Research Article

The Effects of Hesperidin on Ischemia/Reperfusion Induced Arrhythmias and Infarct Size in Isolated Rat Heart

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A B S T R A C T

Background: Hesperidin is a flavonoid and has strong anti-oxidant and anti-inflammatory activities. The aim of the present study is to investigate the effects of hesperidin on ischemic/reperfusion (I/R) induced injuries and arrhythmias.

Methods: Male Wistar rats were anesthetized and then the hearts were removed and cannulated immediately to a langendorff apparatus and prepared for the left coronary artery ligation. The hearts were perfused with Krebs-Henseleit Buffer Solution (KHBS; control) or KHBS plus hesperidin (1, 2.5 & 5µg/ml; treated groups) 5 minutes before coronary occlusion, during the ischemia, and reperfusion period. After reperfusion, double staining strategy (Evans blue and TTC) were used. The percentage of infarct size was determined by Image-j software. Arrhythmia in control group and treated groups were analyzed and compared. Lactate concentration was measured in samples at the end of stabilization, 30 minute after ischemia, and 60 minute after reperfusion. Western blotting was performed for evaluation of pAMPK at the end of the ischemia in the heart tissues.

Results: The results demonstrated that hesperidin caused a significant reduction in ventricular ectopic beats (VEBs) number during ischemia and reperfusion phase ($p<0.01$, $p<0.05$). The infarct size was reduced significantly by all concentration of hesperidin ($p<0.001$) and Lactate concentration at the end of ischemia had a significant reduction in the treatment groups ($p<0.001$). pAMPK/AMPK ratio was reduced by hesperidin at 5 µg/ml.

Conclusion: The results of the study suggest that hesperidin has protective effects against I/R induced injuries and arrhythmias in isolated rat hearts that could be related to its effect on modulating of AMPK activity.

Introduction

Coronary artery diseases are the leading cause of wide spectrum of clinical syndromes. When a coronary artery is blocked for any reason, the restoration of blood flow after a period of ischemia causes myocardial injury. The myocardial damage happens in both ischemic and reperfusion phases. Ischemia leads to an anaerobic metabolism and ATP depletion, followed by arrhythmia, intracellular pH alteration, ionic disturbances, and contractility dysfunction.[1] In the reperfusion phase, overload of reactive oxygen species (ROSs) and calcium ion in mitochondria lead to functional and histological changes and can result in cell death.

According to the previous studies, AMP-activated protein kinase (AMPK) activation protects the heart against ischemia-reperfusion (I/R) injury.[2,3] AMPK is a serine-threonine protein kinase and has a fundamental role in cellular metabolism and function so it is considered as a master regulator of energy homeostasis.[4] AMPK is activated when nutrient supply or adenosine triphosphate (ATP) generation is limited or cellular energy demand is increased. Metabolic inhibitors, hypoxia, myocardial ischemia, hypoglycemia, exercise, heat shock, osmotic stress, peroxynitrite and oxidative stresses are the most important AMPK activators.[5] It has been proved that oxidative stress can activate variety of transcription factors and lead to the expression of a large number of genes and the production of different pro-inflammatory proteins.[3,6] Obviously, antioxidant compounds, especially flavonoids, have a promising role in preventing the damage followed by oxidative stress.[7]

Flavonoids are natural polyphenols, widely distributed in plants. The most important effect of flavonoids is scavenging the oxygen-derived free radicals. Studies

revealed that they can be effective in many clinical disorders producing anti-atherosclerotic, anti-inflammatory, anti-tumor, anti-thrombogenic, anti-osteoporotic and anti-viral effects.[8,9] Citrus flavonoids are examples of flavonoids with all medicinal properties mentioned as above.[10]

In citrus fruits, flavanones and flavones (subclasses of flavonoids) are predominate.[11] Hesperidin (Hsd, Hesperetin 7-rutinoside),[12] a flavanone glycoside, is the most abundant flavonoid in citrus plants[7,10] and is the major flavonoid in sweet orange and lemon.[13] Its aglycone form is called hesperetin (Hst).[7,9] Hsperidin was first discovered in 1827 from citrus peel by the French chemist Lebreton.[7,13] Both Hsperidin and Hesperetin exhibit antioxidant activity. Hsperidin radical scavenging activity has been reported previously and its antioxidative capacity is comparable with that of ascorbic acid and trolox (a vitamin E derivative).[7]

Nowadays, according to the various studies, there is no doubt about the distinct effects of Hsperidin as an anti-allergic,[14] anti-diabetic,[10] anti-cancer,[15] and neuro-protective agent.[16,17] Different studies have shown anti-inflammatory[18,19] effects of hesperidin which could be explained by down-regulation of the over-activated macrophages. Besides, it is believed that hesperidin can up-regulate the function of dysfunctional T lymphocytes and, accordingly, it can have promising anti-inflammatory effects.[7] Since hesperidin has various biological activities, it is also called a bioflavonoid.[7,9,13]

Neohesperidin (hesperetin 7-O-neohesperidoside) is a flavanone glycoside which is found in orange, grapefruit and tomato.[12] Zhang and colleagues in 2012 verified that consumption of glucose is increased in HepG2 cells treated with either naringin or neohesperidin. They claimed that this effect is associated with AMPK activation.[20] Recently Sheng *et al.* proved that neohesperidin may have a specific role in activation of AMPK pathway and regulation of its target gens. According to the western blot results of that study, p-AMPK/AMPK ratio has been increased in the liver of mice treated with neohesperidin.[21]

Considering the previous reports on radical scavenging activities, anti-inflammatory and immunomodulatory effects of antioxidants and flavonoids, research on protective effects of hesperidin in I/R induced arrhythmia and infarct size seems to be beneficial. Besides, regarding to the close structural similarity between hesperidin and neohesperidin, evaluation of the effect of hesperidin on AMPK activation will be promising in cardiovascular disease.

When hesperidin is administered orally, it is hydrolysed by intestinal micro flora to yield a major active metabolite, hesperitin,[22] so, isolated heart model could be a valuable tool to evaluate the direct effects of the hesperidin regardless of its metabolites. So, in this study the effects of hesperidin on I/R induced injuries (arrhythmia and infarct size) and the AMPK activation

in isolated rat heart were evaluated, with the approach of introducing an herbal component with protective effects against ischemic heart diseases.

Materials and Methods
Chemical reagents
Triphenyltetrazolium chloride (TTC) and Evan's blue were purchased from Baker Analyzed (USA). Rabbit monoclonal antibodies against phospho-AMPKα (T172) and AMPKα were obtained from Cell Signaling Technology (Danvers, MA). The other reagents were of a commercial analytical grade.

Plant extraction and preparation
In this study hesperidin was isolated from orange peel. In order to obtain hesperidin from orange peel, 500g dried and powdered peel of *Citrus sinensis* (L.) *Osbeck var.* was defatted with petroleum ether. Then the petroleum extract was discarded and the remnant was exhaustively extracted with 70% methanol in water by maceration at room temperature. The methanol was evaporated at 40˚C under reduced pressure. Subsequently, the resultant aqueous extract was partitioned with EtOAc and n-butanol respectively. The ethyl acetate extract was concentrated and acetone was later added to precipitate hesperidin. The precipitate sucked off with a Buchner funnel and washed with acetone and ethyl acetate. The white amorphous powdery hesperidin was collected and kept in refrigerator for later use.

Animals
Healthy male albino Wistar rats (290±20 g) were used in this study. They were housed in standard polypropylene cages, six per cage, under a 12 h light/dark cycle in temperature of 22±2 °C with 50±10% relative humidity. The animals were given food and water freely. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran which is in line with National Institutes of Health Publication, edition 8, revised 2011.

Preparation and isolated heart perfusion
Materials and techniques of preparation, perfusion, and monitoring of physiological parameters have been described in details, in our previous study.[23] Wistar rats were anesthetized with ketamin/xylasin (60:10 mg/kg, ip). Heparin (1000 IU/kg, ip) was used as the anticoagulant. The ribs at the right and left anterior axillary lines were cut to create a clamshell thoracotomy and the heart was harvested. Each harvested heart was washed immediately with ice cold modified Krebs–Henseleit buffer solution (KHBS) containing (in mmol/L) NaCl 125, KCl 4.3, KH_2PO_4 1.1, $MgCl_2$. $6H_2O$ 1.3, $CaCl_2$. 2 H_2O 2.4, $NaHCO_3$ 25, and 13.32 grs of glucose. Then the heart was cannulated immediately to a non-recirculating

langendorff apparatus. The process duration, from opening the thorax till the complete perfusion of the heart with buffer via langendorff apparatus, was less than 5 minutes, to avoid any damage to the tissue.[24] The hearts were perfused at a constant pressure of 80 mmHg with the KHBS at 37°C and pH 7.38-7.56. The perfusate was gassed with carbogen (5% CO_2/95% O_2). For monitoring coronary perfusion pressure (CPP), the aortic cannula was connected to a pressure transducer (MLT844 physiological pressure, ADInstruments; Australia). To measure left ventricular contractility, a hand-made latex balloon, attached to a second pressure transducer, was inserted smoothly into the left ventricular cavity via the mitral valve after removing the atrial appendage. Left ventricular developed pressure (LVDP) was calculated as the difference between peak-systolic and end-diastolic pressure, the maximum and minimum rate of left ventricular pressure (dP/dt_{max}, dp/dt_{min}) as an index of left ventricular contractility and the heart rate (HR) were continuously recorded by PowerLab 8/35.[24] As soon as the harvested heart was mounted to langendorff setting, the suture is put in place around the left anterior descending artery (LAD) and formed to make a snare. During ischemia, the snare is tightened around LAD and loosed during reperfusion. The protocol was included three phases. The first period was stabilization phase that lasted for 15-20 minutes in a stable steady-state mode. At the end of stabilization time, the hearts with either poor LVDP (was not in range 70-130 mmHg) or prolonged arrhythmia (>3 minutes), were detached and excluded. After stabilization, the heart was perfused with KHBS solution enriched by hesperidin (0 (ischemia group, received just 0.0075 % DMSO as solvent), 1, 2.5 & 5 µg/ml), 5 minutes before ischemia and maintained for the rest of the experiment. The regional ischemia was induced by tightening the snare around LAD artery. This step continued for 30 minutes. The third period was reperfusion phase, in which the flow was re-established by opening the snare and lasted for 120 minutes (Figure 1).

The samples of perfused fluid were collected at the end of stabilization, end of ischemia and 60 minutes after reperfusion for lactate measurement.[2] Ischemia-induced ventricular arrhythmias were determined in accordance with the Lambeth Conventions.[25] Ventricular ectopic beats (VEBs) were defined as identifiable premature QRS complexes. Ventricular tachycardia (VT) was defined as the occurrence of four or more consecutive VEBs at a rate faster than the normal sinus rate. Ventricular fibrillation (VF) was defined as unidentifiable and low voltage QRS complexes. Occurrence of VTs, VFs and any other multipart forms of VEBs such as bigeminy, couplet (two consecutive VEBs), and salvos (three consecutive VEBs) was counted in ischemia and reperfusion phases separately. Ventricular fibrillation may be sustained or revert spontaneously to a normal sinus rhythm. Ventricular fibrillations lasting for more than 5 minutes were considered irreversible.[22]

### *Measurement of myocardial infarct size*
In order to determine the infarct size, double staining strategy is used.[24] In this protocol, after 120 minutes of reperfusion, the ligature around the LAD artery was re-tied. In order to distinguish the perfused area from non-perfused area (area at risk), 1 ml of Evan's solution (0.25% w/v in saline) was perfused slowly through the aortic cannula. This dye turns the non-infarcted zone into dark blue. Then the heart was stored at -20 °C. The frozen heart was sliced from apex to base into 1-2 mm thick slices. The slices were incubated in triphenyl tetrazolium chloride (2% TTC) at 37 °C for 15 min. This procedure makes the viable myocardium turn into red; while, the nonviable and unstained myocardium within the risk zone appears pale. At the end, the slices were fixed in 10% formalin overnight. Digitally photographed slices were imported to Image J software (Wayne Rasband, National Institute of Health, USA) and the infarct size was computed.

Western blot analysis
In another set of experiments, 4 groups (N=3), of control (just vehicle) and hesperidin (5 µg/ml) with or without ischemia, were evaluated for the effect of hesperidin on AMPK activation. The experiment was repeated 2 times, on two sets. In first set the hearts were detached and frozen immediately in liquid Nitrogen, at the end of the ischemia and in the other set they were detached after 60 minute of the reperfusion.

Western blot analyses were performed, as previously described by Soraya *et al*. with minor modifications.[3]

The homogenized samples were centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatants were aliquoted and stored at -70°C for further analysis.
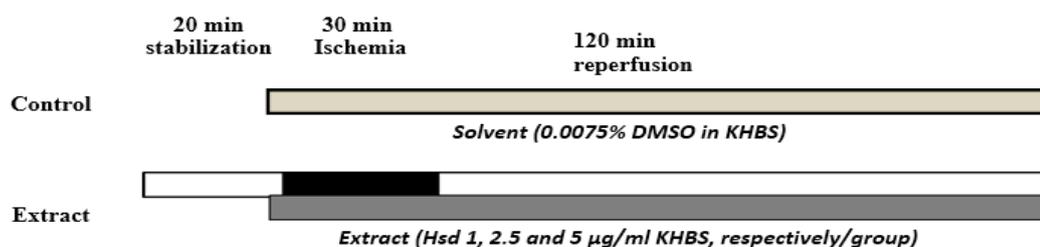
Figure 1. The Protocol of the study of protective effects of hesperidin on ischemia/reperfusion induced injuries in isolated rat heart model.
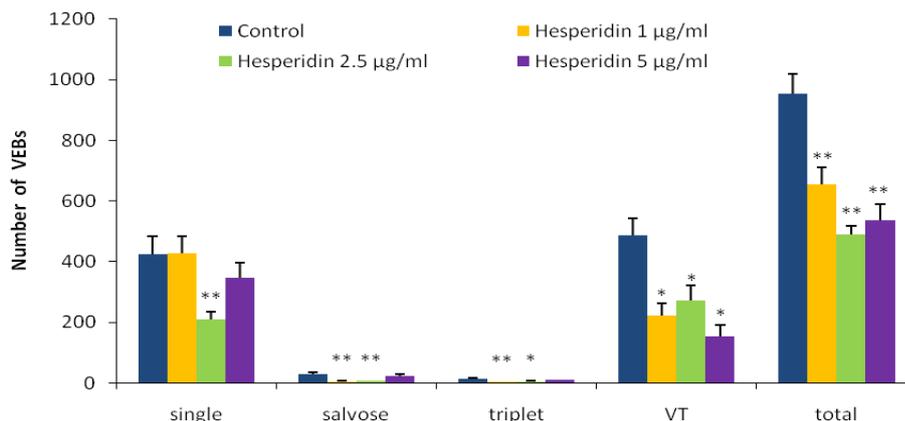
Figure 2. The number of ventricular ectopic beats (VEBs) in the control and treated isolated rat hearts (n=6) perfused with 0, 1, 2.5 & 5 µg/ml hesperidin (Hsd) during ischemia. Data are represented as Mean±SEM. $^*p<0.05$, and $^{**}p<0.01$ versus the control group, respectively. Ventricular tachycardia:VT.

Bradford Protein Assay kit was used for evaluating the protein content of the supernatant. Fifty µg of the homogenate protein was loaded to SDS-Polyacrylamide gel electrophoresis using Bio-Rad mini protean tetra system (Hercules, CA). The separated proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and blocked in 5% non-fat milk. The membranes were probed using a range of primary antibodies raised against AMPK and p-AMPK (1:1000) at 4ºC with gentle shaking, overnight. Then the membranes were washed and incubated with peroxidase-conjugated secondary antibody (1:5000 dilutions) at room temperature with gentle shaking. The antibodies were visualized using the BM Chemiluminescence kit (Roche, Mannheim, Germany). Densitometric analysis of the immunoblots was performed using image j software (Wayne Rasband, National Institute of Health, USA). The densitometric values for AMPK were normalized to p-AMPK.

Statistical Analyses
The data were analyzed by SPSS software (version 20). Number and duration of arrhythmias were evaluated using non-parametric Mann–Whitney U-test and the incidence of VT & VF was analyzed by Fisher exact test. While, the other parameters were analyzed using one-way ANOVA followed by LSD *post hoc* test when the ANOVA analysis indicated significant differences. Continuous variables were expressed as mean± S.EM and qualitative data were presented as percentage. Differences were considered significant at a level of p<0.05.

Results
The effects of hesperidin on ischemia/reperfusion induced cardiac arrhythmia in isolated heart
In order to ascertain the anti-arrhythmic property of hesperidin on ischemic/reperfusion induced cardiac arrhythmias in isolated rat hearts, perfusion of the hearts with hesperidin enriched KHBS was commenced 5 minute before regional ischemia and maintained for the whole period of the experiment. As illustrated in Figure 2, perfusion of KHBS solution containing hesperidin (1, 2.5 & 5 µg/ml) 5 min before the induction of regional ischemia, significantly decreased the number of total VEBs as well as VT during the ischemic period compared to the ischemic group (p<0.01 and p<0.05, respectively). Besides, hesperidin at the concentrations of 1 and 2.5 µg/ml decreased the number of salvoes (p<0.01) and triplet (p<0.01 and p<0.05, respectively) and 2.5 µg/ml of hesperidin significantly reduced the number of single ectopic beats (p<0.01) during the ischemia.
In the first 30 minute of reperfusion time, hesperidin in all concentrations decreased the number of total VEBs significantly (p<0.05, Figure 3). The number of single ectopic beats and VT were reduced by all concentrations of hesperidin, in comparison to the ischemic group (p<0.05). However, there is no significant difference in the number of salvoes and triplet ectopic beats between treated groups and ischemic group (Figure 3). As demonstrated in Table 1, duration of VT, both during ischemia and reperfusion, and its incidence has been reduced significantly by perfusion of hesperidin at all concentrations (1, 2.5 & 5 µ/ml; p<0.05). However, the incidences and duration of reversible VF in both phases and VT incidence in ischemic phase did not show any significant reduction.

*The effect of hesperidin on infarct size in the isolated heart*
As shown in Table 2, hesperidin caused a significant reduction in infarct size. The infarct size in the ischemic group was 80.41±3.08%; while the groups treated by hesperidin at the concentrations of 1, 2.5 and 5 µg/ml, produced the infarct size of 52.33±1.83% (p<0.001), 47.4±6.78% (p<0.001) and 38±1.5% (p<0.001), respectively. In order to show that the ligation place at coronary artery was same in all experiments, the ischemic risk zone was calculated for each group. As shown in Table 2, there is no significant difference between areas at risk in all groups.

The effect of hesperidin on lactate production during ischemia in the isolated heart

Figure 4 shows the percentage of increase in the concentration of lactate at the end of ischemia period compared to the base line value. Perfusion of hearts with hesperidin at conceteration of 1 and 5 μg/ml caused a significant (p<0.001) reduction in the production of lactate from 77.16±5% in control to 20.7±1.7 and 21.78±2.1%, respectively.

Table 2. Risk zone (%) and infarct size (%) in myocardial tissue in the control and treated groups (1, 2.5& 5 μg/ml of Hsd) during 30 min of ischemia followed by 120 min of reperfusion. Data are represented as mean±SEM. ***p<0.001 versus control group. N= 6 rats in each group.

| Groups	Risk zone (%)	Infarct zone (%)
Control (Isch)	44.66±2.78	80.41±3
Hsd (1 μg/ml)	45.83±1.83	52.23±1.8***
Hsd (2.5 μg/ml)	46.5±2.12	47.5±6.3***
Hsd (5 μg/ml)	45.1±3	38.25±4.4***
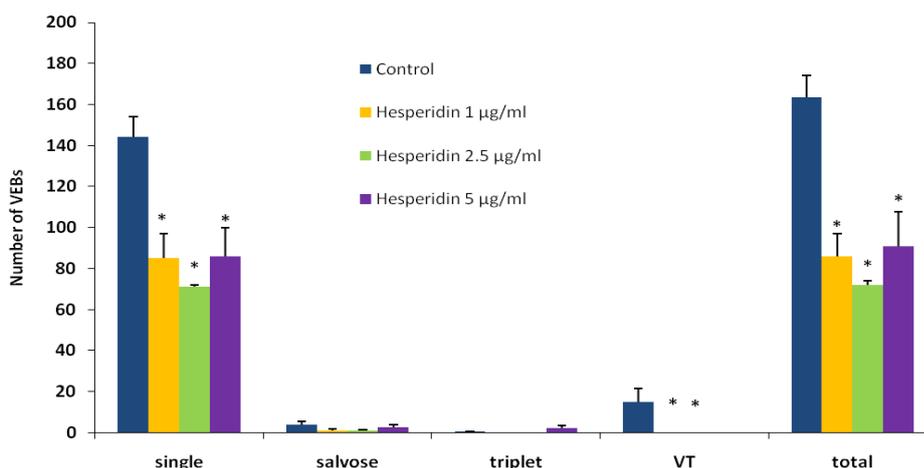
Figure 3. The number of ventricular ectopic beats (VEBs) in the control and treated isolated rat hearts (n=6) perfused with 0, 1, 2.5 & 5 μg/ml hesperidin (Hsd) during first 30 min of reperfusion time. Data are represented as Mean±SEM. *p<0.05 versus the control group. Ventricular tachycardia:VT.

Table 1. Effects of hesperidin (Hsd) (1- 2.5 & 5 μg/ml) on the duration of ventricular tachycardia (VT) and on the duration of reversible VF (Rev VF) and on arrhythmia incidences in ischemia and in the first 30 minutes of reperfusion period. Data are represented as Mean±SEM *p<0.05versus control group. N=6 rats in each group.

Groups	Ischemia time				Reperfusion time			
	Rev VF Duration (sec)	VF incidence (%)	VT Duration (sec)	VT incidence (%)	Rev VF Duration (sec)	VF incidence (%)	VT Duration (sec)	VT incidence (%)
Control	2.3±2	40	66±8	100	0.5	25	5.2±1	100
Hsd (1μg/ml)	0	0	31±7*	100	0.3	25	0*	0*
Hsd (2.5μg/ml)	0	0	42±7*	100	0	0	0*	0*
Hsd (5μg/ml)	0	0	24±4*	100	0	0	0*	0*
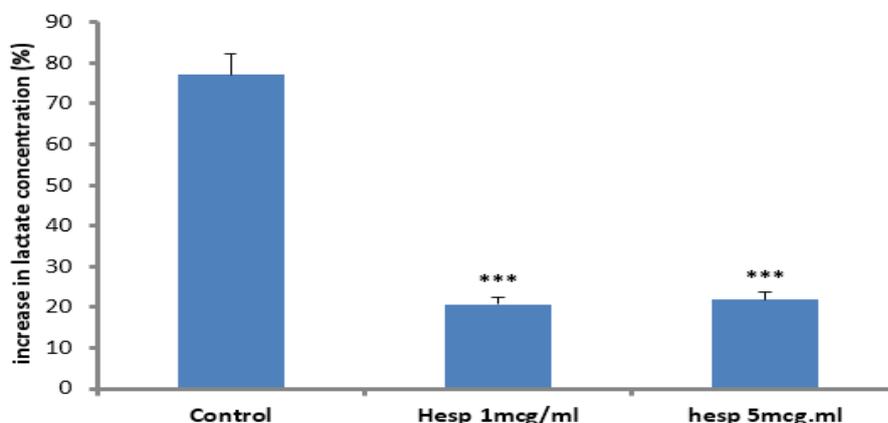
Figure 4. Percentage of increase in lactate concentration at the end of the 30 min of regional ischemia, in the control and treated groups receiving 1 and 5 μg/ml of hesperidin. ***P<0.001 vs. control group.
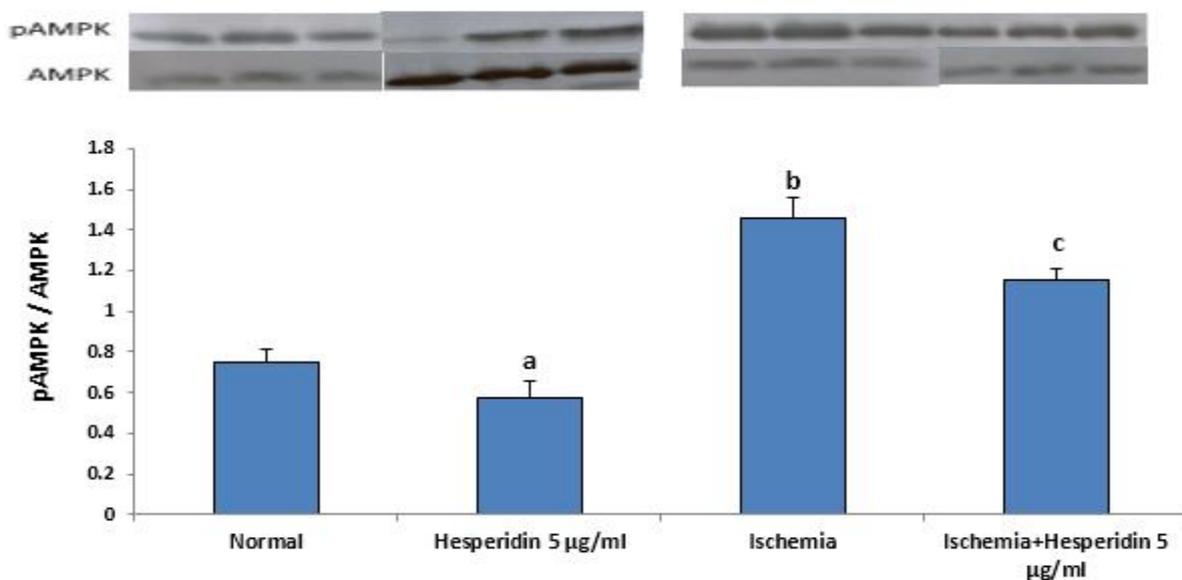
Figure 5. The effect of perfusion hearts with hesperidin at 5 µg/ml for 50 minutes on AMPK phosphorylation in normal and treated groups with and without ischemia. Data are represented as mean± SEM and analyzed by non-parametric Mann–Whitney U-test. [a]$p<0.05$ and [b]$p<0.01$ vs. control normal group and [c]$p<0.05$ vs ischemic group. N=3 in each group.

The effect of hesperidin on AMPK activation in the isolated rat hearts

The phosphorylated form of AMPK is the active form of enzyme. As shown in Figure 5, perfusion of normal hearts without ischemia with hesperidin at 5 µg/ml demonstrated a slight but significant ($p<0.05$) reduction in the activation of AMPK. Of course as expected, ischemia as a stress elevated the phosphorylation of AMPK ($p<0.01$). However, hesperidin similar to the normal group also reduced the activation of AMPK in the ischemic hearts.

Discussion

The key finding the present study is that the administration of hesperidin prior to ischemia caused a significant anti-arrhythmic property both during ischemia and reperfusion time. This antiarrhythmetic effect was associated with the reduction of infarct size. Probably, modulating the metabolism by a relative suppression of AMPK activity is involved in the cardioprotective effects of hesperidin. However, the antioxidative properties of hesperidin should also be considered. These possibilities are based on the following observations in this study: 1) myocardial infarct size was attenuated remarkably by hesperidin which could be accountable for decrease in cardiac arrhythmias, 2) lactate production at the end of ischemia, as an index of anaerobic glycolysis, was diminished by hesperidin, 3) AMPK activity, as a regulator of energy methabolism, was suppressed slightly but significantly by hesperidin.

During myocardial ischemia, sudden thrombotic occlusion of a coronary artery at the site of an atherosclerotic plaque causes tissue hypoxia. It switches the cellular metabolism from aerobic to anaerobic, and leads to decline in the intracellular pH, accumulation of sodium and calcium in the cytosol, and cell injury and/or death. Using thrombolysis to re-perfuse the occluded vessel may cause cellular damages due to ROS formation in the ischemic region.[26] The mitochondrial electron transport chain in myocytes, xanthine oxidase of endothelial cells and NADPH oxidase and myeloperoxidase of neutrophils are the main sources of ROS production that the two former sources are the primary sources in acute phase of ischemia. The elevation of ROS formation, followed by oxidative stress, and the accumulation of calcium in the cytosol and mitochondria are the main factors of ischemia/reperfusion injury and arrhythmia.[22,27]According to Akhlaghi and Bandy, flavonoids have a promising role in declining ischemia/reperfusion injuries.[27] Flavonoids can directly prevent generation of ROS and act as ROS scavenger, or may exert their antioxidant effects indirectly through enhancement of cellular antioxidant enzymes and inhibition of xanthine oxidase and NADPH oxidases. Besides antioxidant effects, flavonoids possess vasorelaxation that lessens the ischemia/reperfusion injury by re-establishing the blood flow.[27]

Hesperidin as a flavonoid[7,10] may also have these properties. In this regard, Parhiz and colleagues in a review about the hesperidin, showed that direct radical scavenging and increasing cellular anti-oxidant defense are the main ways that hesperidin exerts its anti-oxidant properties.[7] In the other study they discussed about the potentiality of hesperidin in prevention of cardio vascular disease.[9] There is very poor data about the effect(s) of hesperidin on ischemia/reperfusion injury in isolated heart model. However, Gandhi *et al.* (2009) proved the protective effects of hesperidin against in vivo focal ischemia/reperfusion induced arrhythmias and myocardial infarction. They confirmed the potent

anti-oxidant properties of hesperidin and claimed that its cardio-protective effects are related to reduction of inflammation and oxidative stress.[22] Likewise, Selvaraj and Pugalendi showed the cardio-protective effects of hesperidin in isoproterenol induced myocardial ischemia, which could be related to its antioxidant properties.[28] Therefore, it can be concluded that hesperidin could reduce the infarct size due to its antioxidant, anti-inflammation and antiarrhythmic effects.[22,28]

Ischemia and hypoxia triggers anaerobic glycolysis due to mitochondrial metabolic dysfunction and reduced aerobic formation of adenosine triphosphate (ATP). This condition causes lactate production, fall in pH[29] and so in AMPK activation. It has been known that ischemic condition triggers AMPK activation,[2,30] which can switch on catabolic pathways to generate ATP, while switching off anabolic pathways to consume ATP.[31] In the present study lactate concentration and infarct size after ischemia/referpusion were attenuated by hesperidin treatment. This observation could be attributed to antioxidant, antiinflammation and antiarrhythmic effects of hesperidin.[22,28] This study also showed that acute treatment with hesperidin, reduced AMPK activity in the ischemic hearts. The present study for the first time showed that hesperiding was able to suppress AMPK activity slightly in the ischemic condition.

Conclusion

The present study demonstrated that perfusion of hesperidin decreased the number of ventricular ectopic beats during ischemia and reperfusion in the isolated rat hearts. In addition, the flavonoid profoundly diminished ischemia/reperfusion induced myocardial infarction. Hesperidin reduced the activation of AMPK in the ischemic hearts. It may be concluded that hesperidin could protect myocardium by suppressing arrhythmia and reducing the infarction injuries following ischemia and reperfusion. The modulation of AMPK activity by hesperidin may be involved in its cardioprotective effects.

Acknowledgments

Conflict of interests

Prof. Alireza Garjani is the Editor-in-Chief of *Pharmaceutical Sciences*. The peer-review process of the submission was supervised by another member of the editorial board. The authors declare no other competing interests with regards to the authorship and/or publication of this article.

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