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Original Article

Cardioprotective effects of pomegranate juice against ischemia and reperfusion in isolated rat heart

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ABSTRACT

Background: The literature regarding the cardiovascular effects of pomegranate has been expanding during recent years. The present study aimed to investigate the cardioprotective effect of pomegranate juice following ischemia and reperfusion.

Methods: The animals were anesthetized with thiopental sodium (60 mg kg^{-1} BW) and their hearts were removed and perfused using Langendorff technique. In the first stage of the experiment, pomegranate juice was dissolved in Krebs solution. The first group was the normoxic control; the remaining 4 groups underwent 30 min global ischemia followed by 90 min reperfusion and received pomegranate juice at 0, 1, 2, and 4% in Krebs solution, respectively. The heart rate, coronary perfusion pressure, left ventricular developed pressure, rate pressure product (RPP), and dp/dtmax were monitored throughout the experiment. The infarct size was also measured via staining with triphenyl tetrazolium chloride. In the second stage, the rats received either placebo or pomegranate juice (4 ml kg^{-1} BW), daily, for 3 weeks. The hearts isolated from these animals were then studied as mentioned earlier, but no juice was added to the perfusion solution.

Results: The hearts treated with the juice showed significantly higher percentages of RPP and increased dp/dtmax during reperfusion, compared to the control group. Moreover, these hearts showed smaller infarct sizes. In the second stage of the study, all studied hemodynamics were significantly higher in the test group both before and after ischemia. The infarct size was also smaller in the test group.

Conclusion: The results of the present study suggest strong cardioprotective effects of pomegranate juice against myocardial ischemia and reperfusion.

Keywords: Pomegranate, *Punica granatum*, Ischemic heart, Reperfusion

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Introduction

Ischemic heart disease, also known as coronary artery disease, is among the leading causes of mortality worldwide (1). Ischemia disrupts ATP production in mitochondria, as a result, the intracellular concentration of sodium is increased, which leads to disturbances in all kinds of secondary active transports across the cell membrane. In addition to accumulation of intracellular lactic acid due to anaerobic metabolism, pH is further declined because of impaired function of Na^+/H^+ counter-transporter. The activity of sodium/calcium exchanger is also inhibited. The resultant intracellular calcium accumulation switches on multiple intracellular cytotoxic pathways (2). The main therapeutic strategy is to restore coronary blood flow, but reperfusion, *per se*, exacerbates the chaos via reperfusion induced-arrhythmias, vascular damage and no-reflow, myocardial functional stunning, and cell necrosis (3). In addition, reperfusion distorts the oxidative balance of the cells, and the subsequent overproduction of reactive oxygen species (ROS) leads to cell damage and necrosis (3). Among different pharmacological interventions to tackle a disease, natural antioxidants have been widely used to minimize the oxidative damage to cardiomyocytes (4, 5).

Pomegranate (*Punica granatum*) has been the target of extensive research over the last decade. A simple search on PubMed shows more than 1,450 related articles since 2007, whereas the overall number of previous articles is only 276. Various therapeutic effects such as antiparasitic, antibacterial, anticarcinogenic, and anti-diabetic properties have been attributed to pomegranate (6). It inhibits platelet aggregation and lowers serum levels of cholesterol. Pomegranate contains strong antioxidant polyphenols such as ellagic acid, punicalagin, and gallic acid (7). It has shown higher free radical scavenging properties in comparison to apple, cranberry, orange, grapefruit, pear, and pineapple (8-10).

The present study aimed to verify the cardioprotective effects of pomegranate juice against ischemia/reperfusion in isolated hearts.

Materials and Methods

Pomegranate juice

Pomegranate juice was prepared by squeezing the arils using a manual juicer. It was then lyophilized and kept dry until use. The prepared powder was dissolved in Krebs solution to yield the initial concentration before being added to the Langendorff perfusion solution.

Chemical analysis

The total polyphenol content of the juice powder was determined using Folin-Ciocalteu (F-C) method (11). One milligram of the sample was dissolved in 2 ml methanol:water (6:4, V/V), and was then further mixed (1:1, V/V) with 10-fold-diluted Folin-Ciocalteu reagent. Two hundred microliters of the mixture were added to 2 ml of 2% sodium carbonate solution. The resultant mixture was left at room temperature for 90 min. The optical absorbance was measured at 760 nm using a spectrophotometer. The tannin equivalent content of the sample was estimated according to the standard curve.

A high-performance liquid chromatography-mass spectroscopy (LC/MS- Agilent Technology-6410-QQQ, US & Japan) technique was used to analyze the main polyphenols of the juice. Using a gradient protocol, the mobile phase consisted of solution A, formic acid in distilled water (1:99, V/V), and solution B, Acetonitrile. The gradient of solution B was as follow: 0-5 min: 5%, 5-10 min: 10%, 10-15 min: 15%, 15-20 min: 20%, 20-25 min: 60%, and ultimately, 25-30 min: 90%.

The rats

The research methods were approved by the Ethics Committee of Ferdowsi University of Mashhad (Ethical code: IR.UM.REC.1400.175). Male Wistar rats (200-250 g) were acclimated in the Animal Unit of the School of Veterinary Medicine, at least one week prior to the experiments.

The experimental procedure

Two separate experiments were performed. The first experiment investigated the cardioprotective effect of pomegranate juice using an *ex-vivo* treatment protocol. The second one employed an *in-vivo* model of intervention.

For the first experiment, the isolated hearts were randomly assigned to five experimental groups (n = 10, each group). The first group was the normoxic control, and the hearts were continuously perfused with oxygenated Krebs solution using the Langendorff apparatus. The hearts in all other experimental groups were allowed to stabilize for 30 min, and then, global hypoxia was induced via turning off the peristaltic pump for 30 min. As a subsequence,

the flow of the Krebs solution to the coronary artery was interrupted. Thereafter, the hearts were reperfused for 90 min. The second group was the ischemic control group and did not receive pomegranate juice. The remaining 3 groups were subjected to ischemia and reperfusion, as described, and received 1, 2, and 4% pomegranate juice in Krebs solution, respectively, throughout the experiment.

In order to supplement the Krebs solution with pomegranate juice in groups 3-5, the lyophilized powder was reconstituted in Krebs solution to yield the initial concentration of the juice. It was then centrifuged to remove the undissolved particles. Afterwards, the solution pH was neutralized using sodium bicarbonate solution. It was finally supplemented to the Krebs reservoir at 1, 2, or 4% (vol/vol). These concentrations were based on our previous pilot studies.

During the second experiment, two groups of rats (n= 10, each group) were employed. The rats in the control group were gavaged with placebo (distilled water), while the rats in the test group were gavaged with pomegranate juice (4 ml kg^{-1} BW), daily for 3 weeks. Afterwards, the hearts were removed under anesthesia and studied using Langendorff technique. All hearts underwent 30 min global hypoxia and 90 min reperfusion. None of them were treated with the juice during Langendorff perfusion of the hearts.

The Langendorff technique

The rats were anesthetized with thiopental sodium (60 mg kg^{-1} BW). The chests were excised, the hearts were removed and immediately mounted on Langendorff apparatus. The hearts were perfused with modified Krebs solution containing NaCl (118 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), KCl (2 mM), $CaCl_2$ (1.23 mM), $MgSO_4$ (1.2 mM), and glucose (11 mM) (gassed with 95% O_2 and 5% CO_2, pH: 7.4, 37°C). The coronary perfusion pressure could be recorded using a pressure transducer (MLT844, ADInstruments, Australia) via a three-way stopcock attached above the aortic cannula. The intraventricular pressure could be monitored using a latex balloon inserted into the left ventricle via another pressure transducer. The heart rate was recorded according to the electrocardiogram (ECG) via two electrodes attached to the apex and the right auricle of the heart (ML865, ADInstruments, Australia). The left ventricular developed pressure (LVDP) was calculated as the difference between the left ventricular systolic pressure and the left ventricular diastolic pressure. The rate pressure product (RPP), used to determine the cardiac workload, was calculated as the heart rate multiplied by LVDP.

Measurement of the infarct size

At the end of the experiments, the hearts were cut into 2 mm thick slices and immersed in 1% triphenyl tetrazolium chloride solution for 10 min. The latter compound is reduced to formazan to give a reddish color to the living cells. The non-stained infarcted myocardial cells remain pale (Figure 1). The heart slices were then photographed and the percentage of infarcted areas were measured using Adobe Photoshop CS5 software (12).

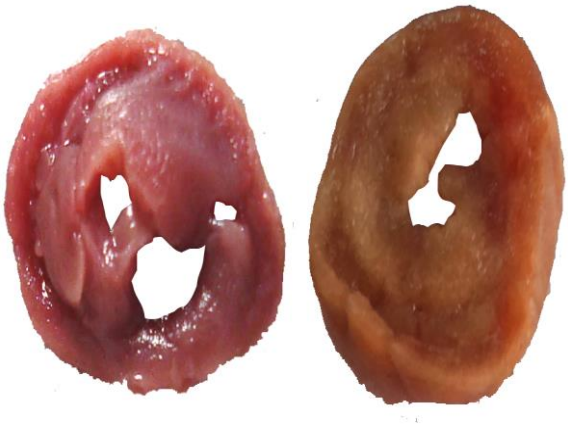

Figure 1. The heart sections stained with triphenyltetrazolium chloride. The left image represents the normoxic control heart, while the right image belongs to the infarcted ischemic heart.

Statistical analysis

Drawing the figures and statistical comparisons were performed using GraphPad Prism Software (GraphPad Software Inc, USA). All values are presented as the mean and standard error of the mean (SEM). Different experimental groups were compared using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The infarcted areas of the hearts were compared using one-way ANOVA followed by Tukey's post-hoc test. In all cases, statistical significance level was considered at $P<0.05$.

Results

The first experiment

During the first stage of the experiment, the juice was supplemented to the perfusion solution of the isolated hearts. The hemodynamic results

are shown in Table 1. The hearts stopped beating during hypoxia, but the heart rate restored upon reperfusion. At some time points during reperfusion, the heart rate was significantly higher with the juice at 1% concentration. Apart from this, however, there were mainly no significant differences among the experimental groups. Obviously, the coronary perfusion pressure declined to zero during global hypoxia. The pressure mainly approached its original value following reperfusion. The perfusion pressure was significantly lower with the highest concentration of the juice at the end of reperfusion period. The mechanical activity of the heart, as represented by LVDP and RPP%, was abolished during hypoxia. During reperfusion, the mechanical activity partially restored in the hypoxic control group, and the percentage of RPP declined to 74% following 90 min reperfusion. This parameter, however, was significantly higher in pomegranate groups during reperfusion. The same was true regarding dp/dtmax with 1% and 2% pomegranate juice.

Table 1. The hemodynamics of the isolated rat hearts. The control group was normoxic, while other groups were subjected to 30 min global ischemia (Isch) and 90 min reperfusion (Rep).

		Stab	Hypoxia			Reperfusion				
	Groups/Time (min)	29	1	15	29	1	15	30	60	90
HR (beats/min)	Control	192±5	202±9[a]	199±9[a]	199±10[b]	193±9[a]	201±9	196±8	183±11	187±12[a]
	Isch/Rep (no Pg)	214±9	2±2[b]	0±0[b]	0±0[b]	219±6	195±6	187±12	204±7	207±9
	Isch/Rep-Pg 1%	227±14	0±0[b]	0±0[b]	0±0[b]	232±21[b]	216±11	223±17	218±14	230±16[b]
	Isch/Rep-Pg 2%	199±11	7±4[b]	0±0[b]	0±0[b]	190±22[a]	205±10	206±9	207±9	219±9
	Isch/Rep-Pg 4%	211±7	1±1[b]	0±0[b]	0±0[b]	198±14	208±7	210±8	215±8	215±5
CPP (mmHg)	Control	54±7	54±6[a]	54±6[a]	57±7[a]	57±7	61±9[a]	61±9	57±6	60±6
	Isch/Rep (no Pg)	43±4	1±1[b]	0±1[b]	-1±1[b]	44±3	41±3[b]	60±6	61±8	67±8[a]
	Isch/Rep-Pg 1%	42±3	-1±0[b]	-2±0[b]	-3±1[b]	49±4	47±4	45±3	50±3	51±3
	Isch/Rep-Pg 2%	50±5	1±0[b]	-1±1[b]	-2±1[b]	55±6	57±7	57±7	61±7	63±7
	Isch/Rep-Pg 4%	45±5	-1±0[b]	-2±0[b]	-2±0[b]	47±4	47±4	46±5	46±5	47±6[b]
LVDP (mmHg)	Control	60±2	59±4[a]	59±4[a]	59±3[a]	61±3	55±4	61±4	60±4	56±5
	Isch/Rep (no Pg)	61±6	14±4[b]	1±1[b]	0±0[b]	56±7	54±7	53±8	49±7[a]	47±7
	Isch/Rep-Pg 1%	67±8	13±3[b]	0±0[b]	0±0[b]	75±5	72±6	72±7	68±7	64±7
	Isch/Rep-Pg 2%	72±7	16±2[b]	0±0[b]	0±0[b]	76±8	69±8	70±6	73±5[b]	65±5
	Isch/Rep-Pg 4%	62±4	11±2[b]	0±0[b]	0±0[b]	72±9	68±5	71±5	67±5	64±5
RPP%	Control	100±0	103±2[a]	103±5[a]	102±5[a]	103±4[a]	97±7	105±6[a]	96±8	92±8
	Isch/Rep (no Pg)	100±0	0±0[b]	0±0[b]	0±0[b]	97±10[a]	79±3[a]	73±7[b]	75±6[a]	74±8[a]
	Isch/Rep-Pg 1%	100±0	0±0[b]	0±0[b]	0±0[b]	136±32[b]	112±13[b]	110±10[a]	102±6	101±8
	Isch/Rep-Pg 2%	100±0	1±1[b]	0±0[b]	0±0[b]	95±7[a]	98±3	102±4	108±4[b]	102±5
	Isch/Rep-Pg 4%	100±0	0±0[b]	0±0[b]	0±0[b]	113±18	110±10[b]	115±8[a]	111±7[b]	107±8[b]
dp/dtmax	Control	1707±94	1752±88[a]	1710±95[a]	1714±104[a]	1777±116	1647±134	1744±135	1721±147	1636±187
	Isch/Rep (no Pg)	1855±209	324±57[b]	26±0[b]	26±0[b]	1620±187	1302±242[a]	1607±210	1546±206[a]	1475±205[a]
	Isch/Rep-Pg 1%	2220±212	316±69[b]	26±0[b]	26±0[b]	2038±123	2008±144[b]	2160±181	2035±202	1928±190
	Isch/Rep-Pg 2%	2101±251	262±19[b]	26±0[b]	26±0[b]	2115±206	1927±202[b]	2128±226	2284±192[b]	2109±194[b]
	Isch/Rep-Pg 4%	1859±132	224±29[b]	26±0[b]	26±0[b]	2044±219	1799±118	1901±153	1966±163	1934±183

The test groups received pomegranate juice (Pg) at 1, 2, and 4% in Krebs solution. The data are shown as mean ± SEM. Non-similar superscript letters indicate statistical significance. Stab: Stabilization, HR: Heart rate, CPP: Coronary perfusion pressure, LVDP: Left ventricular developed pressure, RPP: Rate pressure product

There was an infarcted area of 49% in non-treated hypoxic hearts (Figure 2). The hearts treated with pomegranate juice had dose-dependently lower infarcted areas ($P < 0.0001$).

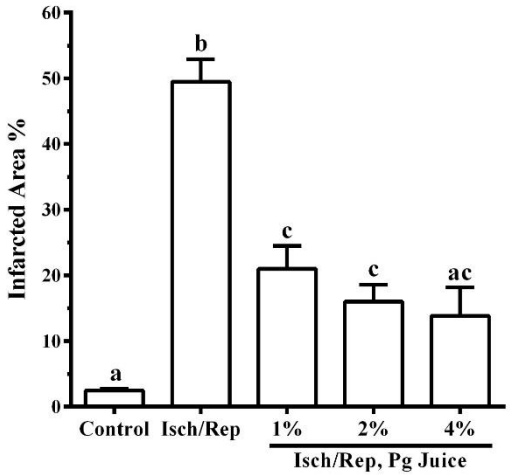

Figure 2. The percentage of the infarcted area of the isolated hearts. The hearts were subjected to 30 min global ischemia (Isch) followed by 90 min reperfusion (Rep). The test groups were treated with 1-4% pomegranate juice (Pg) in Krebs solution. Non-similar letters indicate a significant difference.

The second experiment

The test rats in the second experiment were treated with pomegranate juice for 3 weeks. The isolated hearts from these rats showed significantly higher levels of heart rate, CPP, LVDP, RPP, and dp/dtmax following stabilization compared to the control (Figure 3). All of these parameters approached zero in both groups during global ischemia. Following reperfusion, however, the parameters increased again in both groups but were significantly higher in the test group. The mean infarcted area in the control hearts was 41.6 ± 3.4%, which was about triple that of the test group (13.9 ± 2.8%, $P < 0.0001$, t-test).

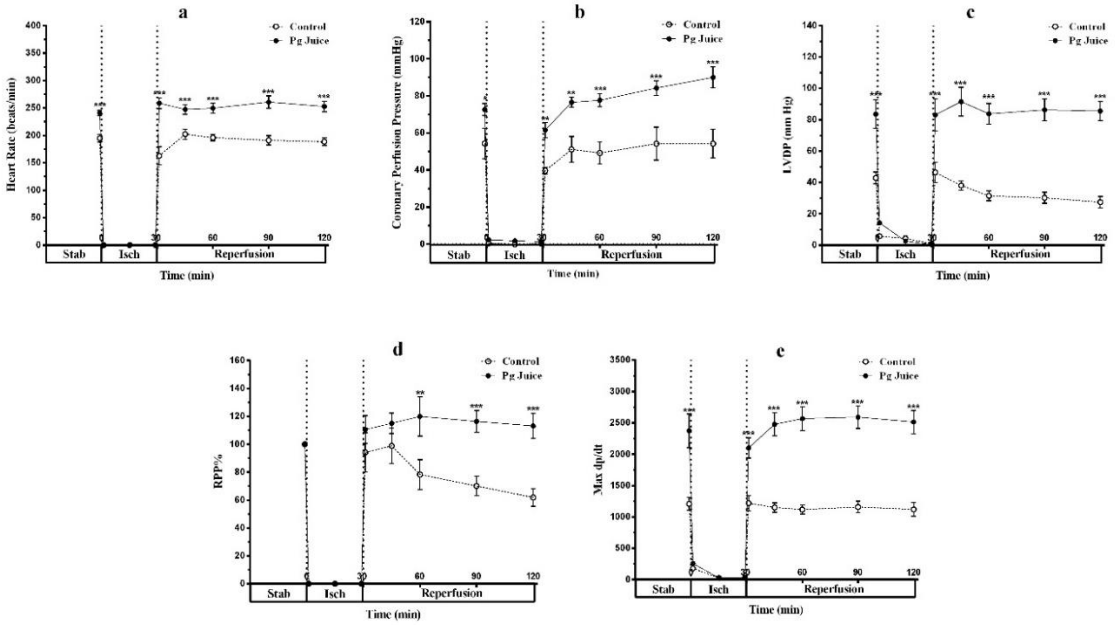

Figure 3. Hemodynamic parameters of isolated rat hearts. Following 30 min stabilization (Stab), the hearts from animals pretreated with pomegranate (Pg) juice (4 ml kg^{-1} BW; 21 d) or placebo (control) were subjected to 30 min ischemia (Isch) and 90 min reperfusion. *P<0.05, **P<0.01, ***P<0.001.

Discussion

To the best of our knowledge, there are few reports regarding the protective effect of pomegranate juice on isolated heart following ischemia and reperfusion. We initially investigated the effect of the juice, supplemented to the Krebs solution, on the performance of perfused rat hearts subjected to ischemia and reperfusion. The changes in the heart rate, CPP, and LVDP were mainly below the threshold of significance. However, the mechanical activity of the heart, as indicated by RPP%, and also, the contractility index, dp/dtmax, were significantly higher in treated hearts. This is while none of these parameters was affected by the juice prior to the induction of ischemia, suggesting minimal effects for the juice, *per se*, in normoxic heart. Subsequent measurement of the infarcted area of the hearts suggests that the observed effects may be, at least partly, due to the protective effects of the juice against cardiac cell damage and death.

In recent years, there has been an extensive body of research, regarding the protective effects of antioxidants with natural origin against myocardial ischemia and reperfusion injury (13-15). Anthocyanins have been shown to reduce myocardial apoptosis and necrosis following global ischemia and reperfusion in isolated rat hearts (16). Using a working heart model, the well-known red grapes phenol, resveratrol, has shown significant myocardial protection against global ischemia and reperfusion (17). Consistent with the present results, resveratrol reduced the infarct size and improved post-ischemic ventricular performance including aortic flow and ventricular developed pressure.

Pomegranate juice contains strong antioxidants such as anthocyanins, ellagic acid, punicalagin, and flavonoids. The juice has shown a stronger antioxidant activity compared to studied fruits (8-10). Despite the vast literature on beneficial cardiovascular effects of the fruit, there is little information regarding its potential protective effects following myocardial ischemia and reperfusion injury. Employing an *in vivo* model of coronary occlusion and reperfusion, the cardioprotective effects of pomegranate polyphenols on rat heart has been studied (18). The treatment has significantly increased left ventricular end-diastolic pressure (LVEDP) and dp/dtmax. In addition, it has reduced the plasma levels of myocardial damage markers, creatine kinase (CK) and lactate dehydrogenase (LDH), and obviously, the infarct size. In a clinical trial on patients with acute coronary syndrome, pomegranate juice was given to the test group, daily, during five days hospitalization period. The heart rate and blood pressure were not affected by the treatment. However, the incidence, intensity, and duration of angina were lower in these patients. Moreover, the marker of cardiac cell damage and death, troponin, was significantly lower in patients with myocardial infarction following 5 days of treatment (19). It is obvious that these results have been observed under different conditions, and despite some similarities, could be compared with *ex-vivo* studies only with great caution. A recent study from our lab focused on the role of nitric oxide in pomegranate-induced cardioprotection (20). A different pomegranate cultivar with dissimilar component quantities was used. However, the results are consistent with the results of the present study. The present study has the advantage of performing complementary *in-vivo* experiments.

During the second stage of this study, the rats were pretreated with pomegranate juice for 21 days. Thereafter, isolated hearts from these animals were subjected to 30 min ischemia followed by 90 min reperfusion, using a Langendorff system. Pretreatment with pomegranate juice significantly increased the heart rate, CPP, LVDP, RPP, and dp/dtmax compared to the control, both before and after the induction of ischemia. The infarct size was also significantly lower in the test group. Except for the rise in CPP, these results suggest cardioprotective properties for the juice against myocardial ischemia and reperfusion. It is noteworthy that the dose of the juice (4 ml kg^{-1} BW) used is roughly extrapolated to 50 ml pomegranate juice in a 76-kg adult human being (21).

Pretreatment of experimental animals with natural antioxidants has shown beneficial effects against ischemia and reperfusion in isolated hearts. In a study by Mokni et al. (2013), supplementation of Krebs solution with resveratrol did not affect contractility of perfused hearts following ischemia/reperfusion. However, when the animals were initially treated with resveratrol for 7 days, the contractile function of the heart was greatly improved (22). In an *in vivo* study, pretreatment of rats with ellagic acid for 10 days improved the heart rate and blood pressure following isoproterenol-induced myocardial infarction. In addition, the serum levels of cardiac cell death biomarkers were significantly decreased, and the extent of

cardiac cell necrosis was also diminished (23). In a rather similar study, the rats were treated with pomegranate for 6 days, then, for the next 6 days, they simultaneously received daunorubicin and pomegranate. The treatment caused a significant reduction in daunorubicin-induced cardiotoxicity as indicated by serum levels of cardiac injury, oxidative stress, and proinflammatory biomarkers (24). Despite the differences in research conditions, these results are consistent with the results of the present study.

In the second stage of this research, the pre-ischemic differences in hemodynamics of the isolated hearts suggest functional/structural changes due to the treatment. The mechanism behind this observation was not further investigated in this study. However, one of the possible explanations may be cardiac hypertrophy. The isolated hearts in the test group were significantly heavier. However, there is no report suggesting such an effect for pomegranate juice. It is noteworthy that we did not manage to reproduce the latter effect in a separate experiment (unpublished), but the chemical composition of the juice, as assessed by LC-mass, was different in these experiments. Further research is needed to validate these findings.

Conclusion

In the present study, the cardioprotective effects of pomegranate against ischemia and reperfusion in isolated rat heart were investigated. Initially, the hearts were treated with pomegranate juice (1-4%) via the perfusion solution. The juice improved cardiac contractility following ischemia and reperfusion and reduced the infarct size. During the second stage of the study, the rats received the juice for 3 weeks. The hearts of these animals were removed and subjected to ischemia and reperfusion using Langendorff system. All studied parameters including the heart rate, CPP, LVDP, RPP, and dp/dtmax were significantly higher, both before and after the induction of ischemia, in the test group compared to the control group. In addition, the pretreatment reduced the infarct size. The results of the present study suggest strong cardioprotective effects for pomegranate juice against myocardial ischemia and reperfusion.

Conflict of interests

The authors declare that there is no conflict of interests.

Acknowledgements

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