Hesperidin, a flavanone glycoside, on lipid peroxidation and antioxidant status in experimental myocardial ischemic rats

Palanisamy Selvaraj, Kodukkur Viswanathan Pugalendi

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar, Tamil Nadu, India

Myocardial infarction continues to be a leading cause of mortality world-wide. Novel therapies are needed to treat the myocardial ischemia. This study was undertaken to evaluate the cardioprotective role of hesperidin on isoproterenol-induced myocardial ischemia in rats. Myocardial ischemia was induced by subcutaneous injection of isoproterenol hydrochloride (85 mg/kg body weight), for two consecutive days. Isoproterenol-administered rats showed elevated levels of cardiac markers (aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, creatine kinase-MB, cardiac troponins T and I) when compared with control and hesperidin treatment groups (100, 200 and 400 mg/kg body weight). The serum levels of cardiac markers were significantly reduced at the doses of 200 mg and 400 mg. All further experiments were carried out at the 200 mg dose. Lipid peroxidation markers (thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes) were elevated significantly in the plasma and heart whereas non-enzymic antioxidants (vitamin C, vitamin E and reduced glutathione) were decreased significantly. Activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase declined significantly in the heart of ischemic rats. However, after hesperidin treatment, all the above parameters reverted to normal levels. This study demonstrated that the cardioprotective effect of hesperidin on ischemic rats could be due to its antilipidperoxidative and antioxidant properties.

Keywords: isoproterenol, hesperidin, myocardial ischemia, antioxidants, lipid peroxidation

Introduction

Myocardial infarction continues to be a leading cause of mortality world-wide. Myocardial infarction will be the most important cause of mortality in India by the year 2015.¹ Isoproterenol-induced myocardial ischemia in rats has been the widely used experimental model to

study the beneficial effects of many drugs on cardiac function. Isoproterenol, a synthetic catecholamine and β -adrenoreceptor agonist, causes severe stress in the myocardial tissue and its high dosages produce acute myocardial necrosis.² Administration of isoproterenol generates reactive oxygen species, which may contribute to oxidative stress and affect the antioxidant system. Previous study suggested that the role of reactive oxygen species in the pathogenesis of ischemia/reperfusion injury.³ Thus, increased production of reactive oxygen species may be a unifying mechanism in ischemic injury progression, and

Correspondence to: K.V. Pugalendi MSc MPhil PhD, Professor and Head, Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar – 608 002, Tamilnadu, India
Tel: +91 4144 238343; Fax: +91 4144 239141; E-mail: pugale@sify.com
Received 5 April 2010, revised manuscript accepted 8 July 2010

Figure 1 Structure of hesperidin

antioxidants may be the therapeutic value in this setting.

Novel therapies are needed to treat myocardial ischemia because current treatments have a limited impact on survival and annual costs. Flavonoids are the major components of traditional medicine. Hesperidin (HDN; 3',5,7-trihydroxy-4'-methoxy flavanone-7-(6- α -L-rhamnopyranosyl- β -D-glucopyranoside) is a naturally-occurring flavonoid found in fruits and vegetables; the structure is given in Figure 1. Flavonoids are polyphenolic compounds which act as potent antioxidants. They can directly quench free radicals and inhibit enzymes of oxygen-reduction pathways and also sequestration of transient metal cations.⁴ Hesperidin possesses significant anti-inflammatory and analgesic,⁵ and anti-oxidant⁶ activities. Previous study reported the antioxidant activity of hesperidin on nicotine-induced oxidative stress in lung toxicity.⁷

The present study aimed to evaluate the cardioprotective role of hesperidin on isoproterenol-induced myocardial ischemia by analyzing biochemical end-points in serum, plasma and heart tissues of Wistar rats.

Materials and methods

Animals

Healthy, male, albino, Wistar rats (160–180 g) were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an air-conditioned room ($25 \pm 3^\circ\text{C}$) with a 12-h light/12-h dark cycle. Feed and water were provided *ad libitum* to all the animals. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA, Proposal number: 541), Annamalai University, Annamalaiagar.

Chemicals

Isoproterenol hydrochloride and hesperidin were

purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, India.

Experimental induction of myocardial ischemia

Myocardial ischemia was induced by subcutaneous injection of isoproterenol hydrochloride (85 mg/kg body weight dissolved in 1 ml of physiological saline) for the first two consecutive days.⁸

Experimental design

Dose fixation study

Thirty-six rats were randomly divided into six groups with six rats each. Hesperidin dissolved in carboxyl methyl-cellulose (0.5%) was administered orally to rats in groups II, IV, V and VI. Carboxyl methyl-cellulose treatment alone acted as a vehicle control.

- Group I Control (0.5% carboxyl methyl-cellulose orally for 7 days)
- Group II Hesperidin control (400 mg/kg body weight, orally for 7 days)
- Group III Isoproterenol control (85 mg/kg body weight, s.c. for first 2 consecutive days)
- Group IV Isoproterenol (85 mg/kg body weight, s.c. for first 2 consecutive days) + hesperidin (100 mg/kg body weight, orally for 7 days)
- Group V Isoproterenol (85 mg/kg body weight, s.c. for first 2 consecutive days) + hesperidin (200 mg/kg body weight, orally for 7 days)
- Group VI Isoproterenol (85 mg/kg body weight, s.c. for first 2 consecutive days) + hesperidin (400 mg/kg body weight, orally for 7 days)

The total duration of the study was 9 days. At day 10, the rats were anaesthetized with intramuscular injection of ketamine hydrochloride (24 mg/kg body weight), and sacrificed by cervical dislocation. Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min. Plasma was separated by collecting blood in tubes containing heparin and centrifuged at 2000 rpm for 10 min. The serum and plasma samples were used for cardiac marker assay.

Further study

The 24 rats were randomly divided into four groups with six rats each. Hesperidin dissolved in carboxyl methyl-cellulose (0.5%) was administered orally to rats

in groups II and IV. Carboxyl methyl-cellulose treatment alone acted as a vehicle control.

- Group I Control (0.5% carboxyl methyl-cellulose orally for 7 days)
- Group II Hesperidin control (200 mg/kg body weight, orally for 7 days)
- Group III Isoproterenol control (85 mg/kg body weight, s.c. for first 2 consecutive days)
- Group IV Isoproterenol (85 mg/kg body weight, s.c. for first 2 consecutive days) + hesperidin (200 mg/kg body weight, orally for 7 days)

The total duration of the study was 9 days. At day 10, the rats were anaesthetized with intramuscular injection of ketamine hydrochloride (24 mg/kg body weight), and sacrificed by cervical dislocation. Blood was collected in a tubes containing heparin and centrifuged at 2000 rpm for 10 min. The separated plasma was used for estimations. Immediately after the sacrifice, the heart was excised and washed in ice-cold isotonic saline, blotted with a filter paper, weighed, and a portion of the tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was used for biochemical estimations.

Biochemical estimations

The activities of aspartate transaminase and alanine transaminase were assayed with the method of Reitman and Frankel.⁹ Lactate dehydrogenase, creatine kinase and creatine kinase-MB were assayed with the methods of King¹⁰ and Okinaka *et al.*,¹¹ respectively, using commercially available kits. Plasma cardiac troponin T and I were quantitatively measured with highly specific enzyme immunoassay kits.^{12,13} The

levels of thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes were estimated with the methods of Niehaus *et al.*,¹⁴ Jiang *et al.*,¹⁵ and Rao and Recknagel,¹⁶ respectively. The levels of vitamin C, vitamin E and reduced glutathione were estimated with the methods of Roe and Kuether,¹⁷ Baker *et al.*,¹⁸ and Ellman,¹⁹ respectively. The protein content was determined with the method of Lowry *et al.*²⁰ Superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase were assayed with the methods of Kakkar *et al.*,²¹ Sinha,²² Rotruck *et al.*,²³ Habig *et al.*,²⁴ and Pinto and Bartley,²⁵ respectively.

Histopathology

Myocardial tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (3 µm) were cut, fixed on glass slides and stained with hemotoxylin and eosin (H&E) and examined under a light microscope.

Statistical analysis

Values are expressed as mean ± SD. The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistical package program (SPSS v10.0 for Windows). *P*-values of less than 0.05 were considered as statistically significant.

Results

Dose fixation study

Table 1 shows the activities of serum aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, creatine kinase-MB and the levels of

Table 1 Effect of hesperidin on serum aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, creatine kinase-MB and plasma cardiac troponin T and I in control and isoproterenol-rats

Groups	Aspartate transaminase (IU/l)	Alanine transaminase (IU/l)	Lactate dehydrogenase (IU/l)	Creatine kinase (IU/l)	Creatine kinase-MB (IU/l)	Cardiac troponin T (ng/ml)	Cardiac troponin I (ng/ml)
Control	75.02 ± 6.99 ^{a,d}	19.18 ± 1.50 ^a	215.11 ± 18.87 ^a	143.81 ± 8.67 ^a	88.11 ± 7.21 ^{a,d}	0.54 ± 0.03 ^a	0.33 ± 0.02 ^a
Hes. control (400 mg/kg BW)	71.60 ± 6.69 ^a	18.46 ± 1.08 ^a	209.71 ± 19.27 ^a	140.73 ± 12.45 ^a	86.04 ± 5.68 ^a	0.51 ± 0.01 ^a	0.30 ± 0.02 ^a
Iso. (85 mg/kg BW)	101.63 ± 9.10 ^b	45.29 ± 4.23 ^b	273.09 ± 17.00 ^b	203.05 ± 16.43 ^b	132.89 ± 9.97 ^b	1.15 ± 0.08 ^b	0.58 ± 0.05 ^b
Iso.+ Hes. (100 mg/kg BW)	93.75 ± 7.08 ^c	41.51 ± 2.42 ^c	251.74 ± 15.56 ^c	184.52 ± 16.61 ^c	121.95 ± 12.41 ^c	0.97 ± 0.05 ^c	0.52 ± 0.04 ^c
Iso. + Hes. (200 mg/kg BW)	81.25 ± 3.78 ^d	23.06 ± 1.05 ^d	222.69 ± 17.14 ^a	135.04 ± 11.75 ^a	96.73 ± 6.59 ^d	0.63 ± 0.02 ^d	0.39 ± 0.02 ^d
Iso. + Hes. (400 mg/kg BW)	80.81 ± 2.98 ^d	21.33 ± 1.91 ^d	220.91 ± 20.15 ^a	134.85 ± 10.88 ^a	95.03 ± 7.38 ^d	0.60 ± 0.06 ^d	0.38 ± 0.01 ^d

BW = Body weight; Iso. = isoproterenol; Hes. = hesperidin

Values are expressed as mean ± SD for six rats in each group>

Values not sharing common superscripts (a, b, c and d) differ significantly at *P* < 0.05 (DMRT).

Table 2 Effect of hesperidin on thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes in the plasma and heart of control and isoproterenol-rats

Groups	Thiobarbituric acid reactive substances		Lipid hydroperoxides		Conjugated dienes	
	Plasma (mmol/dl)	Heart (mmol/100 g wet tissue)	Plasma (mmol/dl)	Heart (mmol/100 g wet tissue)	Plasma (mmol/dl)	Heart (mmol/100 g wet tissue)
Control	0.19 ± 0.01 ^{a,c}	0.46 ± 0.02 ^a	8.74 ± 0.63 ^a	67.91 ± 3.19 ^a	0.75 ± 0.05 ^a	34.50 ± 2.07 ^a
Hesperidin control (200 mg/kg body weight)	0.17 ± 0.01 ^a	0.42 ± 0.03 ^a	8.02 ± 0.23 ^a	65.82 ± 3.99 ^a	0.72 ± 0.03 ^a	32.87 ± 2.90 ^a
Isoproterenol (85 mg/kg body weight)	0.33 ± 0.03 ^b	0.84 ± 0.05 ^b	12.79 ± 1.05 ^b	96.84 ± 8.85 ^b	1.34 ± 0.07 ^b	54.82 ± 5.10 ^b
Isoproterenol + hesperidin (200 mg/kg body weight)	0.21 ± 0.02 ^c	0.53 ± 0.03 ^c	9.95 ± 0.80 ^c	78.50 ± 5.99 ^c	0.85 ± 0.06 ^c	41.87 ± 3.32 ^c

Values are expressed as mean ± SD for six rats in each group.

Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 3 Effect of hesperidin on vitamin C, vitamin E, and reduced glutathione in the plasma and heart of control and isoproterenol-rats

Groups	Vitamin C		Vitamin E		Glutathione	
	Plasma (mg/dl)	Heart (μg/mg protein)	Plasma (mg/dl)	Heart (μg/mg protein)	Plasma (mg/dl)	Heart (μg/mg protein)
Control	2.78 ± 0.11 ^a	0.54 ± 0.04 ^a	2.00 ± 0.13 ^a	2.78 ± 0.21 ^a	36.31 ± 3.45 ^a	8.82 ± 0.69 ^a
Hesperidin control (200 mg/kg body weight)	2.97 ± 0.14 ^a	0.57 ± 0.03 ^a	2.09 ± 0.21 ^a	2.89 ± 0.23 ^a	38.22 ± 2.09 ^a	8.76 ± 0.68 ^a
Isoproterenol (85 mg/kg body weight)	1.89 ± 0.05 ^b	0.38 ± 0.01 ^b	1.12 ± 0.10 ^b	1.91 ± 0.13 ^b	25.66 ± 1.32 ^b	5.88 ± 0.29 ^b
Isoproterenol + hesperidin (200 mg/kg body weight)	2.53 ± 0.12 ^c	0.50 ± 0.03 ^c	1.76 ± 0.08 ^c	2.47 ± 0.22 ^c	31.99 ± 2.58 ^c	7.94 ± 0.59 ^c

Values are expressed as mean ± SD for six rats in each group.

Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

plasma troponin T and I in control and experimental rats. Increased activities of aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, creatine kinase–MB, cardiac troponin T and I were observed in isoproterenol-induced ischemic rats. Oral administration of hesperidin (100, 200 and 400 mg/kg body weight) to ischemic rats decreased the activities of these parameters. Ischemic rats treated with 200 mg and 400 mg hesperidin showed significant difference when compared to isoproterenol control

rats, but no significant difference was observed between these two doses. Hence, the 200 mg dose was used for further study.

Further study

Table 2 shows the levels of thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes in the plasma and heart of control and experimental rats. The levels of thiobarbituric acid reactive substances, lipid hydroperoxides and

Table 4 Effect of hesperidin on the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase in the heart of control and isoproterenol-rats

Groups	Superoxide dismutase	Catalase	Glutathione peroxidase	Glutathione-S-transferase	Glutathione reductase
Control	6.75 ± 0.32 ^a	48.33 ± 4.67 ^{a,c}	6.42 ± 0.56 ^{a,c}	5.69 ± 0.29 ^a	5.61 ± 0.27 ^a
Hesperidin control (200 mg/kg body weight)	6.82 ± 0.29 ^a	49.94 ± 4.65 ^a	6.75 ± 0.39 ^a	5.90 ± 0.23 ^a	5.80 ± 0.37 ^a
Isoproterenol (85 mg/kg body weight)	5.11 ± 0.14 ^b	38.71 ± 3.46 ^b	4.89 ± 0.21 ^b	3.22 ± 0.28 ^b	3.93 ± 0.35 ^b
Isoproterenol + hesperidin (200 mg/kg body weight)	6.16 ± 0.19 ^c	44.65 ± 3.64 ^c	5.96 ± 0.22 ^c	4.65 ± 0.15 ^c	5.06 ± 0.48 ^c

Values are expressed as mean ± SD for six rats in each group.

Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Units: **superoxide dismutase**, enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition; **catalase**, μmole of hydrogen peroxide decomposed/min; **glutathione peroxidase**, μmole of GSH utilized/min; **glutathione-S-transferase**, μg of CDNB conjugate formed/min; and **glutathione reductase**, μg of reduced glutathione formed/min.

Figure 2 Histopathological changes of rat heart (hematoxylin and eosin $\times 400$). (A) Control group showing normal architecture of myocytes. (B) Hesperidin control (200 mg) group showing normal architecture of myocytes. (C) Isoproterenol induced group showing rupture of cardiac muscle fibers with infiltration of acute and chronic inflammatory cells. (D) Isoproterenol + hesperidin (200 mg) group showing mild hypertrophic cardiac muscle fibers

conjugated dienes were significantly elevated in the isoproterenol-induced ischemic rats. Hesperidin treatment significantly decreased the levels of above parameters.

Table 3 shows the levels of vitamin C, vitamin E and reduced glutathione in the plasma and heart of control and experimental rats. Vitamin C, vitamin E and reduced glutathione values significantly lowered in isoproterenol-induced ischemic rats. Hesperidin treatment improved the status of these parameters towards normal.

Table 4 shows the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase and glutathione reductase in the heart of control and experimental rats. Activities of these enzymes significantly decreased in isoproterenol-induced ischemic rats. Hesperidin treatment improved the activities of these enzymes towards normal.

Histopathological examination of isoproterenol-induced myocardial ischemic rats (Fig. 2C) showed rupture of cardiac muscle fibers with infiltration of acute and chronic inflammatory cells. Administration of hesperidin to ischemic rats (Fig. 2D) showed hypertrophic cardiac muscle fibers. Control (Fig. 2A) and hesperidin control (Fig. 2B) groups showed normal architecture of myocytes.

Discussion

The proposed mechanisms of isoproterenol-induced myocardial injury in rats are due to altered sarcolemma permeability and/or the formation of oxygen derived free radicals. In myocardial ischemia, impairment of Ca^{2+} channel in sarcoplasmic reticulum released more Ca^{2+} to cytosol that leads to cytosolic Ca^{2+} overload.²⁶ The oxidative stress may be exerted through quinone metabolites of isoproterenol, which

react with oxygen to produce reactive oxygen species.²⁷ Tappia *et al.*²⁸ have reported the association between Ca^{2+} overload and oxidative stress. When myocardial cells are damaged, the cell membrane becomes permeable, which results in the leakage of marker enzymes (aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase and creatine kinase-MB) into blood.²⁹ This accounts for the increased activities of these enzymes in isoproterenol-induced myocardial ischemic rats. Furthermore, the levels of plasma troponin T and I increased in isoproterenol-induced ischemic rats and our finding was in consonance with an earlier report.³⁰ Hesperidin treatment significantly decreased the activities of serum aspartate transaminase, alanine transaminase, lactate dehydrogenase, creatine kinase, creatine kinase-MB and plasma cardiac troponin T and I levels, indicating the cardioprotective activity. The cardioprotective activity of hesperidin may be due to its calcium channel blocking property. Morita *et al.*³¹ have reported the calcium channel blocking activity of hesperidin.

The isoproterenol-induced myocardial damage in rats can also generate lipid peroxidation products such as thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes.³² Hesperidin treatment reduced the generation of thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes in the plasma and myocardium. Previous study reported the antioxidant and radical scavenging properties of hesperidin.³³

The levels of vitamin C, vitamin E, and reduced glutathione significantly declined in isoproterenol-induced ischemic rats. The decreased levels of vitamin C, vitamin E and reduced glutathione may be due to enhanced utilization during reactive oxygen species scavenging function. Vitamin C is a potent free radical scavenger which prevents radical-mediated lipid

peroxidation.³⁴ α -Tocopherol, the major membrane constituent, suppresses membrane damage, thus terminating the chain reactions of lipid peroxidation. The restoration of α -tocopherol radical is achieved by reaction with ascorbic acid. Both vitamin C and vitamin E protect tissues from injury caused by reactive oxygen species.³⁵ Karthikeyan *et al.*³⁶ have reported the decreased levels of reduced glutathione in heart tissue of isoproterenol-induced ischemic rats. Reduced glutathione is a powerful cellular antioxidant, which is directly involved in the removal of superoxide radicals, peroxy radicals and singlet oxygen.³⁷ Hesperidin treatment significantly increased the levels of vitamin C, vitamin E, and reduced glutathione. It has been demonstrated that hesperidin treatment restored the levels of reduced glutathione in the liver of lipopolysaccharide-administered rats.⁶

Antioxidant activities of superoxide dismutase and catalase significantly decreased in the heart tissue of isoproterenol-induced ischemic rats. Superoxide dismutase is usually found in the plasma membrane, which protects cells from oxidative stress. Catalase is a tetrameric hemoprotein which acts as a catalyst for the removal of hydrogen peroxide. Oxygen species and utilization of these enzymic antioxidants counter lipid peroxidation. Decreased activities of superoxide dismutase and catalase in myocardium of isoproterenol-administered rats have been reported.³⁸ Hesperidin administration significantly increased the activities of superoxide dismutase and catalase. The increased activities of these enzymes might be due to the reactive oxygen species scavenging property of hesperidin.

The glutathione-dependent enzymes, glutathione peroxidase, glutathione *S*-transferase and glutathione reductase activities significantly declined in the heart of ischemic rats. Karthikeyan *et al.*³⁶ have shown that isoproterenol-intoxication can lead to depletion of glutathione peroxidase and glutathione *S*-transferase activities in rat myocardium. Glutathione peroxidase acts as a catalyst in the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxides. Glutathione *S*-transferase detoxifies reactive oxygen species and other xenobiotic molecules by the utilization of reduced glutathione.³⁹ Regeneration of reduced glutathione from oxidized glutathione in the cell is catalysed by glutathione reductase.⁴⁰ Glutathione peroxidase and glutathione *S*-transferase activities decreased in isoproterenol-induced ischemic rats which might be due to decreased availability of reduced glutathione. Suppression of

these enzymes leads to the accumulation of reactive oxygen species and makes the cardiomyocytes more susceptible to oxidative injury. In our study, the administration of hesperidin restored the activities of glutathione-dependent enzymes close to normal, which is attributed to the therapeutic nature of hesperidin against peroxidative injury.

Histopathological examination of isoproterenol-induced myocardial ischemic rats showed rupture of cardiac muscle fibers with infiltration of acute and chronic inflammatory cells. Administration of hesperidin to ischemic rats showed mild hypertrophy of cardiac muscle fibers. Control and hesperidin control groups showed normal architecture of myocytes.

Conclusions

Hesperidin treatment to isoproterenol-induced ischemic rats proved to be effective in reducing the extent of myocardial damage by decreasing lipid peroxidation and increasing antioxidant status in experimental rats, and administration of hesperidin at 200 mg dose brought the changes to near normal. Thus, the histopathological studies served as a direct evidence of efficacy of hesperidin as protectant.

References

1. Gilski DJ, Borkenhagen B. Risk evaluation in action for cardiovascular health. *Crit Care Nurse* 2005; **25**: 26–28, 30–37.
2. Jia YX, Pan CS, Zhang J *et al.* Apelin protects myocardial injury induced by isoproterenol in rats. *Regul Pept* 2006; **133**: 147–154.
3. Singh N, Dhalla AK, Seneviratne C, Singal PK. Oxidative stress and heart failure. *Mol Cell Biochem* 1995; **147**: 77–81.
4. Chen YT, Zheng RL, Jia ZJ, Ju Y. Flavonoids as superoxide scavengers and antioxidants. *Free Radic Biol Med* 1990; **9**: 19–21.
5. Galati EM, Monforte MT, Kirjavainen S, Forestieri AM, Trovato A, Tripodo MM. Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco* 1994; **40**: 709–712.
6. Kaur G, Tirkey N, Chopra K. Beneficial effect of hesperidin on lipopolysaccharide-induced hepatotoxicity. *Toxicology* 2006; **226**: 152–160.
7. Balakrishnan A, Menon VP. Antioxidant properties of hesperidin in nicotine-induced lung toxicity. *Fund Clin Pharmacol* 2007; **21**: 535–546.
8. Seth SD, Maulik M, Katiyar CK, Maulik SK. Role of Lipistat in protection against isoproterenol-induced myocardial necrosis in rats: a biochemical and histopathological study. *Indian J Physiol Pharmacol* 1998; **42**: 101–106.
9. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamate oxaloacetic and glutamate pyruvic transaminases. *Am J Clin Pathol* 1957; **28**: 56–63.
10. King J. The dehydrogenases or oxidoreductase. Lactate dehydrogenase. In: Van Nostrand E. (ed) *Practical Clinical Enzymology*. London: 1965; 106.
11. Okinaka S, Kumagai H, Ebashi S *et al.* Serum creatine phosphokinase. Activity in progressive muscular dystrophy and neuromuscular diseases. *Arch Neurol* 1961; **4**: 520–525.

12. Katus HA, Remppis A, Neumann FJ, Scheffold T, Diederich KW, Vinar G. Diagnostic efficacy of troponin T measurements in acute myocardial infarction. *Circulation* 1991; **83**: 902–912.
13. Larue C, Calzolari C, Bertinchant JP, Leclercq F, Grolleau R, Pau B. Cardiac-specific immunoenzymomimetic assay of troponin I in the early phase of acute myocardial infarction. *Clin Chem* 1993; **39**: 972–979.
14. Niehaus WG, Samuelson B. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem* 1968; **6**: 126–130.
15. Jiang ZY, Hunt JV, Wolff SP. Detection of lipid hydroperoxides using the 'fox method.' *Anal Biochem* 1992; **202**: 384–389.
16. Rao KS, Recknagel RO. Early onset of lipid peroxidation in rat liver after carbon tetrachloride administration. *Exp Mol Pathol* 1968; **9**: 271–278.
17. Roe JH, Kuether CA. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J Biol Chem* 1943; **11**: 145–164.
18. Baker H, Frank O, DeAngelis B, Feingold S. Plasma α -tocopherol in man at various times after ingesting free or acetylated tocopherol. *Nutr Res Inst* 1980; **21**: 531–536.
19. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82**: 70–77.
20. Lowry OH, Rosebrough NJ, Farr AL, Randell RJ. Protein measurement with Folin's phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
21. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* 1984; **21**: 130–132.
22. Sinha AK. Colorimetric assay of catalase. *Anal Biochem* 1972; **47**: 389–394.
23. Rotruck T, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973; **179**: 588–590.
24. Habig WH, Pabst MJ, Jakoby WBC. Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130–7139.
25. Pinto RE, Bartley W. The effect of age and sex on glutathione reductase and glutathione peroxidase activities on aerobic glutathione oxidation in rat liver homogenate. *Biochem J* 1969; **12**: 109–115.
26. Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol Rev* 1997; **49**: 1–51.
27. Rathore N, John S, Kale M, Bhatnagar D. Lipid peroxidation and antioxidant enzymes in isoproterenol-induced oxidative stress in rat tissues. *Pharmacol Res* 1998; **38**: 297–303.
28. Tappia PS, Hata T, Hozaima L, Sandhu MS, Panagia V, Dhalla NS. Role of oxidative stress in catecholamine-induced changes in cardiac sarcolemmal Ca^{2+} transport. *Arch Biochem Biophys* 2001; **387**: 85–92.
29. Nasa Y, Sakamoto Y, Sanbe A, Sasaki H, Yamaguchi F, Takeo S. Changes in fatty acid compositions of myocardial lipids in rats with heart failure following myocardial infarction. *Mol Cell Biochem* 1997; **176**: 179–189.
30. Acikel M, Buyukokuroglu ME, Erdogan F, Aksoy H, Bozkurt E, Senocak H. Protective effects of dantrolene against myocardial injury induced by isoproterenol in rats: biochemical and histological findings. *Int J Cardiol* 2005; **98**: 389–394.
31. Morita O, Sasaki H, Sato S. Calcium antagonists containing phenols. *Patent Japan Kokai Tokkyo Koho* 1992; 04, 243, 822.
32. Senthil S, Sridevi M, Pugalendi KV. Cardioprotective effect of oleanolic acid on isoproterenol-induced myocardial ischemia in rats. *Toxicol Pathol* 2007; **35**: 418–423.
33. Kim JY, Jung KJ, Choi JS, Chung HY. Hesperetin: a potent antioxidant against peroxynitrite. *Free Radic Res* 2004; **38**: 761–769.
34. Choi SW, Benzie IF, Collins AR, Hannigan BM, Strain JJ. Vitamins C and E: acute interactive effects on biomarkers of antioxidant defence and oxidative stress. *Mutat Res* 2004; **551**: 109–117.
35. Gester H. β -Carotene, vitamin-E and vitamin C in different stages of experimental carcinogenesis. *Eur J Clin Nutr* 2002; **49**: 155–168.
36. Karthikeyan K, Bai BR, Devaraj SN. Cardioprotective effect of grape seed proanthocyanidins on isoproterenol-induced myocardial injury in rats. *Int J Cardiol* 2007; **115**: 326–333.
37. Abidi P, Afaq F, Arif JM, Lohani M, Rahman Q. Chrysotile-mediated imbalance in the glutathione redox system in the development of pulmonary injury. *Toxicol Lett* 1999; **106**: 31–39.
38. Sumitra M, Manikandan P, Kumar DA *et al.* Experimental myocardial necrosis in rats: role of arjunolic acid on platelet aggregation, coagulation and antioxidant status. *Mol Cell Biochem* 2001; **224**: 135–142.
39. Coles B, Ketterer B. The role of glutathione and glutathione transferases in chemical carcinogenesis. *Crit Rev Biochem Mol Biol* 1990; **25**: 47–70.
40. Voet D, Voet JG. *Biochemistry*, 2nd edn. New York: Wiley, 1995; 400–406.