of heart in PJ-supplemented IP-treated rats were merely 25.22 and 18.07%, respectively (Fig. 2). Control rats treated with PJ alone did not register any significant alterations in heart weight and infarct size compared to CON rats (Figs. 1, 2).

Microscopic Evaluation of Cardiac Tissue

The histoarchitecture of the cardiac tissue of CON and PJ2 rats appeared to be normal, as there was no visible necrotic damage to the myocytes (Fig. 3a, b). However, extensive

Table 1 Effect of PJ supplementation on plasma markers of cardiac injury

	CON	PJ2	IP	IP+PJ1	IP+PJ2
CK-MB ¹	100.78 \pm 9.44	98.76 \pm 7.20	186.12 \pm 10.56 ^C	143.33 \pm 11.01 ^a	129.01 \pm 9.02 ^b
LDH ¹	79.55 \pm 6.09	77.09 \pm 5.02	177.37 \pm 11.00 ^C	143.11 \pm 9.02 ^a	100.34 \pm 8.99 ^c

Where, ¹ IU/L, ^A ($P < 0.05$), ^B ($P < 0.01$) and ^C ($P < 0.001$) when CON vs. PJ2 & IP, and ^a ($P < 0.05$), ^b ($P < 0.01$) and ^c ($P < 0.001$) when IP vs. PJ2, IP+PJ1 & IP+PJ2

Table 2 Effect of PJ supplementation on cardiac antioxidants and lipid peroxidation level

	CON	PJ2	IP	IP+PJ1	IP+PJ2
SOD ¹	6.19 ± 0.66	5.87 ± 0.76	2.87 ± 0.51 ^B	4.80 ± 0.58 ^a	5.67 ± 0.55 ^b
CAT ²	8.00 ± 0.89	7.10 ± 0.71	4.00 ± 0.66 ^B	6.37 ± 0.76 ^a	7.68 ± 0.56 ^b
GSH ³	5.02 ± 0.42	4.99 ± 0.31	2.00 ± 0.39 ^C	3.47 ± 0.29 ^a	4.29 ± 0.49 ^b
AA ⁴	3.01 ± 0.19	2.76 ± 0.11	1.20 ± 0.13 ^C	2.00 ± 0.14 ^b	3.19 ± 0.14 ^c
LPO ⁵	3.04 ± 0.33	3.00 ± 0.41	5.18 ± 0.29 ^B	4.00 ± 0.33 ^a	3.11 ± 0.27 ^b

Where, ¹ Units/mg protein, ² μmoles of H₂O₂ consumed/mg protein, ³ μg of GSH/mg protein, ⁴ mg/g, and ⁵ nmol of MDA/mg protein

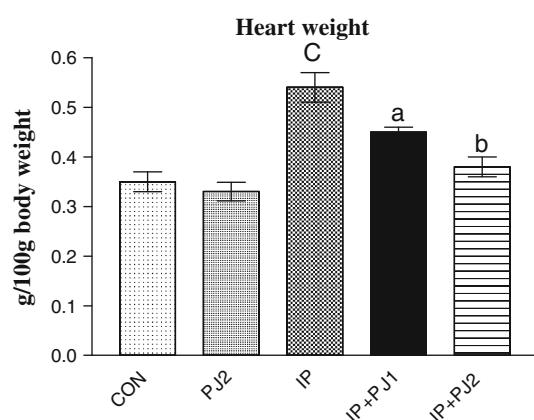
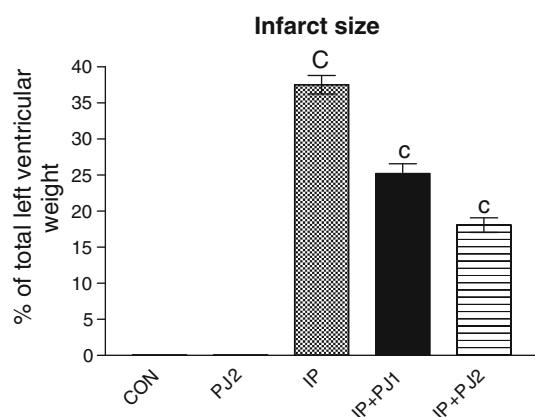
^A ($P < 0.05$), ^B ($P < 0.01$) and ^C ($P < 0.001$) when CON vs. PJ2 & IP, and ^a ($P < 0.05$), ^b ($P < 0.01$) and ^c ($P < 0.001$) when IP vs. PJ2, IP+PJ1 & IP+PJ2

Table 3 Effect of PJ supplementation on cardiac ATPase activity levels

	CON	PJ2	IP	IP+PJ1	IP+PJ2
Na ⁺ -K ⁺ ATPase ¹	5.10 ± 0.22	4.98 ± 0.17	3.03 ± 0.21 ^C	4.11 ± 0.27 ^a	4.36 ± 0.19 ^c
Mg ²⁺ ATPase ¹	2.99 ± 0.19	2.76 ± 0.11	1.67 ± 0.17 ^C	2.23 ± 0.14 ^a	2.61 ± 0.15 ^b
Ca ²⁺ ATPase ¹	3.29 ± 0.25	3.17 ± 0.12	6.98 ± 0.28 ^C	5.11 ± 0.27 ^b	4.00 ± 0.23 ^c

Where, ¹ μmoles of pi liberated/min/mg protein

^A ($P < 0.05$), ^B ($P < 0.01$) and ^C ($P < 0.001$) when CON vs. PJ2 & IP, and ^a ($P < 0.05$), ^b ($P < 0.01$) and ^c ($P < 0.001$) when IP vs. PJ2, IP+PJ1 & IP+PJ2

**Fig. 1** Effect of PJ supplementation on heart weight**Fig. 2** Effect of PJ supplementation on infarct size

myofibrillar degeneration (33.33% of group's subject), myonecrosis and infiltration of inflammatory cells (41.67% of group's subjects) were observed in IP-treated rats (Fig. 3c and Table 4). Pre-supplementation with PJ in IP+PJ rats showed significantly lesser extent of damage with maximal protective effect with the higher dose of PJ supplementation (Fig. 3d, e and Table 4).

Discussion

Deficient glucose supply and/or tissue hypoxia caused by IP have been reported to result in increased cell permeability and damage leading to leakage of myocardial CK-MB and LDH into plasma. This leakage is due to non-specific alteration in the integrity plasma membrane and/or permeability of myocytes [28]. Significantly elevated activity levels of plasma CK-MB and LDH in our IP-treated rats are in accordance with previous reports [29]. Interestingly, pre-supplementation with PJ significantly prevented IP-induced elevation in the plasma activity levels of these diagnostic marker enzymes. Apparently, PJ seems capable of resisting membrane destabilizing activity of IP to a great degree and thereby minimizing leakage of these enzymes.

Glutathione is known to protect myocardium against free radical-mediated injury by reduction of hydrogen peroxide radicals, leading to decreased glutathione levels during induction of CN [30]. This protective ability of glutathione against oxidative stress during CN is likely

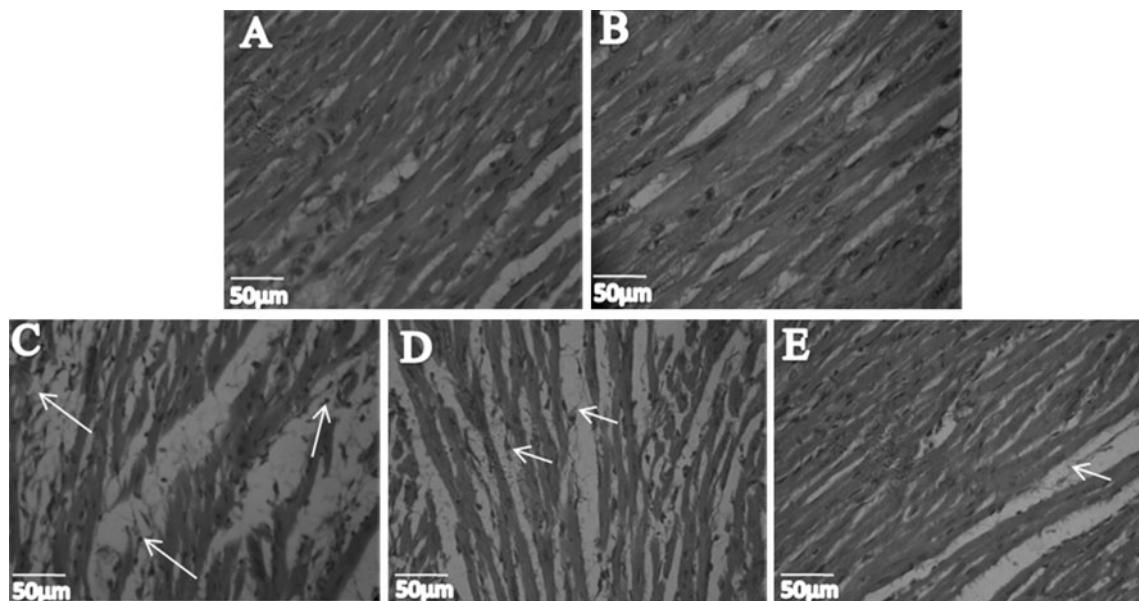


Fig. 3 **a** Photomicrograph of rat heart from control group showing normal architecture of heart. **b** Photomicrograph of rat heart from PJ2 group showing normal architecture of heart. **c** Photomicrograph of rat heart of isoproterenol-treated group showing focal myonecrosis with myophagocytosis and lymphatic infiltration, vacuolar changes and

oedema are prominent with chronic inflammatory cells visible (arrows) (H X E; $\times 100$). **(D & E)** Photomicrographs of rat heart of pomegranate juice supplemented and IP-treated groups showing lesser degree of myonecrosis and infiltration of inflammatory cells (arrows) (H X E; 100X)

Table 4 quantitation of inflammatory infiltration in experimental groups

Pathology score	Experimental groups					Total
	CON	PJ2	IP	IP+PJ1	IP+PJ2	
0 Count	12	12	0	4	6	22
% within group	100	100	0	33.33	50	45.82
1 Count	0	0	0	2	3	5
% within group	0	0	0	16.67	25	10.42
2 Count	0	0	3	5	3	11
% within group	0	0	25	41.67	25	22.92
3 Count	0	0	4	1	0	5
% within group	0	0	33.33	8.33	0	10.42
4 Count	0	0	5	0	0	5
% within group	0	0	41.67	0	0	10.42
Total Count	12	12	12	12	12	60

Pathology scores: 0 nil, 1 minimum (focal myocytes damage), 2 mild (small multifocal degeneration with slight degree of inflammatory process), 3 moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process), 4 severe (necrosis with diffuse inflammatory process)

depress reduced glutathione GSH levels. The observed decrease in GSH levels in IP-treated rats could possibly be due to its conversion to oxidized glutathione (GSSG) or due to decreased synthesis under oxidative stress. Ascorbic acid (Vit C) can directly scavenge singlet oxygen, superoxide and hydroxyl radicals [31]. In the present study, we observed significant decrease in cardiac Vit C content in

IP-treated rats. Pre-supplementation with PJ minimized the depletion in GSH and Vit C content induced by IP, suggesting the probable ability of PJ to act as a scavenger of FRs and thereby sparing the endogenous non-enzymatic antioxidants.

Concurrently, we have also recorded decrement in the activity levels of SOD and CAT in IP-treated rats. Superoxide dismutase protects cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which gets further metabolized by CAT to molecular oxygen and water [32]. It is suggested that the decrease in the activities of these antioxidant enzymes might be related with enhanced lipid peroxidation [33]. Pre-supplementation with PJ significantly protected the activity levels of cardiac SOD and CAT in IP-treated rats. It is possible that potent superoxide scavenging ability of PJ could contribute to maintaining the levels of endogenous SOD and CAT activity under oxidative stress.

Lipid peroxidation, a type of oxidative deterioration of polyunsaturated fatty acids has been linked with altered membrane structure, and inactivation of SOD and CAT resulting in accumulation of superoxide anion, which further damages the myocardium [34]. In this context, the observed depletion in GSH and Vit C and decreased activity levels of SOD and CAT can be correlated with significant increment in cardiac LPO in IP-treated rats and indicate higher susceptibility of myocardium to oxidative damage. The currently observed significant decrease in LPO with PJ supplementation in IP-treated rats could be a

consequence of the direct FR scavenging ability of PJ as well, as its ability to conserve the endogenous antioxidant machinery of the cardiac tissue, thus imparting cardioprotection.

$\text{Na}^+ \text{-K}^+$ ATPase is the 'SH' group containing enzymes responsible for the active transport of Na^+ and K^+ across cell membrane, while Ca^{2+} ATPase is responsible for the maintenance of normal intracellular calcium levels [6]. Currently, we have observed significant decrement in $\text{Na}^+ \text{-K}^+$ and Mg^{2+} ATPase activity levels in IP-treated rats. Inactivation of $\text{Na}^+ \text{-K}^+$ ATPase could be due to FR-mediated lipid peroxidation on β -adrenergic stimulation, while enhanced Ca^{2+} ATPase and intracellular Ca^{2+} overload in IP-treated rats could be due to activation of adenylate cyclase, as cAMP-dependent phosphorylation of Ca^{2+} channel protein is known to cause increased influx of Ca^{2+} into myocardium following IP treatment [34]. In our study, pre-supplementation with PJ could resist to a greater extent the perturbations in $\text{Na}^+ \text{-K}^+$, Mg^{2+} and Ca^{2+} ATPase caused due to IP. It is likely that the protective effect of PJ could be due to its ability to preserve 'SH' group and inhibit membrane lipid peroxidation and consequent alterations in the activity of various ATPases.

Significant increase in the heart weight of IP-treated rats has been attributed to increased water content, formation of oedematous intramuscular spaces, extensive necrosis of cardiac muscle fibres and invasion by inflammatory cells [29]. In the current study, pre-supplementation with PJ could significantly minimize increase in heart weight possibly due to prevention of oedema [34]. Heart sample of IP-treated rats registered higher infarct size, whereas in PJ-supplemented IP-treated rats it was significantly minimized. This observation is suggestive of salvation of higher viable area in PJ-supplemented rats and protection against IP-induced cardiac damage. These results can be attributed to the high content of polyphenols in PJ [14] that help protect the cardiac tissue against IP-induced oxidative damage [34]. The histoarchitecture of cardiac tissue of CON rats appeared to be normal as there was no visible necrotic damage to the myocytes. However, extensive myocyte membrane damage, myonecrosis, fibroblastic proliferation and infiltration of inflammatory cells were observed in IP-treated rats. Pre-supplementation of PJ to IP-treated rats showed significantly lesser extent of myocyte damage.

Conclusion

It can be concluded from the results showcased herein that PJ may act as a free radical scavenger and conserve the endogenous antioxidant system in IP-treated rats, thus inhibiting peroxidation of membrane lipids, consequent

leakage of soluble enzymes and oxidation of -SH groups of enzymatic proteins. Antioxidant and free radical scavenging property of PJ [11, 14, 35] seem to protect the myocardium against IP-induced oxidative damage. Presence of polyphenols such as ellagitannins and anthocyanins [14] and flavonoids such as kaempferol, myricetin, rutin, naringenin, luteolin, luteolin glycosides, quercetin, and quercetin glucosides in pomegranate juice [36–38] imparts free radical scavenging and antioxidant effects, thereby protecting the myocardium against IP-induced cardiac damage. Overall, it can be concluded that PJ has the potential to protect against myocardial damage by overcoming oxidative stress and associated biochemical and structural distortions.

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