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Research Report

The anti-arrhythmic effects of pomegranate (*Punica granatum*) are mainly mediated by nitric oxide

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Abstract.

BACKGROUND: Pomegranate juice (PJ) contains strong antioxidant polyphenols, and cardiovascular beneficial effects have been reported for the fruit.

OBJECTIVE: Using a Langendorff's model, we hypothesized that PJ has antiarrhythmic effects on isolated rat hearts.

METHODS: The hearts in the control group ($n = 10$) were perfused with Krebs solution. The test groups ($n = 10$, each) were further perfused with PJ, L-NAME (L), or both (PJL). Following 30 min stabilization, all hearts experienced 30 min global ischemia and 120 min reperfusion. The hearts were monitored for the occurrence of single and salvo arrhythmias, ventricular tachycardia (VT), and ventricular fibrillation (VF). The cardiac release of creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and nitrite were measured. In addition, the markers of oxidative stress including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and malondialdehyde (MDA) were assayed in the homogenates of the hearts.

RESULTS: The PJ group showed a significant reduction in the occurrence of arrhythmias, an improvement in redox markers, and a rise in nitrite release, compared to the control. Simultaneous treatment with L-NAME significantly reduced, but not fully abolished, these effects.

CONCLUSIONS: The current study suggests strong anti-arrhythmic effects for PJ, which are mainly mediated by nitric oxide.

Keywords: pomegranate, *Punica granatum*, ischemia and reperfusion, ventricular arrhythmia, antioxidant

List of abbreviations

PJ	Pomegranate juice
L	L-NAME
PJL	Pomegranate juice and L-NAME
VT	Ventricular tachycardia

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VF	Ventricular fibrillation
CK-MB	Creatine kinase
LDH	Lactate dehydrogenase
SOD	Superoxide dismutase
GPX	Glutathione peroxidase
CAT	Catalase
MDA	Malondialdehyde
VPMBs	Ventricular premature beats
ECG	Electrocardiogram
NO	Nitric oxide

1. Introduction

Cardiovascular diseases are the most prevalent causes of mortality worldwide. Among different cardiovascular disorders, ventricular arrhythmia following coronary occlusion and reperfusion plays a major role in this regard [1, 2]. A wide variety of intracellular as well as cell-membrane bound proteins are involved in the pathophysiology of these arrhythmias [3]. For instance, the lack of ATP and the subsequent inhibition of Na^+/K^+ ATPase leads to the disruption of Ca^{2+} transport via Na^+/Ca^{2+} exchanger. This leads to intracellular Ca^{2+} overload, which is a key determinant in the development of delayed afterdepolarization and the occurrence of ectopic beats [4, 5]. Disturbed homeostasis of *K*+ [1], Na^+ [6], and *H*+ [7] ions are among other trigger mechanisms of ischemia and reperfusion-induced arrhythmias. In addition to the mentioned mechanisms, reactive oxygen species (ROS) play an important role in the pathophysiology of post-ischemic arrhythmias [8–10].

A variety of different antioxidants have been successfully used to eliminate the harmful effects of ischemia and reperfusion on the heart [5, 11]. Among these free radical scavengers, plant-origin polyphenols have drawn specific attention in recent decades [12, 13]. Pomegranate (*Punica granatum*) is a rich source of strong phenolic antioxidants such as ellagic acid, punicalagin, and gallic acid [14]. In addition to antioxidant properties, anti-carcinogenic [15], anti-inflammatory, and anti-nociceptive [16] effects have been reported for the fruit. The recent growing literature suggests promising cardioprotective effects for the juice [17]. However, the mechanisms behind these beneficial effects are still obscure.

2. Materials and methods

2.1. The juice

Pomegranate juice (PJ) was prepared using a manual juicer. It was lyophilized (Zirbus, Germany) and kept under dark and dry conditions until used. On the day of the experiment, the lyophilized powder was dissolved in Krebs solution (1.8 mg ml^{-1}) to yield the original concentration and then was added to the perfusion solution (2%: v/v) of the test groups.

The phenolic content of the dried juice was analyzed using Folin-Ciocalteu procedure [18]. Briefly, 50 mg of the sample was dissolved in 100 ml of mixed acidified methanol (containing 3% HCl) and distilled water (60/40: v/v). Following filtration, 100 μl of the mixture was added to 2 ml of 2% sodium bicarbonate solution and 100 μl of Folin-Ciocalteu reagent (Sigma, USA). The final solution was left at room temperature for 2 h before being measured for optical absorbance (750 nm). A gallic acid standard curve (0-10 mg ml^{-1}) was used for the measurement of the total phenol content.

A high-performance liquid chromatography-mass spectroscopy (LC-MS, Agilent Technology 6410-QQQ, USA & Japan) method was used for the determination of the main polyphenols of the juice. Chromatographic separation was achieved using a C18 column (250 × 4.6 mm, particle size: 5 μm, 25ºC). A gradient procedure was employed: Solution A consisted of 1% formic acid in distilled water and solution B was acetonitrile. The flow rate was 0.5 ml min^{-1}. The percentage of solution B was 5% for the first 5 min and then experienced 5% increases every 5 min, up to 20 min. Solution B was maintained at 90% during 25–30 min. The column eluant passed through the photodiode array detector (760 nm) before being directed toward the electrospray ionization (ESI) interface and the triple quadrupole mass spectrometer. A negative mode and a full scan mass spectrum (100–1500 m/z) were employed for the ESI. The temperature of the electrospray source was set at 100ºC. The cone and the capillary voltages were set at 40 V and 4.0 kV, respectively. The nebulizing gas (N_2) had a velocity of 6 L min^{-1} (25 psi, 300 ºC). Agilent 6410 Triple Quad LC/MS Software was used for data processing.

2.2. The animals

Adult Wistar rats weighing 250–300 g were used for the experiment. The animals were raised in the Animal Unit of the School of Veterinary Medicine, Ferdowsi University of Mashhad. All experimental procedures were in accordance with the guidelines of the Animal Ethics Committee of the Ferdowsi University of Mashhad (IR.UM.REC.1398.127).

2.3. The experimental groups

The hearts from anesthetized rats were removed and were randomly assigned to one of the four experimental groups ($n = 10$, each). The control group was perfused with Krebs solution. For the test groups, the Krebs solution was supplemented with 2% PJ (v/v), N^G-nitro-L-arginine methyl ester (L-NAME) 100 μM (the L group), or 2% PJ plus L-NAME 100 μM (the PJL group) throughout the experiment.

2.4. Perfusion of the hearts

The rats were anesthetized via intraperitoneal injection of thiopental sodium (50 mg k^{-1}). Following i.v. injection of heparin (50 IU), the chest was opened and the heart was excised. It was immediately mounted on Langendorff apparatus. The heart was perfused with modified Krebs solution consisted of (in mM): NaCl 118, KCl 7.4, $NaHCO_3$ 25, KH_2PO_4 2.1, $CaCl_2$ 1.25, $MgSO_4$ 1.2, and glucose 11. The solution had a pH of 7.4 and the flow rate was 10 ml min^{-1} (37ºC). Aeration of the solution with oxygen and carbon dioxide (95%:5%, v:v) started 15 min before the beginning of the experiment and continued throughout the experiment. The heart was first allowed to stabilize for 30 min and then was subjected to global ischemia (30 min) via turning off the peristaltic pump. Reperfusion continued for 120 min, thereafter.

2.5. Monitoring and classification of ventricular arrhythmias

Throughout the experiment, the electrocardiogram (ECG) was monitored using a PowerLab Electrophysiological Instrument (ML11, Australia) via two electrodes attached to the apex and the right auricle of the heart. Identification and classification of ventricular arrhythmias were conducted according to the Lambeth Convention [19]. Accordingly, the separate single ventricular premature beats (VPMBs) were regarded as single arrhythmias. Two or three consecutive VPMBs were recognized as salvo arrhythmias, while more sequential PMVBs were considered as ventricular tachycardia. According to Lambeth convention, ventricular fibrillation (VF) was characterized as ECG signals where no distinct QRS complexes could be recognized.

2.6. Biochemical parameters

To measure some cardiac biomarkers, the perfusate of the heart was sampled at specific time-points. The markers of myocardial damage including lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were evaluated using a spectrophotometric assay kit (Pars Azmun, Iran). The effluent level of nitrite, a metabolite of nitric oxide (NO), was measured at 2 min reperfusion using the Griess method (Sib Biotec Co., Iran; [20].

The markers of oxidative stress were assayed in the homogenates of the hearts. The level of superoxide dismutase (SOD) activity was assayed using the Ransod assay kit (Randox, Germany). The method is based on the oxidation of xanthine to generate superoxide radicals, which lead to the formation of a red formazan dye. The SOD activity was estimated by the level of inhibition of this reaction [21]. The levels of glutathione peroxidase (GPX) in cardiac samples were measured as described by Paglia and Valentine [22]. The level of catalase (CAT) activity was assayed via the reaction of hydrogen peroxide with ammonium molybdenum. The optical absorbance of the resultant yellowish color was detected at 410 nm. Malondialdehyde (MDA) was measured as an indicator of lipid peroxidation. This method was based on MDA reaction with thiobarbituric acid as previously described [23]. The Biuret method was employed to measure the total protein content of the homogenate (Pars Azmun, Iran).

2.7. Statistical analysis

GraphPad Prism V6.0 software (GraphPadPrism Software, USA) was used for drawing the figures and statistical analysis. The results regarding ventricular arrhythmias were expressed as median and quartiles and were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparisons post-test. All other results were expressed as mean + SEM. The statistical comparisons for CK-MB, LDH, and nitrite were carried out using a two-way analysis of variance (two-way ANOVA). However, one-way ANOVA was used for the markers of oxidative stress. Both tests were followed by Bonferroni's multiple comparison post-test. In all cases, differences with $p < 0.5$ were considered as statistically significant.

3. Results

3.1. Chemical analysis of pomegranate juice

The total polyphenol content of the juice was estimated to be 4200 mg/L. The estimated percentages of the main polyphenol contents including ellagic acid, gallic acid, punicalin, and gallagic acid were 58.1%, 13.6%, 12.8%, and 15.5%, respectively (Fig. 1).

3.2. Ventricular arrhythmias

The results regarding different types of arrhythmias including single and salvo arrhythmias, ventricular tachycardia, and ventricular fibrillation in different experimental groups are shown in Fig. 2. Accordingly, only sparse arrhythmias have occurred before the induction of ischemia, and there were no significant differences among the studied groups. During ischemia, however, there were higher rates of arrhythmias, but both the incidence and the duration of ventricular fibrillation were significantly lower in the PJ group, compared to the control. The highest rates of arrhythmias were recorded during reperfusion, especially in the control and L-NAME groups. However, ventricular fibrillation had a lower incidence during reperfusion, compared to the ischemic period. The frequencies of single, salvo, and VT, as well as the total duration of VT, were significantly lower in the PJ group, compared to the control. Nevertheless, when L-NAME accompanied PJ, these arrhythmias occurred significantly more often.

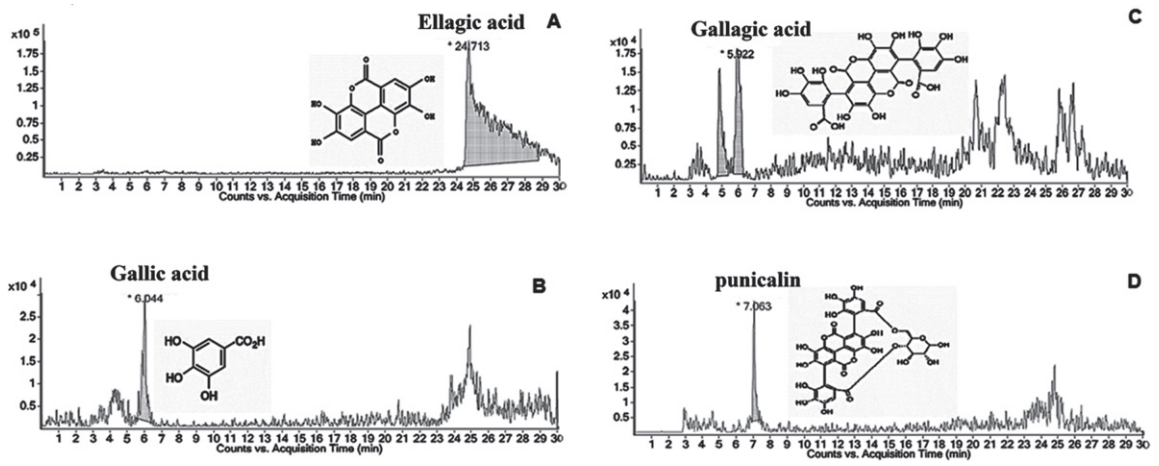

Fig. 1. The LC-MS chromatograph of pomegranate juice.

3.3. The biochemical markers

The markers of myocardial cell injury, including CK-MB and LDH, were measured in the coronary effluent (Table 1). Before the induction of ischemia, there were no significant differences among the studied groups. However, during both ischemia and reperfusion, there were statistical differences among all experimental groups. The lowest amounts of these markers were found in the PJ group, while the highest marker levels were detected in the L group. In the PJL group, the markers were significantly higher compared to the PJ group, though the levels were statistically higher in the control group.

The markers of oxidative stress including SOD, GPX, and catalase showed significant increases in the PJ group (Fig. 3). The addition of L-NAME to PJ significantly diminished this increase. The level of SOD in the PJL group, however, did not decline to that of the control group. This is while a reverse pattern was observed for MDA levels, i.e. the lowest amount was observed in the PJ group, then the PJL group and finally the control and the L groups.

The nitrite levels were measured in the heart perfusate both at the beginning and at the end of the reperfusion period (Fig. 4). In both cases, the highest amounts were observed in the PJL group, while the lowest concentrations were detected in the L group ($p < 0.05$). There was no statistical difference between the PJL and the control groups in this regard.

4. Discussion

In this study, we initially investigated the protective effect of pomegranate juice against ischemia and reperfusion-induced arrhythmias. Arrhythmias occurred during both ischemia and reperfusion periods. However, while ventricular fibrillation predominantly occurred during the ischemic period, single and salvo arrhythmias, as well as ventricular tachycardia, were mainly observed during reperfusion. Pomegranate juice had astonishing effects on the occurrence of all studied arrhythmias. Since one of the main outcomes of coronary occlusion and reperfusion is myocardial cell injury, we also assessed this damage via measuring the related biomarkers, CK-MB and LDH. Once again, the juice showed a remarkable cardioprotective effect. In addition, the indices of oxidative stress including SOD, GPX, CAT, and MDA were evaluated in this research. Consistent with our other results, treatment with PJ significantly improved the status of these markers.

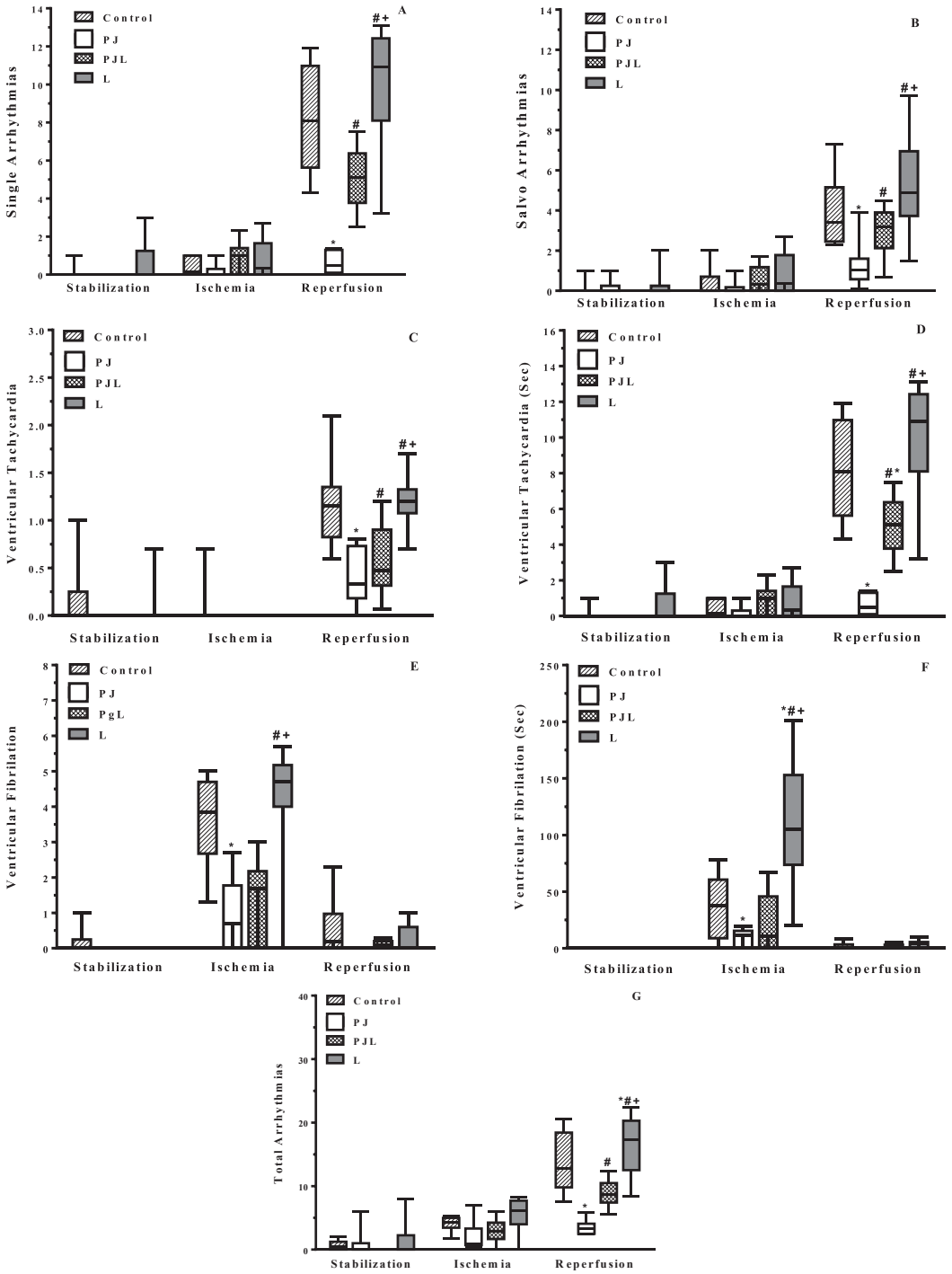

Fig. 2. The effects of pomegranate juice on different types of ventricular arrhythmias. The perfused isolated hearts in the control (C), 2% pomegranate juice (PJ), PJ+ L-NAME (PJL), and L-NAME (L) groups underwent 30 min global ischemia followed by 120 min reperfusion. Data are presented as median ± Q1-Q3: *: $p < 0.05$ vs control, #: $p < 0.05$ vs PJ, +: $p < 0.05$ vs PJL.

Table 1
The levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in the effluent of isolated perfused heart. All hearts were allowed to stabilize (Stab) for 30 min and then experienced 30 min global ischemia and 120 min reperfusion

		Stab	Reperfusion	
	Time/Groups	28 min	5 min	120 min
CK-MB (μg/L)	C	22.8 ± 3.1	83 ± 4	64.8 ± 2.8
	PJ	16.3 ± 0.4	$31.2 \pm 1^{*}$	$22.4 \pm 1.6^{*}$
	PJL	21 ± 1.4	$57.3 \pm 1.9^{*\#}$	$40.8 \pm 1.9^{*\#}$
	L	24.5 ± 1.4	$129.7 \pm 1.4^{*\#+}$	$102 \pm 2.5^{*\#+}$
LDH (μg/L)	C	122.1 ± 1.6	259.3 ± 4	229.8 ± 7
	PJ	118 ± 1.3	$148.8 \pm 2.4^{*}$	$136.2 \pm 1.6^{*}$
	PJL	122.7 ± 2.1	$232.3 \pm 2.7^{*\#}$	$188.6 \pm 5.3^{*\#}$
	L	126.2 ± 1.8	$316 \pm 4.8^{*\#+}$	$304.5 \pm 2^{*\#+}$

The experimental groups consisted of the control (C), 2% pomegranate juice (PJ), 2% PJ + L-NAME (PJL), and L-NAME (L). *: $p < 0.05$ vs control, #: $p < 0.05$ vs PJ, +: $p < 0.05$ vs PJL.

We further investigated the involvement of NO in the above protections using the competitive NO synthase inhibitor, L-NAME. The results suggest a pivotal role for NO in the antiarrhythmic effects of PJ. Nitric oxide was also involved in the improvement of oxidative status and reduced cardiac cell damage in the PJ group. The results regarding the nitrite levels in the effluent of the isolated hearts further supported this conclusion. While L-NAME declined nitrite release to that of the control group, it did not fully abolish the antiarrhythmic effect of the juice. Rather consistent results were observed regarding the other studied parameters. These observations suggest that, in addition to NO as the main mediator of cardioprotection, other mechanisms seem to be also involved.

Excessive production of ROS plays an important role in the occurrence of ischemia and reperfusion-induced arrhythmias. These highly destructive chemicals are produced via the respiratory chain, lipid metabolism, and ischemia-activated xanthine/hypoxanthine oxidase [24]. The resultant oxidative stress leads to lipid peroxidation and oxidation of proteins and thiol groups. As a consequence, the configuration and permeability of the cell membrane, as well as the function of proteins, are disturbed. This way, the activities of sarcolemmal Ca^{2+}-ATPase and Na^{+}-K^{+} ATPase are depressed. These changes lead to intracellular Ca^{2+} overload, which is a pivotal player in the development of cardiac arrhythmias and cell death [25]. It has been shown that mitochondrial permeability transition pore (mPTP), a protein that is formed in the inner membrane of mitochondria under certain pathological situations, can open in response to ROS buildup and myocardial ischemia and reperfusion. This phenomenon seems to play a crucial role in the occurrence of cardiac arrhythmia, myocardial cell necrosis, and apoptosis [26].

Antioxidants have been widely used for their cardioprotective properties following myocardial ischemia and reperfusion. Superoxide dismutase mimetics have been successfully used to alleviate cardiac damage following ischemia and reperfusion under both *in-vivo* and *ex-vivo* conditions [27]. Using an isolated rat heart model, in addition to SOD, glutathione and ascorbic acid significantly reduced the incidence of reperfusion-induced arrhythmias [5]. Recently, the researchers have paid close attention to plant-origin antioxidants. For instance, anti-arrhythmic effects have been reported for the natural polyphenol extracted from the grape seed, proanthocyanidin [28]. Consistently, several studies have investigated the cardioprotective effects of some Chinese traditional medicines. In this regard, the effects of tanshinone from *Salvia miltiorriza*, EGb 761 from *Ginkgo biloba*, and chinonin from *Anemarrhena rhizome* against myocardial ischemia and reperfusion-induced injury were evaluated. These active ingredients mitigated cardiac damage and decreased the incidence of cardiac arrhythmias. The effects were attributed, at least partly, to the antioxidant properties of the studied chemicals [24]. Similar

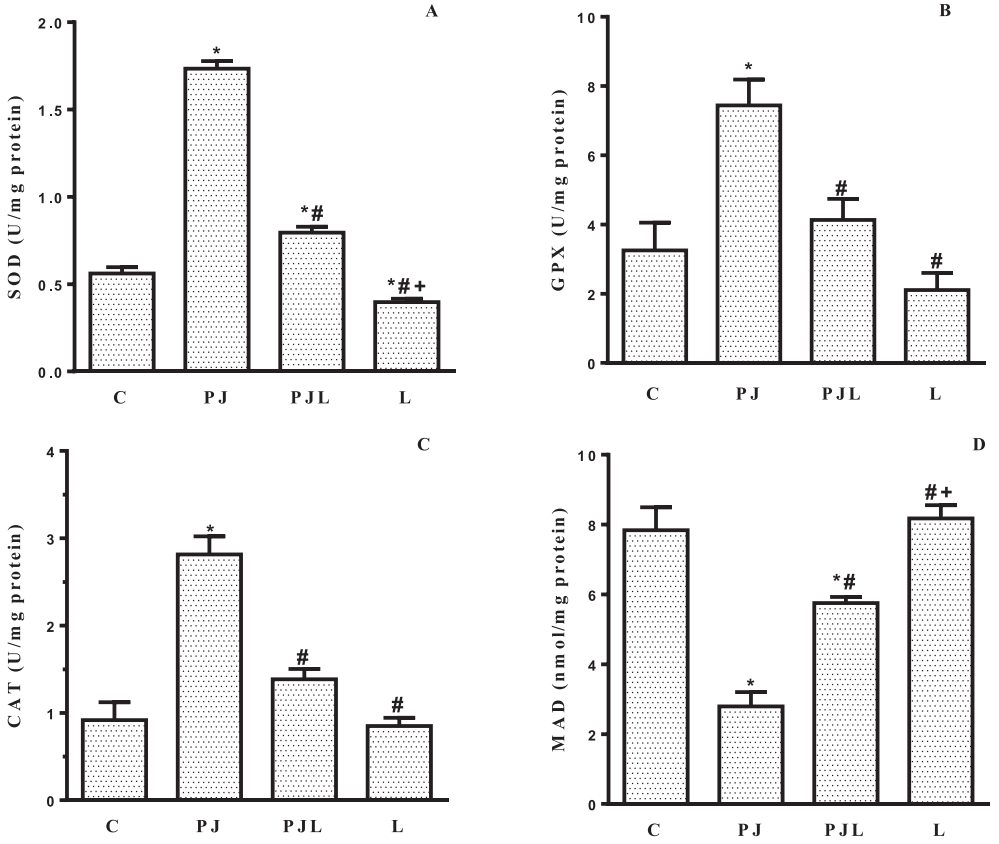

Fig. 3. The effect of pomegranate juice on the redox status of isolated hearts. The hearts in different experimental groups including the control (C), 2% pomegranate juice (PJ), PJ + L-NAME (PJL), and L-NAME (L) underwent 30 min ischemia and 120 min reperfusion. SOD: superoxide dismutase, GPX: glutathione peroxidase, CAT: catalase, MDA: malondialdehyde, *: $p < 0.05$ vs control, #: $p < 0.05$ vs PJ, +: $p < 0.05$ vs PJL.

results have been reported using extracts of green tea [29] or *Scrophularia frigida* [30] on isolated rat hearts subjected to ischemia and reperfusion. Resveratrol is a potent antioxidant derived from some plants such as red grapes. There is a tremendous amount of evidence suggesting cardioprotective and anti-arrhythmic effects for this natural polyphenol [31–35]. Similar effects have been reported for piceatannol, a derivative of resveratrol [36, 37].

Pomegranate has been subject to intensive research during the last decade due to its remarkable antioxidant properties. The fruit has shown a higher antioxidant activity compared to other studied fruit juices, red grapes, or green tea [38, 40]. Pomegranate polyphenols caused a significant reduction in the serum markers of myocardial cell damage, LDH and CK-MB in rats subjected to 45 min coronary occlusion and 180 min reperfusion [41]. Consistent results were obtained in isoproterenol-induced cardiac infarction in rats [42]. Our team performed a clinical trial on patients hospitalized with symptoms of ischemic heart disease. Five-days of treatment with PJ significantly decreased the serum levels of MDA and troponin in these patients [43]. Furthermore, using Langendorff-perfused rat-hearts, we recently reported cardioprotective effects for PJ following 30 min global ischemia and 90 min reperfusion [44]. To the best of our knowledge, there is no previous report regarding the anti-arrhythmic effects of PJ. However, ellagic acid, one of the main polyphenols found in the fruit, has shown consistent results to the present study in reducing the incidence of $CaCl_2$-induced arrhythmias in anesthetized rats [45].

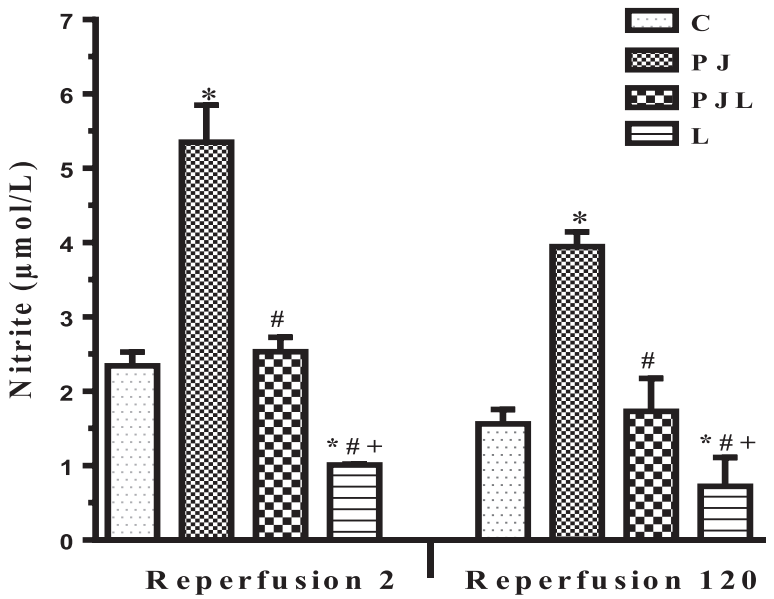

Fig. 4. The nitrite levels in the effluent of isolated hearts. The hearts in the control group (C) were perfused with Krebs solution, the perfusion solution in the test groups was further supplemented with 2% pomegranate juice (PJ), PJ and L-NAME (PJL), or L-NAME (L). All hearts were subjected to 30 min ischemia followed by 120 min reperfusion. The symbols *, #, and + indicate $p < 0.05$ compared to the control, PJ, and PJL groups, respectively.

The current study suggests a significant role for NO in the anti-arrhythmic effects of PJ. Nitric oxide is well known for its vasodilator effect. However, it plays a key role in the modulation of heart rate and ventricular contractility [46, 47]. In fact, anti-arrhythmic effects have been reported for NO [48]. It modulates several ion channels in the heart, such as ATP-sensitive potassium (K_{ATP}) channels, and L-type Ca^{2+} channels [49]. Hereby, NO may protect the heart against ischemia/reperfusion-induced cell damage and arrhythmias. For instance, an increase in *K+* current via K_{ATP} channels may protect the heart against ischemic damage via shortening the duration of the action potential. This leads to a shorter contraction, which results in a lower ATP demand, and hence, less cardiac damage [50]. In addition to sarcolemmal K_{ATP} channels, nitric oxide is also a modulator of mitochondrial K_{ATP} channels, through which it may exert anti-arrhythmic effects [51, 52]. Calcium channel blockers are routinely prescribed for patients with ventricular arrhythmias [48, 53]. However, conflicting effects have been reported for the effect of NO on calcium current in myocardial cells. These controversies have been attributed to the differences in the experimental conditions [49, 54]. We have recently reported a central role for NO in cardioprotective effects of PJ following ischemia and reperfusion [44]. The anti-arrhythmic effects of some plant-origin antioxidants have been attributed to NO [55, 56]. These reports are consistent with the results of the current research. However, the anti-arrhythmic effect of resveratrol has not been related to NO [57].

In this research, despite the significant role of NO in the protective effect of PJ, regarding both cell damage and arrhythmias, the effects were not fully abolished in the presence of NO synthase inhibitor, L-NAME. This suggests other mechanisms are also involved. The antioxidant property of PJ may be regarded as a strong candidate. However, further research is needed to fully understand these mechanisms.

This study had both advantages and disadvantages. Among the advantages, this research was performed on isolated hearts. This way, most of the interfering parameters present in in vivo studies, such as the modulating effects of the autonomic nerves, are abolished. Besides, the use of PJ, instead of the active ingredients, provides more applied results. Different PJ phytochemicals may have conflicting effects, and the overall effect of PJ will

remain obscure in studies using the individual active compounds. Using PJ, however, has the disadvantage of uncertainty regarding the main involved ingredient. As another shortcoming, PJ was directly supplemented to the perfusion solution. This is while PJ is normally ingested and may undergo several biological modifications within the digestive system or following absorption. It is obvious that ex vivo and in vivo studies are complementary and can not replace each other. Therefore, the results from the current study can not be extrapolated to clinical conditions in human beings unless other supplementary studies are performed.

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Conflict of interest

The authors have no conflict of interest to report.

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