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ANTIARRHYTHMIC EFFECTS OF POMEGRANATE (*Punica granatum*) JUICE ON ISOLATED RAT HEARTS FOLLOWING ISCHEMIA AND REPERFUSION

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Cardioprotective effects of pomegranate *(Punica granatum*) have been discovered in recent years. This study was aimed to assess the potential antiarrhythmic activity of pomegranate juice in isolated rat hearts subjected to hypoxia and reperfusion. The antiarrhythmic effects of the juice were studied in two phases: first, the isolated hearts of anesthetized rats were subjected to 30 min global ischemia and 90 min reperfusion. In four groups (n = 9 each), hearts received the pomegranate juice via Krebs solution at 0 (control), 1, 2 or 4% concentration. For the second phase, two groups of rats (n = 10) were gavaged with placebo (control) or pomegranate juice (4 mL kg^{-1} BW d^{-1}) for three weeks. The isolated hearts from these animals underwent 30 min ischemia and 90 min reperfusion. The occurrence of single and salvo arrhythmias, ventricular tachycardia, and ventricular fibrillation were significantly lower in the juice-treated test groups in both phases of the study. Thus, results suggest strong protective effects of pomegranate juice against ischemia and reperfusion-induced arrhythmias in isolated rat hearts.

Keywords: pomegranate; *Punica granatum*; arrhythmia; anti-arrhythmic effect; ischemia and reperfusion.

1. INTRODUCTION

Cardiac arrhythmias occurring as a result of myocardial ischemia and reperfusion are the main causes of deaths in patients with coronary artery disease. Several electrophysiological disturbances contribute to this disorder [1]. During ischemia, the lack of oxygen and diminished intracellular adenosine triphosphate (ATP) level disrupt Na^+/K^+-ATPase activity. Intracellular sodium concentration is increased, and upon disturbed sodium/calcium exchange, calcium ions are accumulated within the cells. Calcium overload is a key player in the occurrences of fatal arrhythmias [2]. On the other hand, extracellular K^+ concentration rises due to leakage of K^+ out of the hypoxic cardiomyocytes, leading to a less negative resting membrane potential. The duration of the action potential is also shortened because of K_{ATP} channel activation [3]. Anaerobic metabolism and inhibition of Na^+/H^+ exchanger lead to accumulation of H^+ within the cells. Both H^+ and Ca^{2+} ions are believed to contribute to ischemic uncoupling of gap junction channels in cardiomyocytes. The conduction velocity, a key player in cardiac arrhythmias, is reduced as a result [4]. In addition to all mentioned disturbances, recent studies suggest a pivotal role for reactive oxygen species (ROS), and the beneficial use of natural anti-oxidants are the subject of intensive research in this regard [5].

Several therapeutic effects have been reported for pomegranate (*Punica granatum*) fruit [6]. There is a growing body of research regarding anti-carcinogenic effects of the fruit. It has shown anti-metastatic, anti-proliferative and anti-invasive effects against different cancerous cells, in animal models or *in vitro* studies, as well as in human clinical trials [7]. Anti-nociceptive and anti-inflammatory effects have been also reported for the fruit [8]. A polyphenol-rich extract of the fruit has been able to inhibit the expression of interleukin (IL)-6, IL-8, mitogen-activated protein kinases (MAPKs), and the nuclear factor kappa B (NF-κB) in human KU812 cells [9]. In addition, ellagic acid, punicalagin, and gallic acid, isolated from pomegranate, were able to inhibit lipopolysaccharide (LPS) induced production of nitric oxide (NO), prostaglandin E2 and IL-6 [10]. Powerful antioxidant properties have been reported for pomegranate [11]. The antioxidant capacity of the fruit juice has been reported to be three times higher than those of red wine and green tea [12]. Among other therapeutic properties, cardiovascular protective effects have been also proposed for the fruits [13].

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The present study aimed to assess the potential antiarrhythmic effects of pomegranate juice in isolated rat hearts subjected to hypoxia and reperfusion.

2. MATERIALS AND METHODS

2.1. Fruit Juice Preparation

Pomegranate juice was extracted from arils using a manual pomegranate squeezer. The juice was lyophilized and kept in a cool dry container until use. The powder was reconstituted with Krebs solution to yield the original concentration for use.

2.2. Chemical Analysis

Total polyphenols of the lyophilized juice were measured using Folin-Ciocalteu method [14]. One milligram of the sample was dissolved in 2 ml methanol: distilled water (6:4, V/V) and was then added to 10 fold diluted Folin-Ciocalteu reagent (1:1, V/V). Two hundred microliters of the latter mixture were added to 2 mL of 2% sodium carbonate solution and were then allowed to stand at room temperature for 90 min. The optical absorbance of the final solution was measured at 760 nm using a spectrophotometer. The concentration of tannic acid equivalent was estimated using a standard curve.

Main polyphenols of the juice were analyzed using high-performance liquid chromatography-mass spectroscopy system (HPLC/MS, Agilent Technology 6410-QQQ, US & Japan) in a gradient protocol regime. The column (C18, 25 cm × 4.6 mm, 5 μm particles) was eluted at 0.5 mL min^{-1} rate by a mobile phase consisted of solution A (1% formic acid in distilled water) and solution B (acetonitrile). Solution B percentage was varied according to the following gradient protocol: 0 – 5 min, 5%; 5 – 10 min, 10%; 10 – 15 min, 15%; 15 – 20 min, 20%; 20 – 25 min, 60%; and final 25 – 30 min; 90%.

2.3. Experimental Animals

All experimental procedures were approved by and performed in compliance with the Animal Ethics Committee of the Ferdowsi University of Mashhad. Male Wistar rats (weighing 200 – 250 g) were used for the experiments. All animals were acclimated and housed at the temperature of 24 ± 2°C and in the relative humidity of 40 – 60% in a 12-hour dark/light cycle in the Animal Unit of the School of Veterinary Medicine, the Ferdowsi University of Mashhad for at least 5 days before commencement of the experiments.

2.4. Experiment Planning

The study was performed in two phases. During the first phase, isolated rat hearts, in groups of 9, were perfused with different concentrations of pomegranate juice (1, 2 and 4%). The fourth group (the hypoxic control) was not treated with the juice. All four groups underwent 30 min global hypoxia followed by 90 min reperfusion. The fifth group (the normoxic control) was not treated with the juice and was not subjected to hypoxia and reperfusion. In the second phase, two groups of 9 rats each were gavaged with placebo or the juice (4 mL kg BW^{-1}) daily for 3 weeks. The isolated hearts of all rats were then removed under anesthesia and were studied using Langendorff setup. All hearts underwent 30 min hypoxia and 90 min reperfusion under the same conditions.

2.5. Perfusion of Isolated Hearts

Rat hearts were carefully removed from anesthetized animals (thiopental sodium, 60 mg/kg BW) and were immediately mounted to the aortic cannula of the Langendorff apparatus. The coronary perfusion pressure was monitored using a pressure transducer (MLT844, AD Instruments, Australia) connected above the aortic cannula via a three-way stopcock. Intra-ventricular pressure could be measured via another pressure transducer using a latex balloon inserted into the left ventricle through the left atrium. The electrocardiogram (ECG) was recorded via electrodes attached to the apex and the right atrium (ML865, AD Instruments, Australia). The perfusion solution represented modified Krebs solution containing (in mM) NaCl 118, KCl 2, $CaCl_2$ 1.23, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11 at 37°C. The solution was saturated with 95% O_2 and 5% CO_2 and pH was maintained at 7.4. The hearts were allowed to stabilize for 30 min and then subjected to 30 min global hypoxia by stopping the Krebs flow to the hearts. Then, the hearts were reperfused for 90min.

Evaluation of cardiac arrhythmias was performed in accordance with Lambeth Convention [18]. Accordingly, the arrhythmias were categorized into four classes: single premature beats (single), two or three successive premature beats (known as salvo), ventricular tachycardia (VT) and ventricular fibrillation (VF).

2.6. Statistical Analysis

Statistical analysis and drawing the graphs were achieved using GraphPad Prism Software (GraphPad Software Inc., USA). All data are presented as the mean values with standard error of the mean (mean ± SEM). The data were analyzed using two-way analysis of variance (2-way ANOVA) followed by Bonferroni post-test. In all cases, statistical differences with $p < 0.05$ were considered as significant.

3. RESULTS

During the first phase of the study, the hearts in the normoxic control group showed minimal arrhythmias throughout the experiment. Compared to all other experimental groups, the non-treated hypoxic/reperfused group had

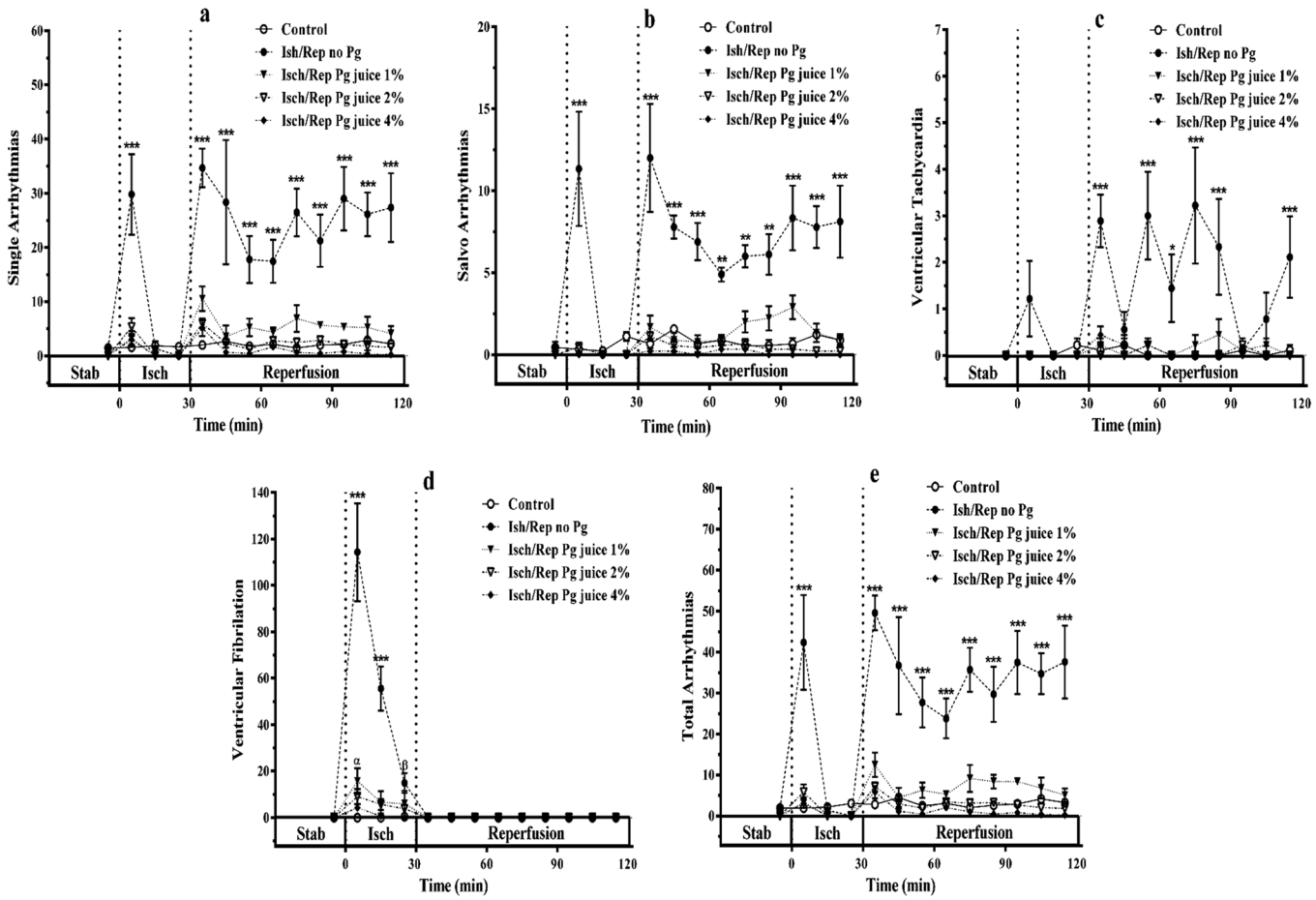

Fig. 1. The incidence of arrhythmias in isolated rat hearts. Following 30 min stabilization (Stab), all hearts, except for the normoxic control group, were subjected to 30 min global ischemia (Isch) and 90 min reperfusion (Rep). Test groups were perfused with Krebs solution containing 1 – 4% pomegranate (Pg) juice; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to all other groups; α: $p < 0.05$ compared to the control; β: $p < 0.05$ compared to the control and Pg 4% juice.

a significantly higher incidence of single arrhythmias at the beginning of ischemia, as well as throughout the reperfusion period (Fig. 1*a*). The prevalence of single arrhythmias in the test groups treated with pomegranate juice was significantly lower and was not statistically different from that of the control group. A rather similar pattern was observed for salvo arrhythmias and VT (Figs. 1*b* and 1*c*). VT mainly occurred during the ischemic period in the non-treated group (Fig. 1*d*). The total number of arrhythmias was consistent with the single, salvo and VT, being more common at the beginning of ischemia and throughout the reperfusion period (Fig. 1*e*).

In the second phase of the study, the isolated hearts from both groups underwent ischemia and reperfusion. Single, as well as salvo arrhythmias, were observed at the beginning of ischemia and throughout reperfusion (Figs. 2*a* and 2*b*). The incidence of these arrhythmias was significantly lower in the test group. The prevalence of VT was low in both groups but the difference was still significant (Fig. 2*c*). VF was mainly detectable during ischemia (Fig. 2*d*). The total incidence of arrhythmias was significantly lower in the test group throughout the reperfusion period (Fig. 2*e*).

4. DISCUSSION

In this work, we investigated the potential antiarrhythmic effect of pomegranate juice in isolated rat hearts subjected to ischemia and reperfusion. During the first phase of the study, the hearts of the test groups were perfused with Krebs solution containing different concentrations of pomegranate juice. During the second phase of the study, the rats were pre-treated with the juice for 3 weeks. Then, the hearts were removed under anesthesia and subjected to ischemia and reperfusion. Both methods, i.e., the treatment of hearts and pre-treatment of the animals with pomegranate juice, resulted in significant protection against ischemia and reperfusion-induced arrhythmias compared to the control groups.

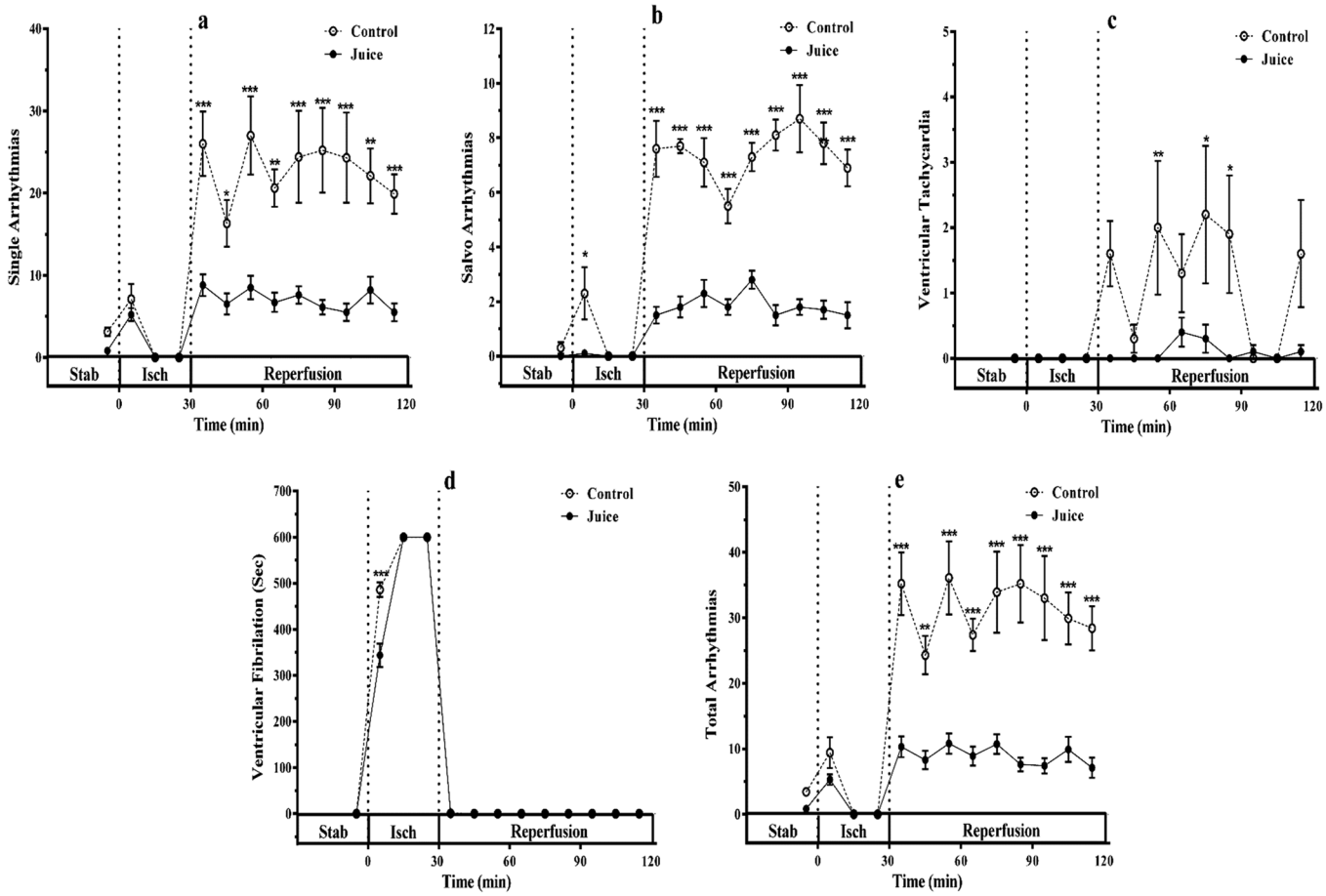

Fig. 2. Different types of arrhythmias in perfused rat hearts. The hearts from rats pre-treated with pomegranate juice (4 mL kg^{-1} BW d^{-1}, 3 weeks) were stabilized for 30 min and then subjected to 30 min ischemia (Isch) and 90 min reperfusion (Rep). The perfusion solution in the test groups contained 1 – 4% pomegranate (Pg) juice; Stab: stabilization; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ compared to the control group.

To the best of our knowledge, there are no reports on a similar research in the literature. However, antiarrhythmic effects have been reported for natural antioxidants [13]. For instance, employing an *in vivo* model of coronary occlusion and reperfusion, the methanol extract of Chilean blackberry *Aristotelia chilensis* showed antiarrhythmic effects in rats [15]. Consistently, potential protective effects against ischemic arrhythmias were proposed for the extract of Ginkgo biloba and its active ingredient ginkgolide via inhibition of I_{Kr} and $I_{Ca\text{-}L}$ currents [16]. Similar effects have been reported for the plant-derived alkaloid, berberine [17] and the red grapes antioxidant, resveratrol [18]. In fact, the latter compound has diminished both the duration and the incidence of VT and VF in anesthetized rats subjected to regional ischemia [19].

Pre-treatment of animals with plant-derived antioxidants has shown protective effects against ischemia and reperfusion-induced arrhythmias. In one study, the rats were gavaged with total flavonoids from Ganshanbian (*Herba Hyperici Attenuati*) for 7 days. Regional ischemia was then induced via ligation of the left main coronary artery under anesthesia. The incidence and duration of ischemia and reperfusion-induced arrhythmias were significantly lower in pre-treated rats compared to the control group [20]. In a similar study, the rats were pre-treated with olive leaf extract for 1, 3, 7, 14, and 28 days before the induction of regional ischemia under anesthesia. In this research, pre-treatment of the animals for more than 7 days, and particularly for 28 days, attenuated the severity and occurrence of arrhythmias [21]. In another study, 10 days of pre-treatment with ellagic acid, a potent antioxidant present in pomegranate, has resulted in cardioprotection against isoproterenol-induced arrhythmias in rats [22].

This study did not investigate the underlying mechanisms for the antiarrhythmic effects of pomegranate juice. Several mechanisms, such as modulation of some potassium or calcium channels, have been proposed for antiarrhythmic effects of plant antioxidants [23]. There is not enough evidence to evaluate these effects of pomegranate. However, protection of myocardial cells against ischemia and

reperfusion-induced damage seems to be among the rational explanations. Using an isolated heart model of global ischemia and reperfusion, anthocyanins have shown cardioprotection against apoptosis and necrosis [24]. Similar results have been reported following 10 days of pre-treatment with ellagic acid in rats [22]. Our unpublished data on isolated rat heart shows similar protective effects for pomegranate juice. In addition, we have shown a significant reduction in the serum levels of troponin in patients with myocardial infarction following five days of treatment with pomegranate juice [25].

This study evaluated the potential antiarrhythmic effects of pomegranate juice in isolated rat hearts subjected to ischemia and reperfusion. The juice was either supplemented to the Krebs solution at 1, 2, or 4% or it was gavaged to the rats daily for 3 consecutive weeks. Treatment of the hearts or pre-treatment of the animals with pomegranate juice seems to protect against ischemia/reperfusion-induced arrhythmias in isolated rat hearts.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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