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# Protection against *in vivo* focal myocardial ischemia/reperfusion injury-induced arrhythmias and apoptosis by *Hesperidin*

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**Abstract**
Among the heart diseases, ischemia and reperfusion (I/R) induced arrhythmias contribute to episodes of sudden death. Cardiac arrhythmias during ischemia reperfusion are believed to be related to oxidative stress. Therefore, the aim of this study was to examine whether treatment with Hesperidin alleviates arrhythmias and infarct size in experimentally-induced myocardial I/R injury using an *in vivo* rat model. In this study haemodynamics parameters, markers of inflammation, biomarkers of oxidative stress and tissue nitrite level and infarct size of the heart were estimated in various groups. I/R showed a significant decrease in tissue nitrite and antioxidant level and significant increase in arrhythmias, inflammation and myocardial cell apoptosis. Treatment with Hesperidin showed a significant increase in tissue nitrite, antioxidant level and reduction in inflammation, arrhythmias and apoptosis. In conclusion, the protecting effect of Hesperidin in I/R induced arrhythmias is due to reduction in inflammation and oxidative stress.

**Keywords:** *Antioxidant, Hesperidin, reperfusion arrhythmias, ischemia reperfusion injury*

## Introduction

Heart disease is a primary killer of people in the advanced countries of the world. Among the heart diseases, ischemia and reperfusion induced arrhythmias contribute to many episodes of sudden-death [1]. However, reperfusion is the only way to restore blood flow of coronary arteries and prevent the myocardium suffering from necrosis [2]. The immediate and full restoration of coronary blood flow results in arrhythmias, contractile dysfunction (stunning), microvascular injury and irreversible myocardial damage through apoptosis and necrosis [3]. Cardiac arrhythmias during ischemia reperfusion (I/R) are believed to be related to generation of reactive oxygen species (ROS) [4]. Defense against free radical injury is provided by enzymatic (catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic ($\alpha$-tocopherol, vitamin C, allopurinol and dimethyl sulphoxide) free radical scavengers [5,6]. I/R-induced arrhythmias have been shown to be ameliorated in animal models by many free radical scavengers or antioxidants, such as mannitol, superoxide dismutase, catalase, ascorbate, allopurinol, methionine, glutathione and desferoxamine [1].

*Hesperidin* is a major and active flavanone glycoside, mainly isolated from citrus fruits [7]. It is reported to have anti-allergic, radio protective and anti-oxidant properties [8,9]. Moreover, hesperidin is shown to possess immunomodulator and anti-hypertensive activities [10]. When hesperidin is administered orally, it is hydrolysed by intestinal micro flora to yield a major active metabolite *hesperitin* [7].

There is no evidence to show that treatment with hesperidin could suppress I/R-induced arrhythmias. In this study, we examined whether treatment with *Hesperidin* alleviate arrhythmias and infarct size in experimentally-induced myocardial I/R injury using an *in vivo* rat model.

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## Materials and methods

### Drugs

*Hesperidin* (97%) was purchased from ACROSS lab (New Jersey, USA) and other chemicals used in the study were of laboratory grade.

### Animal preparation

Sprague-Dawley rats of either sex with body weight between 230–260 g were housed in an air-conditioned room with 12-h light and dark cycles, with free access to food and water *ad libitum* during the experiments. The institutional animal ethics committee approved the experimental protocol. All the experiments were conducted as per norms of CPCSEA.

### Grouping of animals

The rats were divided into four groups, each consisting of six animals.

- *Group 1*: Sham operated animals were administered 0.05% sodium CMC (vehicle) for 15 days and on the 16$^{th}$ day, the animals were subjected to the entire surgical procedure as given below and thread was passed beneath the coronary artery but the LAD (the left anterior descending) coronary artery was not ligated (Sham).
- *Group 2*: The rats were administered 0.05% sodium CMC for 15 days and on the 16$^{th}$ day the animals were subjected to 30 min LAD coronary artery ligation (ischemia) and 60 min reperfusion (I/R control).
- *Group 3*: The rats were administered Hesperidin (100 mg/kg, p.o.) [8] for 15 days and on the 16$^{th}$ day the animals were subjected to 30 min LAD coronary artery ligation (ischemia) and 60 min reperfusion (I/R + HSP).
- *Group 4*: The rats were administered Vit.E (100 mg/kg/day, p.o) [11] for 15 days and on the 16$^{th}$ day the animals were subjected to 30 min LAD coronary artery ligations (ischemia) and 60 min reperfusion (I/R + Vit.E).

### Surgical preparation

Rats were anaesthetized with intraperitoneal injection of ketamin (100 mg/kg, i.p). Tracheotomy was performed and an intubation cannulated which was connected to a rodent ventilator (67 strokes/min, UGO BASILE rodent ventilator). The right carotid artery was cannulated by advancing PE-50 tubing to measure left ventricular pressure rate. Body temperature was maintained at or near 37°C. The left thoracotomy was performed at the fifth intercostals space. The pericardium incised and the heart was exteriorized. A thin silk thread (No. 6/0) was then placed around the left anterior descending coronary artery (LAD). The heart was then replaced into the thoracic cavity with the thread ends exteriorized. A small plastic snare was threaded through the ligature and placed in contact with the heart. The coronary artery could then be occluded by applying tension to the ligature and reperfusion was achieved by releasing tension. The ECG was continuously recorded on (SS2L) BIOPAC MP30 data acquisition system by Lead III electrode.

Successful coronary artery occlusion was evidenced by ST-segment elevation, decrease in arterial pressure and increase in R-wave amplitude. Successful reperfusion was indicated by recovery from reversal of the ECG changes. At the end of the reperfusion period animals were euthanized and blood and tissues samples were collected for various biochemical analysis.

### Exclusion criteria

Experiments were terminated or excluded from the final data analysis if any of the following conditions occurred: absence of signs of successful coronary artery occlusion, severe arrhythmias prior to LAD occlusion, mean arterial pressure (MAP) less than 60 mmHg prior to induction of ischemia or severe atrioventricular block during the first 5 min ischemia.

### Cardiac function measurements

Heart rate (HR), MAP, cardiac contractility rate of rise and decline of LVP ($\pm dp/dt_{max}$) were recorded through a pressure transducer on a MP30 data acquisition system (BIOPAC).

### Assessment of ventricular arrhythmias

Ischemia-induced ventricular arrhythmias were determined in accordance with the Lambeth Conventions [12]. 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 resting sinus rate. Ventricular fibrillation (VF) was defined as unidentifiable and low voltage QRS complexes. In the case of other multipart forms of VEBs such as bigeminy, couplet (two consecutive VEBs) and salvos (three consecutive VEBs), they were counted at separate episodes (Figure 1). Ventricular fibrillation may be sustained or may revert spontaneously to a normal sinus rhythm. VF lasting for more than 5 min was considered as irreversible.

The severity of arrhythmias was quantified by the following scoring system [13]:

- 0: 0–50 VEBswith no other arrhythmias over the 25-min ischemia period,
- 1: Only 50–500 VEBs,

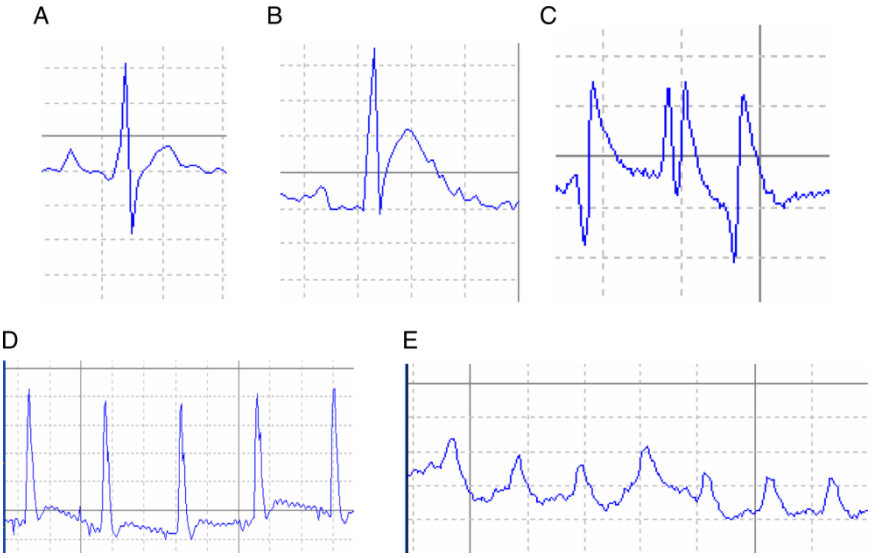


Figure 1. Electrocardiogram recording. (A) During baseline; (B) during coronary artery occlusion; (C) ventricular ectopic beat (VEB); (D) ventricular tachycardia (VT); (E) ventricular fibrillation (VF).

- 2. More than 500 VEBs or one episode of spontaneously reversible VT or VF,
- 3. 2–30 episodes of spontaneously reversible VT and/or VF,
- 4. More than 30 episodes of spontaneously reversible VT and/or VF, and
- 5. Irreversible VF.

*Evaluation of QT interval*

The ECG tracings were analysed visually and the following ECG parameters were examined:

(1) RR interval (the interval between the apex of two consecutive adjacent R-waves),
(2) QT interval (the interval between beginning of Q-wave and T-wave apex), and
(3) Corrected QT interval (QTc, defined as the QT interval corrected for the heart rate by means of Bazett's equation: corrected QTc = QT (ms)/RR $(s)^{1/2}$) [14].

*Estimation of serum for TNF-α (marker of inflammation) and CK-MB (marker of myocyte necrosis)*

*TNF-α quantitation by ELISA.* Levels of TNF-α in serum were determined using an enzyme-linked immunosorbent assay (ELISA) (Endogen, Mouse TNF-α kit, USA) according to the manufacturer's instructions.

*CK-MB.* Levels of CK-MB in serum were determined using a kit according to the manufacturer's instructions based on the principle of immunoinhibition (Reckon diagnostics, Baroda).

*Evaluation of heart tissue*

*MPO activity (marker of inflammation).* MPO activity was measured in tissues in a procedure similar to that documented by Hillegas et al. [15]. Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41 400 g (10 min); pellets were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles with sonication between cycles, the samples were centrifuged at 41 000 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM phosphate buffer, o-dianisidine and 20 mM $H_2O_2$ solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/gm of tissue.

*Tissue nitrite levels.* The nitrite (NO) was estimated by the method of Lepoivre et al. [16]. To 0.5 ml of tissue homogenate, 0.1 ml of sulphosalicylic acid was added and vortexed well for 30 min. The samples were then centrifuged at 5000 rpm for 15 min. The protein-free supernatant was used for estimating nitrite levels. To 200 μL of the supernatant, 30 μL of 10% NaOH was added, followed by 300 μL of Tris-HCl buffer and mixed well. To this, 530 μL of Griess reagent was added and incubated in the dark for 10–15 min and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

*Determination of infarct size and area at risk.* After completion of reperfusion, the animals were sacrificed and the heart was quickly removed into ice cool phosphate buffered saline for measuring area of infarction.

The suture was tied again and Evans Blue solution (2% in PBS) was forced retrogradely through the aorta to demark the area at risk (AAR). The heart was frozen at $-20^{\circ}$C and ~8–10 thin sections were cut (~1.5–2 mm) from apex to base. The sections were placed in triphenyl tetrazolium chloride (TTC) solution (2% in PBS) and kept at 37°C for 20 min. Then sections were fixed with 10% formalin. Then sections were put on a slide and scanned (Hewlett Packard Scanner). The resultant slides were examined by ImageJ software for measurement of area of infarction. The area free from blue stain was area at risk. The portion seen red or pink was salvaged myocardium and unstained whitish portion was infarct zone. Infarct size (IS) was expressed as a percentage of the AAR (% IS/AAR). Each area total area, area at risk and infracted area were calculated for each slide, the sum of these areas was done and percentage of area calculated accordingly.

*Myocardial water content.* Samples of heart were weighed after blotting. They were then dried at ~80°C for 48 hor until their weights were static. Myocardial water content was then calculated as [(Weight (wet) − Weight (dry))/Weight (wet)] [17] and expressed as a proportion.

### *Statistics*

All the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test using computer-based fitting program (prism, Graphpad). Differences were considered to be statistically significant when $p < 0.05$.

## Results

A total of 76 rats were used in this study. Three rats were excluded for low MAP before LAD occlusion and one for failing to occlude the LAD.

### *Effects of hesperidin on MAP, HR and ± dp/dt$_{max}$*

There were no significant differences at baseline (10 min before ischemia) values for MAP HR and $\pm dp/dt_{max}$ among the experimental groups. Animals subjected to I/R showed significant ($p < 0.01$, $n = 6$) decline (from $104.7 \pm 3.283$ to $82.33 \pm 5.783$ mmHg) in MAP at the end of ischemia, which was further reduced ($63.83 \pm 3.544$ mmHg, $p < 0.001$, $n = 6$) at the end of reperfusion. Also, in animals subjected to I/R, there was a significant ($p < 0.001$, $n = 6$) decline in the $\pm dp/dt_{max}$ as compared to the sham-operated group. In addition, animal subjected to I/R showed a significant ($p < 0.05$, $n = 6$) increase (from $362.8 \pm 5.648$ to $396.7 \pm 4.883$ BPM) in HR during ischemia and significant ($p < 0.05$, $n = 6$) reduction ($322.3 \pm 5.719$ BPM) in HR during reperfusion as compared to the sham operated group. However, in the *Hesperidin* treated I/R control group there was a significant improvement in MAP during ischemia ($p < 0.05$, $n = 6$) and reperfusion ($p < 0.001$, $n = 6$) as compared to the I/R control group. Also, in the *Hesperidin* treated I/R control rats there was a significant elevation in the $+dp/dt$ ($p < 0.01$, at the end of ischemia and $p < 0.05$ at the end of reperfusion, $n = 6$) and $-dp/dt$ ($p < 0.001$, $n = 6$, at the end of ischemia and reperfusion) as compared to the I/R control group. Moreover, in the *Hesperidin* treated I/R control rats there was a significant decrease in HR during ischemia ($p < 0.05$, $n = 6$) and significant ($p < 0.01$, $n = 6$) increase in HR during reperfusion as compared to the I/R control group (Tables I and II). Treatment with Vit.E also showed improvement in cardiac function, but not as effective as hesperidin.

### *Effects of hesperidin on reperfusion-induced arrhythmias*

*Incidences of VT, VF & VEBs.* In the I/R control group, VT, VF and VEBs were observed in 100, 83.34 and 100% of the rats, respectively. Animals subjected to I/R showed significant increase in the incidences of VT + VF ($p < 0.01$, $n = 6$) and non-significant increase in the incidences of VEBs as compared to the sham-operated group. In the *Hesperidin* treated I/R control group, there was a significant attenuation of VT + VF (~3-fold, $p < 0.01$, $n = 6$) as compared to the I/R control group. In the *Hesperidin* treated animals, a non-significant increase in the incidences of VEBs was observed as compared to the I/R control group (Figure 2). Treatment with Vit.E also showed reduction in incidences of arrhythmias, but not as effective as hesperidin.

*Duration of VF and VT.* In the I/R control group there was a significant increase in the duration of VT (~3-fold, $p < 0.001$, $n = 6$) and VF (~5-fold, $p < 0.01$, $n = 6$) as compared to the sham operated group. In the *Hesperidin* treated I/R control group, there was a significant reduction in duration of VT (~2-fold, $p < 0.001$, $n = 6$) and VF (~2.5-fold, $p < 0.05$, $n = 6$) as compared to the I/R control group (Figure 3). Treatment with Vit.E also showed a reduction in duration of arrhythmias, but not as effective as hesperidin.

*Severity (score) of arrhythmias.* In the I/R control group, there was a significant ($3.00 \pm 0$ to $4.50 \pm 0.22$, $p < 0.05$, $n = 6$) increase in the severity of

Table I. Value of haemodynamic parameter (MAP & HR) in rats subjected to I/R injury. Values are expressed as mean ± SEM for six animals in each group.

| | Baseline | End of ischemia | End of reperfusion |
|---|---|---|---|
| *MAP* (mmHg) | | | |
| Sham | 105.2 ± 2.197 | 104.7 ± 3.283 | 104.3 ± 2.092 |
| I/R control | 106.5 ± 3.222 | 82.33 ± 5.783** | 63.83 ± 3.544*** |
| I/R + HSP | 106.0 ± 3.109 | 88.33 ± 3.783# | 101.0 ± 1.528### |
| I/R + Vit.E | 110.3 ± 2.011 | 66.50 ± 3.990 | 83.83 ± 3.701 |
| *HR* (beats/min) | | | |
| Sham | 370.3 ± 6.396 | 362.8 ± 5.648 | 354.7 ± 6.505 |
| I/R control | 370.0 ± 5.416 | 396.7 ± 4.883* | 322.3 ± 5.719# |
| I/R + HSP | 365.2 ± 3.736 | 364.5 ± 4.877# | 357.8 ± 4.868## |
| I/R + Vit.E | 368.5 ± 7.995 | 388.2 ± 10.00 | 330.7 ± 5.327 |

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for sham vs I/R control.
#$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ for I/R control vs I/R + HSP.



arrhythmias as compared to the sham operated group. In the *Hesperidin* treated I/R control group, there was a significant ($3.16 \pm 0.47$, $p < 0.05$, $n = 6$) reduction in severity of arrhythmias as compared to the I/R control group (Figure 4).

*Corrected QT (QTc) interval.* The change in QTc interval was measured between the baseline, at the end of ischemia and at the end of reperfusion periods within groups. In the I/R control group, QTc interval was significantly (from $89.27 \pm 5.679$ to $80.39 \pm 5.735$ ms, $p < 0.001$, $n = 6$) decreased at the end of ischemia and it was significantly ($94.31 \pm 3.759$ ms, $p < 0.001$, $n = 6$) increased at the end of reperfusion, as compared to baseline QTc in the I/R control group. In the *Hesperidin* treated I/R control group, there was a significant elevation (from $93.47 \pm 5.666$ to $98.93 \pm 2.690$ ms, $p < 0.001$, $n = 6$) in the QTc interval at the end of ischemia which was further increased ($101.7 \pm 4.168$ ms, $p < 0.01$, $n = 6$) at the end of reperfusion as compared to the I/R control group (Table III).

*Estimation of serum for TNF-α (marker of inflammation) and CK-MB (marker of myocyte necrosis)*

*TNF-α quantitation by ELISA.* The serum TNF-α level was significantly ($p < 0.001$, $n = 6$) increased by ~1.75-fold ($762.2 \pm 27.53$ pg/ml) in the I/R control group as compared to the sham operated group ($431.3 \pm 22.62$ pg/ml). In *the Hesperidin* treated I/R control group, there was a significant reduction in serum TNF-α level ($p < 0.01$, $n = 6$) as compared to the I/R control group (Figure 5). Treatment with Vit.E also showed a reduction in serum TNF-α level, but not as effective as hesperidin.

*CK-MB.* The serum CK-MB level was significantly ($p < 0.001$, $n = 6$) increased by 41.5% ($1125 \pm 41.56$ U/L) in the I/R control group as compared to the sham operated group ($795.0 \pm 26.51$ U/L).

In the *Hesperidin* treated I/R control group, there was a significant reduction in serum CK-MB level ($p < 0.001$, $n = 6$) as compared to the I/R control group (Table IV).

*Evaluation of heart tissue*

*Biomarkers of oxidative stress.* I/R produced a significant increase in tissue MDA level (from $100.0 \pm 4.70$ to $143.5 \pm 3.74$ nmol/g of tissue, $p < 0.001$, $n = 6$) in the I/R control group as compared to the sham-operated group. In the *Hesperidin* treated I/R control group, there was a significant reduction in MDA level ($116.8 \pm 1.27$ nmol/g of tissue) as compared to the I/R control ($p < 0.001$, $n = 6$). I/R produced significant

Table II. Value of left ventricular pressure rate in rats subjected to I/R injury. Values are expressed as mean ± SEM for six animals in each group.

| | $+dp/dt$ (mm/Hg$^1$) | | | $-dp/dt$(mm/Hg$^1$) | | |
|---|---|---|---|---|---|---|
| Groups | Baseline | End of ischemia | End of reperfusion | Baseline | End of ischemia | End of reperfusion |
| Sham | 2497 ± 41.10 | 2489 ± 30.08 | 2462 ± 36.72 | 2059 ± 41.10 | 2047 ± 29.99 | 2005 ± 27.95 |
| I/R control | 2521 ± 48.07 | 1660 ± 31.91*** | 1505 ± 61.27*** | 2042 ± 46.76 | 1640 ± 30.94*** | 1523 ± 25.52*** |
| I/R + HSP | 2442 ± 35.46 | 1974 ± 66.21## | 1774 ± 74.79# | 2054 ± 28.80 | 1918 ± 30.94### | 1848 ± 29.37### |
| I/R + Vit.E | 2460 ± 19.85 | 1830 ± 79.74 | 1535 ± 61.27 | 2074 ± 26.28 | 1777 ± 30.94 | 1695 ± 29.37 |

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for sham vs I/R control.
#$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ for I/R control vs I/R + HSP.

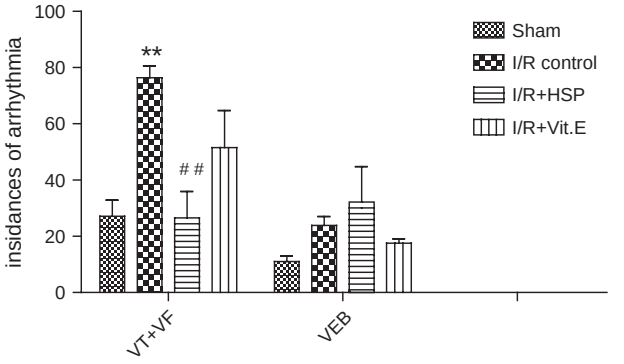


Figure 2. Incidences of arrhythmias in rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Values are expressed as mean ± SEM for six animals in the group. $^{**}p < 0.01$ for sham vs I/R control. $\#\#p < 0.01$ for I/R control vs I/R+HSP.

reduction in the anti-oxidant enzymatic activity of GSH (from 479.0 ± 11.71 to 366.0 ± 20.84 μg/gm of tissue, $p < 0.001$, $n = 6$), CAT (from 1349 ± 30.27 to 1087 ± 53.04 nmols of $H_2O_2$ consumed/g of tissue, $p < 0.01$, $n = 6$) and SOD (from 106.2 ± 4.37 to 54.50 ± 5.81 U/g of tissue, $p < 0.001$, $n = 6$) in the I/R control group as compared to the sham-operated group. In the *Hesperidin* treated I/R control group, there was a significant increase in the level of GSH (452.2 ± 19.50 μg/gm of tissue, $p < 0.01$, $n = 6$), CAT (1263 ± 39.30 nmols of $H_2O_2$ consumed/g of tissue, $p < 0.05$, $n = 6$) and SOD (92.17 ± 9.094 U/g of tissue, $p < 0.01$, $n = 6$) as compared to the I/R control group (Figure 6A–D).

*MPO activity.* I/R produced a significant ($p < 0.001$, $n = 6$) increase (~3-fold) in tissue MPO activity in the I/R control group as compared to the sham-operated animals. In the *Hesperidin* treated I/R control group, there was a significant reduction in MPO activity ($p < 0.001$, $n = 6$) as compared to the I/R control group (Table IV). Treatment with Vit.E also showed a reduction in MPO activity, but not as effective as hesperidin.

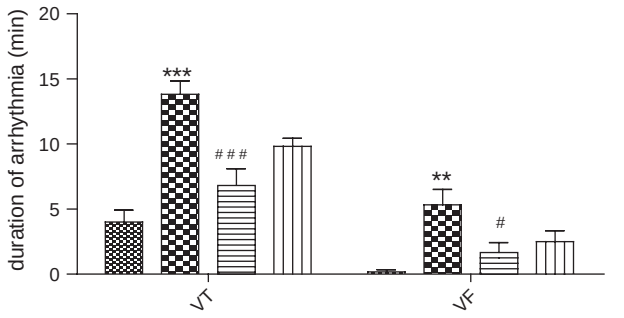


Figure 3. Duration of arrhythmias in rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: Nos of arrhythmias/min. Values are expressed as mean ± SEM for six animals in the group. $^{**}p < 0.01$ and $^{***}p < 0.001$ for sham vs I/R control. $\#p < 0.05$ and $\#\#\#p < 0.001$ for I/R control vs I/R+HSP.

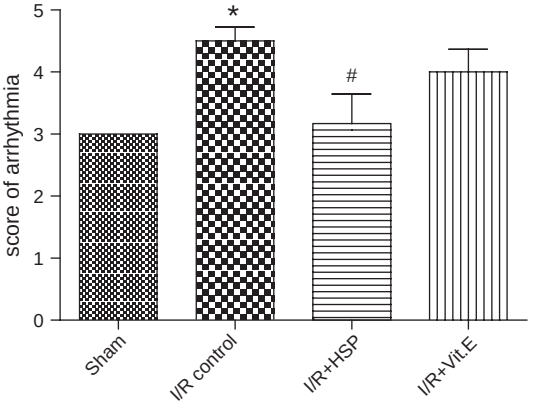


Figure 4. Score of arrhythmias in rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Values are expressed as mean ± SEM for six animals in the group. $^{*}p < 0.05$ for sham vs I/R control. $\#p < 0.05$ for I/R control vs I/R+HSP.

*Tissue nitrite levels.* I/R resulted in a significant decrease in the tissue level of nitrite (135.5 ± 4.38 nmol/g tissue, $p < 0.05$, $n = 6$) as compared to values obtained from the tissue of sham-operated animals (157.3 ± 6.22). However, in the *Hesperidin* treated I/R control group, there was a significant (160.2 ± 3.37 nmol/gm tissue, $p < 0.05$, $n = 6$) increase in tissue nitrite level as compared to the I/R control group (Table IV). Treatment with Vit.E in rats subjected to I/R improved tissue NO level, but not as significantly as hesperidin.

*Determination of infarct size and area at risk.* The area at risk (AAR/heart) and the infarct size (IS/AAR) expressed as percentages are shown in Figure 7. There was a significant increase in the of AAR (0.733 ± 0.47 to 43.18 ± 2.45, $p < 0.001$, $n = 6$) and infarct size (0 ± 0 to 40.64 ± 3.00, $p < 0.001$, $n = 6$) in the I/R control group as compared to the sham-operated group. The *Hesperidin* treated I/R control group showed a significant decrease in the AAR (30.99 ± 1.03, $p < 0.01$, $n = 6$) and infarct size (29.62 ± 1.65, $p < 0.01$, $n = 6$) as compared to the I/R control group (Figure 7A and B). Treatment with Vit.E also showed a reduction in AAR and infarct size, but not as effective as hesperidin.

*Myocardial water content.* I/R resulted in a non-significant increase in the myocardial tissue water content as compared to the sham-operated animals. However, in the *Hesperidin* treated I/R control group, there was a non-significant reduction in myocardial tissue water content as compared to the I/R control group (Figure 8).

## Discussion

An important consequence of myocardial I/R is the occurrence of cardiac arrhythmias, which is often life

Table III. Value of ECG parameters in rats subjected to I/R injury. Values are expressed as mean±SEM for six animals in each group.

| Groups | Baseline | | | End of ischemia | | | End of reperfusion | | |
|---|---|---|---|---|---|---|---|---|---|
| | QT interval (ms) | RR interval (s) | QTc | QT interval (ms) | RR interval (s) | QTc | QT interval (ms) | RR interval (s) | QTc |
| Sham | 36.00±1.065 | 166.8±3.591 | 88.16±2.438 | 33.33±1.874 | 151.5±5.858 | 85.87±5.059 | 37.50±2.232 | 154.3±4.177 | 95.77±6.411 |
| I/R control | 29.33±1.382 | 156.2±5.747 | 89.27±5.679 | 31.67±2.044 | 75.83±6.779*** | 80.39±5.735*** | 25.67±0.8819 | 109.7±5.678*** | 94.31±3.759*** |
| I/R+HSP | 36.67±2.011 | 154.7±3.836 | 93.47±5.666 | 33.50±0.7638 | 109.5±4.349## | 98.93±2.690*** | 37.00±0.5774 | 140.5±4.006## | 101.7±4.168## |
| I/R+Vit.E | 35.67±1.892 | 151.5±5.858 | 91.90±5.223 | 25.17±0.3073 | 100.7±6.422 | 79.98±2.953 | 30.17±0.7032 | 110.8±3.754 | 90.71±1.715 |

$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ for sham vs I/R control.
$\#p<0.05$, $\#\#p<0.01$ and $\#\#\#p<0.001$ for I/R control vs I/R+HSP.

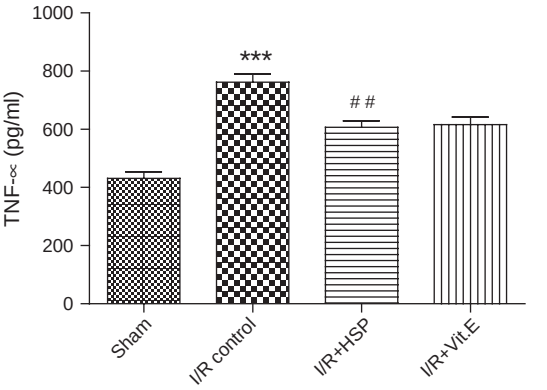


Figure 5. TNF-$\alpha$ level in rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: pg/mL. Values are expressed as mean±SEM. for six animals in the group. Values are expressed as mean±SEM for six animals in the group. $^{***}p<0.001$ for sham vs I/R control. $\#\#p<0.01$ for I/R control vs I/R+HSP.

threatening [18,19]. Notable evidence suggests ROS are involved in the pathogenic mechanism of I/R-induced arrhythmia [20]. Therefore, searches for preventive alternate measures are needed. It is reasonable to assume that a reinforcement of anti-oxidant defense of cardiac tissue should be a strategy to protect the heart from the oxidative damage. In this study, *Hesperidin* effectively reduced tissue MDA level and increased tissue levels of antioxidant enzymes like SOD, CAT and GSH. Therefore, in this study, *Hesperidin*, a flavanone glycoside with an antioxidant activity, is studied for its protective effects in an *in vivo* rat model of I/R-induced arrhythmias [21].

The release of ROS in the early phase of reperfusion, in combination with the ischemia-induced decrease in anti-oxidant activity, renders the myocardium vulnerable [22]. In this study, I/R caused a significant increase in the tissue MDA level and depleted the anti-oxidant enzyme pool, as evident from the significant declined levels of biomarker of oxidative stress enzymes like SOD, CAT and GSH in I/R control animals as compared to sham-operated animals, which indicates an increase in oxidative stress. In addition, the results show a significant reduction in tissue nitrite level in the I/R control group as compared to the sham-operated group. Decreased NO level indicates a rise in peroxynitrite (reactive nitrogen species) concentration after I/R. Schulz et al. [23] showed the cardiac depressant effect of peroxynitrite. In this study, fall in MAP and $\pm\, dp/dt_{max}$ during I/R and decrease in HR at the end of reperfusion indicates the cardiac depressant effect. There was a significant reduction in the levels of MDA and increase in the levels of biomarker of oxidative stress in the *Hesperidin* treated I/R control group, which had ultimately improved the level of tissue NO. Reduction in oxidative stress and increase in NO level in the *Hesperidin* treated I/R control

Table IV. Value of tissue MPO activity, nitrite level and serum CK-MB level in rats subjected to I/R injury. Values are expressed as mean ± SEM for six animals in each group.

| Groups | MPO (U/g tissue) | CK-MB (U/L) | Nitrite (nM/g tissue) |
|---|---|---|---|
| Sham | 3.50 ± 0.84 | 795.0 ± 26.51 | 157.3 ± 6.22 |
| I/R control | 10.83 ± 0.47*** | 1125 ± 41.56*** | 135.5 ± 4.38* |
| I/R+HSP | 5.16 ± 0.94### | 913.2 ± 19.76### | 160.2 ± 3.37# |
| I/R+Vit.E | 7.00 ± 0.73 | 999.0 ± 13.38 | 143.5 ± 5.43 |

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for sham vs I/R control.
#$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ for I/R control vs I/R+HSP.

group showed significant improvement in MAP and $\pm dp/dt_{max}$ during I/R and HR at the end of I/R. Previous studies have shown that ROS in sufficiently high concentrations damage the sarcoplasmicreticulum, causing calcium overload [24]. These increased $Ca^{2+}$ levels shift the inositol phosphate response from inositol 1,4-bisphosphate to inositol 1,4,5-trisphosphate receptor activation, thus potentially increasing $Ca^{2+}$ levels even further and producing arrhythmias [25]. Moreover, peroxynitrite formation mediates the effects of the ischemic metabolite lysophosphatidylcholine on cardiac late cardiac $Na^{+}$ current and produces arrhythmias [26]. Numerous observations have confirmed the roles of ROS and peroxynitrite in aggravation of inflammation during I/R [27–31]. NF-*Ќβ* produced during I/R stimulates

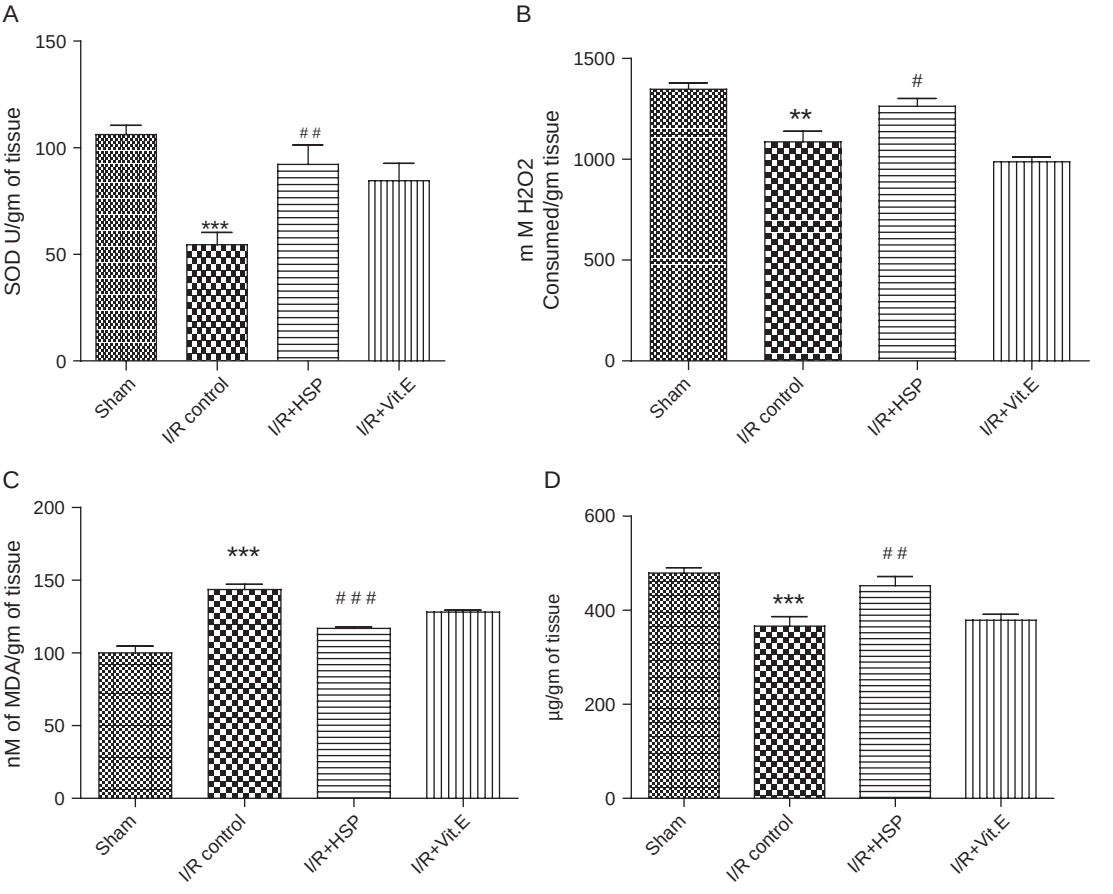


Figure 6. (A) SOD level in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: SOD U/gm of tissue. Values are expressed as mean ± SEM. for six animals in each group. ***$p < 0.001$ for sham vs I/R control. ##$p < 0.01$ for I/R control vs I/R+HSP. (B) CAT level in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: nmols of $H_2O_2$ consumed/g of tissue. Values are expressed as mean ± SEM for six animals in each group. **$p < 0$ for sham vs I/R control. # $p < 0.05$ for I/R control vs I/R+HSP. (C) MDA level in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: nM OF MDA/g tissue. Values are expressed as mean ± SEM for six animals in each group. ***$p < 0.001$ for sham vs I/R control. ###$p < 0.001$ for I/R control vs I/R+HSP. (D) GSH level in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit: µg/gm of tissue. Values are expressed as mean ± SEM for six animals in each group. ***$p < 0.001$ for sham vs I/R control. ##$p < 0.01$ for I/R control vs I/R+HSP.

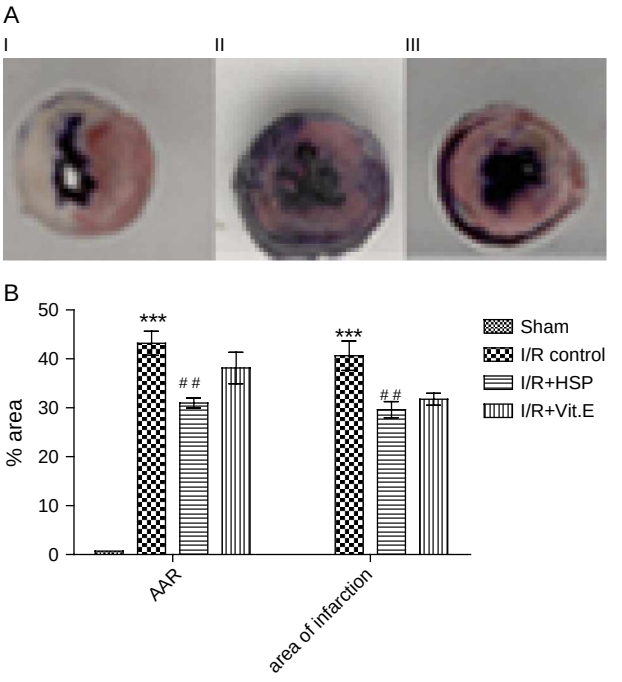


Figure 7. (A) Images of AAR and area of infarction of the heart subjected to I/R in (I) I/R control, (II) I/R+HSP and (III) I/R+Vit.E groups. (B) AAR and area of infarction in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Unit:% of area. Values are expressed as mean ± SEM for six animals in each group. ***$p < 0.001$ for sham vs I/R control. ##$p < 0.01$ for I/R control vs I/R+HSP.

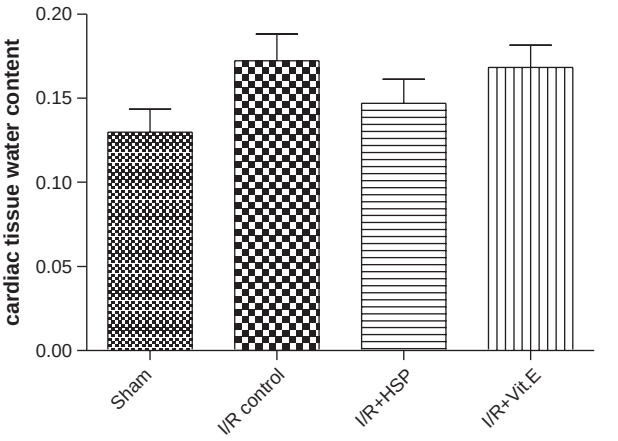


Figure 8. Cardiac tissue water content in heart tissue of rats subjected to I/R in sham-operated, I/R control, I/R+HSP and I/R+Vit.E groups. Values are expressed as mean ± SEM for six animals in each group. $p > 0.05$ for sham vs I/R control (non-significant). $p > 0.05$ for I/R control vs I/R+HSP (non-significant).

specific protein transcription such as TNF-$\alpha$ [31]. Over-expression of TNF-$\alpha$ contributes to the initiation of rapid re-entrant arrhythmias [32]. Studies also proved that neutophils involved in I/R, which releases HOCl, a product of MPO, increase the susceptibility of the myocardium to reperfusion induced arrhythmias [33]. In this study also, significant increases in levels of serum TNF-$\alpha$ and tissue MPO activity were observed, which ultimately enhance incidences, duration and severity of arrhythmias (VT, VF and VEBs) in the I/R control group. In addition, in this experiment I/R shortened the corrected QT (QTc) interval. It is believed the QTc indicates the action potential duration (APD). Refractoriness may correspond to APD and shortening of APD during ischemia will be accompanied by a corresponding shortening of refractoriness, which would be pro-arrhythmic [34]. However, treatment with *Hesperidin* in the I/R control group showed a significant decrease in incidences and duration of arrhythmias (VT, VF, VEBs and prolongation of QTc) as compared to the I/R control group, the effect may be secondary to decreased levels of oxidative stress, serum TNF-$\alpha$ and tissue MPO activity.

In this study, ischemia was associated with a greater degree of oedema formation and might associate with myocardial dysfunction. Laine and Allen [35] proposed that 1% increase in myocardial water content could be expected to result in possibly a 10% reduction in myocardial function. This finding is consistent with the current study. In the present study, the I/R control group showed\non-significant increase in myocardial tissue water content and a reduction in cardiac function (MAP, $\pm$ d$p$/d$t$). However, *Hesperidin* treatment in the I/R control group showed a non-significant reduction in myocardial tissue water content and improved cardiac functions.

Previous studies proved that ROS produced during I/R could trigger myocyte apoptosis by activating MAPK and producing DNA damage by activation of the nuclear enzyme poly(ADP-ribose) polymerase, which consumes cellular nicotinamide dinucleotide and adenosine triphosphate [36–38]. It has been previously shown that $H_2O_2$ administration to cultured adult rat cardiomyocytes led to a decrease in Bcl-2 (an anti-apoptotic protein) levels and an increase in Bax (a pro-apoptotic protein) levels, whereas cells concomitantly exhibit apoptotic features [38]. Similar results were obtained in the current study. In this study also, the I/R control group showed a significant increase in AAR and area of infarction. In addition, increased serum CK-MB level also confirms marked myocardial damage. However, evidence proved that the polyphenolic agents have the ability to inhibit the expression of the apoptosis-related genes Fas and Fas-L [39]. Studies also proved that anti-oxidant treatment suppresses cardiac oxidative stress and hence myocardial apoptosis. Similar results were observed in our study. *Hesperidin* treatment significantly reduced AAR and area of infarction as compared to the I/R control group [40,41].

In conclusion, oxidative stress is a broad term, which influences many pathways during I/R. The current study has examined various parameters which

can aggravate this diverse pathological condition after myocardial I/R. The mechanism of oxidative stress-induced apoptosis and arrhythmias has not been fully elucidated so far. Therefore, it seems that *Hesperidin*, by prolonging the QTc interval, action potential duration and subsequent refractoriness, could improve the I/R-induced arrhythmias and, by reducing oxidative stress and inflammation, protect myocardial apoptosis. However, further clinical studies are necessary to characterize the effects of the *Hesperidin* on the heart and to predict its potential therapeutic use.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.



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