

Preventive effect of Fisetin on Cardiac markers, Lipid peroxides and Antioxidants in normal and Ischemia-Reperfusion induced Myocardial infarction in rats

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Abstract: Cardioprotective effects of Fisetin may be attributed to its antioxidant, anti-inflammatory and anti-apoptotic activities. Wistar albino rats were randomly cleaved into 5 groups (n=10 in each group): Group I (Sham group), Group II (Control group), Group III (Vehicle treated), Group IV (Fisetin 10 mg/kg) and Group V (Fisetin 20 mg/kg), left coronary artery occluded for 30 min, followed by 4 hours of anesthetized rat reperfusion. The concentration of myocardial enzymes (LDH, CKMB), antioxidants (SOD, CAT, GSH, GPx, GST) and MDA levels were measured after the model was developed. Results showed that the rates of infarction, serum cardiac markers (CKMB and LDH), antioxidant tissue enzymes (SOD, CAT, GPx, GSH, and GST) have increased, the levels of lipid peroxides have decreased and the ultrastructural changes in the myocardial organization have improved significantly in Group IV and V, when compared with the Group I. Fisetin's cardioprotective effect in this model was slightly changed via reduced infarct volume, decreased CK-MB and LDH release increased the capacity of antioxidants (SOD, CAT, GPx, GSH, and GST), decreased lipid peroxidation (MDA) and further confirmed by histopathological reports.

Keywords: Cardioprotection, Fisetin, ischemia-reperfusion, cardiac markers, anti-inflammatory

Introduction

Ischemic heart disease is the predominant cause of mortality globally; hence, several therapeutic approaches to protect the ischemic myocardium have been studied (1). When coronary blood circulation to the myocardium is reduced, either the absolute flow rate or relative to increased tissue demand, myocardial ischemia occurs. Irreversible myocardial injury is the most severe outcome of depreciated blood circulation to the heart, which befalls when ischemia is prolonged exceeding 20 min (1-3). Reperfusion, the accelerated return of blood circulation to the ischemic region of the myocardium, provides oxygen and metabolic substrates and washes out ischemic metabolites. Myocardial ischemia accompanied by reperfusion effects in myocardial ischemia/reperfusion (I/R) injury and is characterized by diminished myocardial function, increased incidence of arrhythmias and the growth of tissue necrosis. The reperfusion phase is propitious to the heart but is still correlated with myocardial injury (4). Reperfusion of ischemic myocardium can result in additional cellular damage; still, the pathogenesis of I/R injury is not completely known.

Despite, enormous experimental knowledge and numerous promising statements in both experimental and clinical studies, there is no pharmacological therapy yet that is acknowledged to be of the 'gold standard' for using in the treatment of I/R injury. Therapeutic strategies that can reduce I/R injury, especially during cardiac surgery, are required. Consequently, one of the significant therapeutic aims of current studies related to heart is to propose tactics intended at restoring the myocardium from the ravages of I/R injury and to enhance the benefits of reperfusion therapy. Numerous mechanisms have been suggested to describe the myocardial injury perceived after I/R.

Flavonoids have been found to show antioxidant activity and to possess cardioprotective potential. The link between flavonoid ingestion and long-term mortality effects was subsequently studied and it was exhorted that flavonoid ingestion is conversely proportional with mortality due to heart disease (5). Experimental evidence is lacking on

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Fisetin's biochemical role in coronary artery ligation-induced myocardial infarction. In this context, an attempt was made to clarify the preservation of myocardial integrity in the presence of Fisetin on ischemic-reperfusion injury caused by coronary artery ligation in rats regarding biochemical markers, lipid peroxides, antioxidants and histology.

Materials and Methods

Experimental animals:

The experiment was conducted in compliance with recommendations from the Committee for Animal Experimental Control and Supervision (CPCSEA), New Delhi, India and approval sought by Institutional Animal Ethical Committee (IAEC) of Acharya & BM Reddy college of Pharmacy with Regd. No: 997/c/06/CPCSEA. For cardioprotective behavior testing, 50 male Wistar albino rats weighing 200-250gm were chosen. The animals were held in a polypropylene cage under 12 h light / dark conditions at 25 ± 2 ° C and provided with standard diet and water *ad libitum* one week before and during the experimental time.

Preparation of drug:

For cardioprotective effect, two doses of 10 and 20 mg/kg of Fisetin have been identified.

Drugs and Chemicals:

Sigma Aldrich, INDIA, procured Fisetin while Swemed Diagnostics Ltd. purchased enzyme kits. All other chemicals used in the analysis are quantitative.

Induction of myocardial infarction:

Rats are intraperitoneally anaesthetized with thiopentone sodium (45 mg/kg). The neck was incised with a ventral midline and cannulated with a techno positive pressure respirator through a tracheotomy and ventilation with room air. A left thoracotomy and pericardiotomy were made and the left anterior descending coronary artery was found. A thread made up of silk was passed behind the artery and was obscured for 30 minutes by a knot. After 30 minutes, the silk thread was removed by making two-knot releasers to allow heart reperfusion for 4 hours.

Experimental design:

Rats were divided into five groups at random, each consisting of TEN animals.

Group I: Sham group (**without I/R**).

Group II: Rats were received 0.2 ml of saline and served as a control subjected to ischemia reperfusion (**with I/R**).

Group III: Rats were received 10% DMSO 10 minutes before reperfusion (vehicle group) subjected to ischemia reperfusion (**with I/R**).

Group IV: Rats were received Fisetin 10 mg/kg 10 minutes before reperfusion, subjected to ischemia reperfusion (**with I/R**).

Group V: Rats were received Fisetin 20 mg/kg 10 minutes before reperfusion, subjected to ischemia reperfusion (**with I/R**).

Parameters:

To quantify infarct size:

In every group, the heart was quickly expunged from the thorax after the animal was sacrificed and the larger vessels were removed. The left ventricle was estranged and weighed from the heart. It was sliced to 0.1 cm thick analogous to the atrioventricular rut and the slices were incubated at 37 °C in 1 per cent TTC solution prepared in phosphate buffer pH 7.4 for 30 min. Dehydrogenase enzymes are converted into a red formazan pigment that stains dark red tissue in viable myocardium TTC.

The myocardium infarction which did not take TTC stain where the enzymes of dehydrogenase are drained off remains white. Separated from the stained portions, the pale necrotic tissue was weighed on an electronic balance. The scale of myocardial infarction was quantitatively expressed as a percentage of left ventricular necrosis (PLVN).

Preparation of tissue homogenate and serum:

Heart tissues were homogenized with an ice-cold phosphate buffer at a volume of 10% of tissue weight. At 4 ° C, the homogenate was centrifuged for 15 minutes at 5,000 rpm. For the calculation, the supernatant was taken. For serum separation, 2 ml of blood was collected from the left ventricle before euthanizing the animals at the end of 4 h of reperfusion. The blood samples were for 10 minutes centrifuged at 5,000 rpm. The resulting upper layer containing serum was collected and stored for the estimations of serum cardiac markers such as creatinine kinase (CK- MB), lactate dehydrogenase (LDH) and antioxidant parameters such as superoxide dismutase

(SOD) (6), Catalase (CAT) (7), Glutathione Peroxidase (8), Reduced glutathione (9), Glutathione-S-transferases(10), Malonaldehyde (MDA) in tissue (11), MDA in serum and protein estimation (12).

Statistical analysis

Results are expressed as mean ± SD. Differences in infarct size, cardiac markers (CK-MB, LDH), serum and tissue lipid peroxide levels, SOD, CAT, GST, GSH, and GPx were determined by one-way ANOVA. Individual groups were compared using Dunnett’s test. Differences

were considered to be statistically significant at P<0.05.

Results

FT-IR studies:

The FT-IR spectra of Fisetin shows the band assigned to O-H stretching between 3490.0 cm⁻¹, 3145.0 cm⁻¹ as C= H stretching, 1774.0 cm⁻¹ as C= O stretching and 1265.0 cm⁻¹ as C-O stretching vibration. This finding indicates the existence of a group of hydroxyls, ketones, unsaturated carbons, and carboxyls.

Figure 1: FTIR for Fisetin

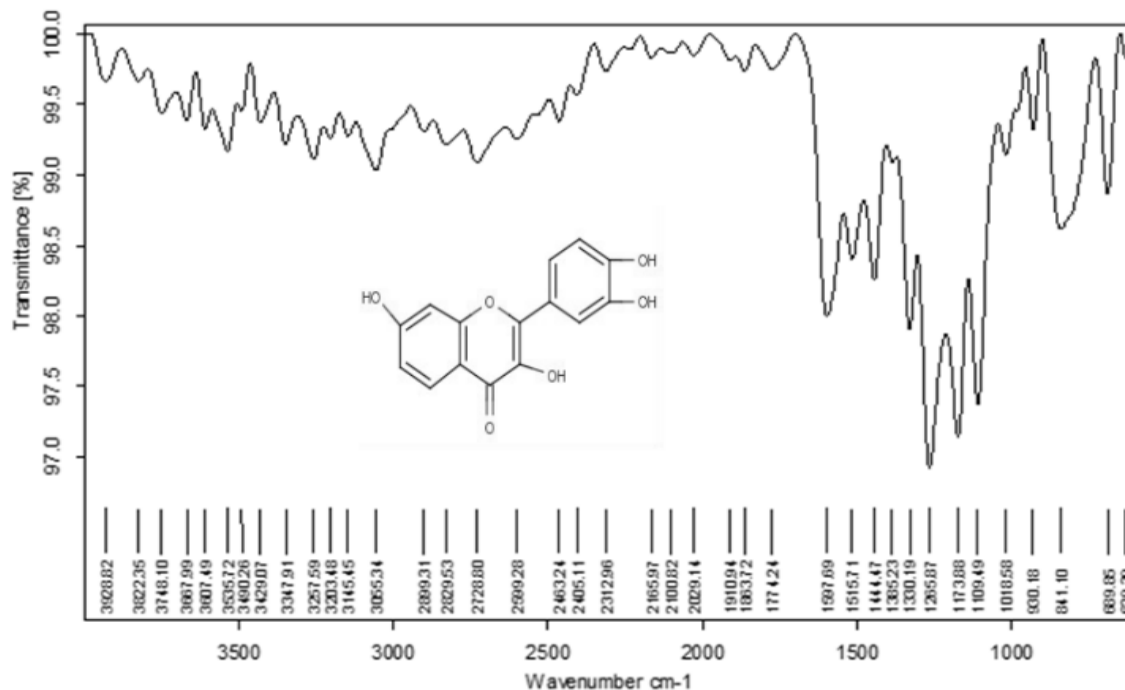


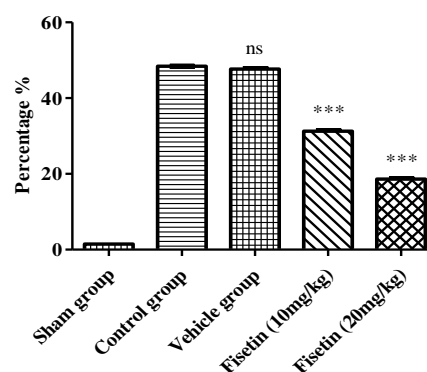
Table 1: Functional groups vibration range of the Fisetin

Sl. No	Molecular vibration	Frequency cm ⁻¹
1	-O-H Stretch	3490
2	-C=C Stretch	3145
3	-C=O Stretch	1774
4	-C-O Stretch	1265

Myocardial infarct size:

The control group's risk of myocardial infarction was significantly higher in the placebo group (P<0.05). Compared to the control group, Fisetin 10 mg / kg and 20 mg / kg classes of myocardial infarction are significantly reduced (P < 0.05).

Figure 2: Effect of Fisetin on Infarct size (% left ventricle necrosis) in normal and ischemia-reperfusion induced myocardial infarction in rats

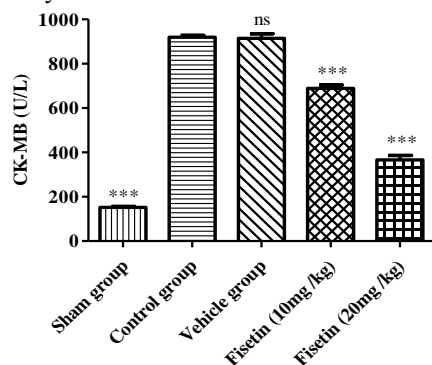


All values are expressed as Mean ± SD (n=6), ***=p<0.001 as compared to control; ns=p>0.05 as compared to control.

Serum cardiac enzymes (CK-MB and LDH) concentrations:

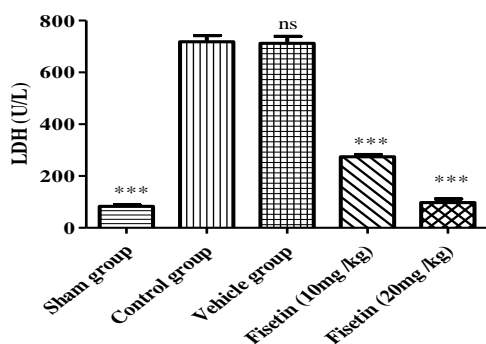
Experimental results showed that compared with sham group, serum CK-MB and LDH levels were significantly increased in control group ($P < 0.05$). Compared with control group, serum CK-MB and LDH levels were all decreased in Fisetin 10 mg/kg and 20 mg/kg ($P < 0.05$).

Figure 3: Effect of Fisetin on serum CK-MB levels in normal and ischemia reperfusion induced myocardial infarction in rats



All values are expressed as Mean \pm SD ($n=6$), ***= $p < 0.001$ as compared to control; ns= $p > 0.05$ as compared to control.

Figure 4: Effect of Fisetin on serum LDH levels in normal and ischemia reperfusion induced myocardial infarction in rats.

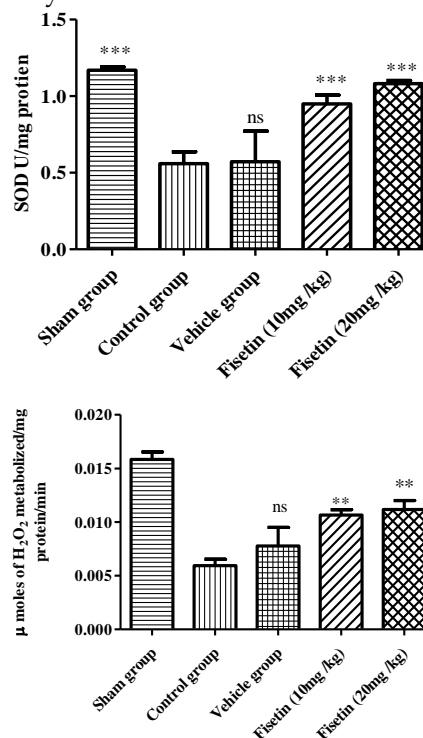


All values are expressed as Mean \pm SD ($n=6$), ***= $p < 0.001$ as compared to control; ns= $p > 0.05$ as compared to control.

Tissue anti-oxidant enzyme (SOD, CAT, GPx) concentrations:

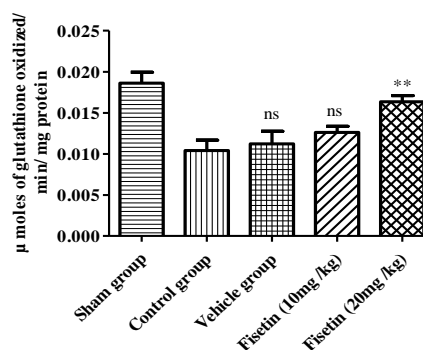
Experimental results showed that compared with sham group, tissue SOD, CAT, GPx levels were significantly decreased in control group ($P < 0.05$) and significantly increased in Fisetin 10 mg/kg and 20 mg/kg ($P < 0.05$), compared to control group.

Figure 5: Effect of Fisetin on tissue SOD and CAT levels in normal and ischemia-reperfusion induced myocardial infarction in rats



All values are expressed as Mean \pm SD ($n=6$), ***= $p < 0.001$ as compared to control; ns= $p > 0.05$ as compared to control.

Figure 6: Effect of Fisetin on tissue GPx levels in normal and ischemia-reperfusion induced myocardial infarction in rats

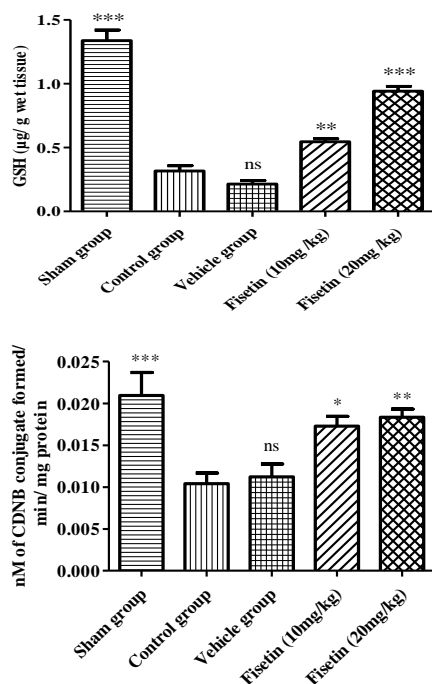


All values are expressed as Mean \pm SD ($n=6$), **= $p < 0.01$ as compared to control; ns= $p > 0.05$ as compared to control.

Tissue anti-oxidant enzymes (GSH and GST) concentrations:

Experimental results showed that compared with sham group, tissue GSH, GST levels were significantly decreased in control group ($P < 0.05$). Compared with control group, tissue GSH, GST levels were all increased in Fisetin 10 mg/kg and 20 mg/kg ($P < 0.05$).

Figure 7: Effect of Fisetin on tissue GSH and GST levels in normal and ischemia-reperfusion induced myocardial infarction in rats

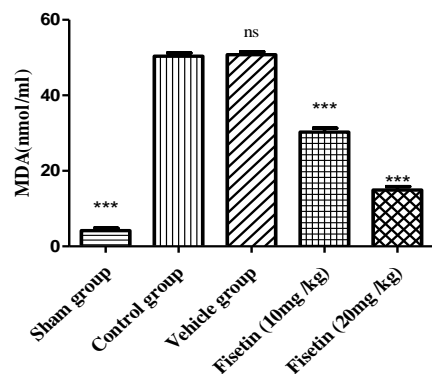


All values are expressed as Mean ± SD (n=6), ***=p<0.001 as compared to control; **=p<0.01 as compared to control; ns=p>0.05 as compared to control.

Serum and tissue lipid peroxide (MDA) concentrations:

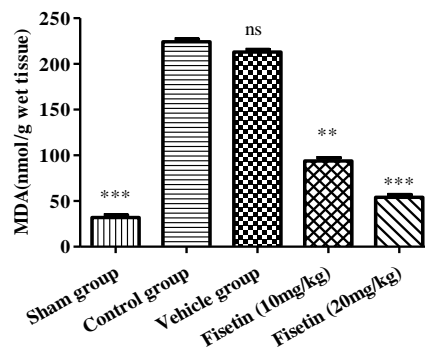
Experimental results showed that compared with sham group, tissue and serum MDA levels were significantly increased in control group (P<0.05). Compared with control group, tissue and serum MDA levels were all decreased in Fisetin 10 mg/kg and 20 mg/kg (P<0.05).

Figure 8: Effect of Fisetin on serum MDA levels in normal and ischemia-reperfusion induced myocardial infarction in rats



All values are expressed as Mean ± SD (n=6), ***=p<0.001 as compared to control; ns=p>0.05 as compared to control.

Figure 9: Effect of Fisetin on tissue MDA levels in normal and ischemia-reperfusion induced myocardial infarction in rats

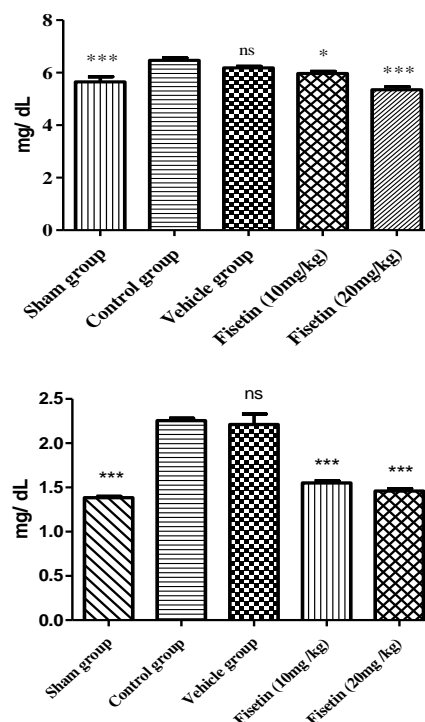


All values are expressed as Mean ± SD (n=6), ***=p<0.001 as compared to control; ns=p>0.05 as compared to control.

Serum and tissue protein concentrations:

Experimental results showed that compared with sham group, tissue and serum MDA levels were significantly increased in control group (P<0.05). Compared with control group, tissue and serum MDA levels were all decreased in Fisetin 10 mg/kg and 20 mg/kg (P<0.05).

Figure 10: Effect of Fisetin on serum protein levels in normal and ischemia-reperfusion induced myocardial infarction in rats

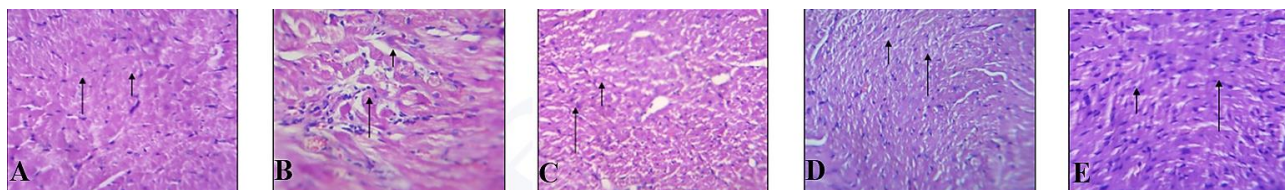


All values are expressed as Mean ± SD (n=6), ***=p<0.001 as compared to control; * =p<0.05 as compared to controls; ns=p>0.05as compared to control.

Histopathological studies:

Histopathological studies showed that section of heart tissues A: Sham group showed normal architecture; B: Control group-myocardial infarcted rat showed degenerative changes, hyalinization of muscle fibers and cellular infiltration; C: Vehicle group (DMSO treated) rats showing degenerative changes,

hyalinization of muscle fibers and cellular infiltration; groups D & E (Fisetin treated) rats showed less cellular infiltration, normal muscle fibers. The cardio-protective effect is evident from reduced myocardial damage even after ischemia-reperfusion injury.



A: Sham Group: Section of the cardiac muscle showing cardiac muscle fibers have intact integrity of myocardial cell membrane (long arrow) myofibrillar structure with striations and continuity with adjacent myofibrils (short arrow). B: Control Group: Section of the cardiac muscle showing cardiac muscle fibers have loss of integrity of myocardial cell membrane (long arrow) myofibrillar structure with intact striations and continuity with adjacent myofibrils (short arrow). C: Vehicle Group: Section of the cardiac muscle showing cardiac muscle fibers have loss of integrity of myocardial cell membrane (long arrow) myofibrillar structure with intact striations and continuity with adjacent myofibrils, The interstitial space appears increased with inflammatory infiltration (short arrow). D: Fisetin (10 mg/kg) Group: Section of the cardiac muscle showing cardiac muscle fibers have loss of integrity of myocardial cell membrane with variable waviness of fibers (long arrow) myofibrillar structure with intact striations and continuity with adjacent myofibrils, the interstitial space appears mildly increased (short arrow). E: Fisetin (20 mg/kg) Group: Section of the cardiac muscle showing most of the cardiac muscle fibers have intact integrity of myocardial cell membrane (long arrow) myofibrillar structure with striations and continuity with adjacent myofibrils, the interstitial space appears intact (short arrow).

Discussion

In this current study of global cardiac ischemia-reperfusion injury, we conducted only a transient and not perpetual ligation of the left coronary artery to achieve effects closer to the clinical circumstances in which nearly all patients with myocardial infarction are reperfused. Treatment with Fisetin through the last ten minutes of ischemia and the comprehensive reperfusion phase significantly diminished the infarct size. Investigations infer that there is a linear relationship between fatality and the degree of infarct size because the myocardium cannot regenerate (13). Cardioprotective effects of Fisetin may be due to its anti-oxidant, anti-inflammatory and anti-apoptotic activities. Reperfusion of the ischemic myocardium results in cardiomyocyte apoptosis and heart dysfunction (14, 15).

In the current study, a significant increase in serum LDH and CK-MB endorsing the acute myocardial infarction in rats. The myocardial

cells contain numerous cardiac enzymes like lactate dehydrogenase, aspartate transaminase, creatinine kinase, etc., upon LAD ligation, the oxygen demand of the heart increased with raise in inotropic effect in the heart, showing long-term ischemia and glucose deprivation. The cells are injured with prolonged muscle contractility, which results in enhanced membrane permeability facilitating cardiac enzymes to ooze out into the blood circulation (16-19).

Creatinine kinase is an enzyme proficient of reversibly transferring a phosphate group from the energy storage form of creatinine phosphate to a molecule of ADP, producing ATP. CK-MB is confined effectively in the heart and this makes it a valuable diagnostic tool for MI since injury specific to the myocardium would result in elevation of CK-MB levels. CK-MB has estimated to the standard to which all cardiac biomarkers were compared to (20-22 and 23).

LDH has been used traditionally as a nonspecific diagnostic tool for myocardial infarction (22, 24). A rise in the proportion of LDH in the serum can be diagnostic of myocardial infarction. Upon treatment with Fisetin, there is a significantly decreased level of CK-MB and LDH.

Bioflavonoids have been reported to have antioxidant activity and to possess cardioprotective potential (25). Because oxidative damage is a potential culprit in the injury of viable myocardium, it would be beneficial to limit oxidative damage to a minimum and reinforce the antioxidant defense mechanism by supplementing antioxidants. Indeed, several clinical studies and experimental studies reported that the administration of antioxidants resulted in beneficial outcomes in I/R injury (26).

Free radical scavenging enzymes such as superoxide dismutase, glutathione peroxidase and catalase are the first line cellular defense against oxidative stress, eliminating reactive oxygen radicals such as superoxide ($\bullet\text{O}_2^-$) and hydrogen peroxide (H_2O_2) and preventing the formation of more reactive radical of hydroxyl radical ($\bullet\text{OH}$) (26).

In the present study, SOD activity decreased significantly ($p < 0.05$) in the control group (I/R injury) as compared with the sham group. This may be due to the excessive formation of superoxide anions. Administration of Fisetin (10 and 20 mg/kg) to ischemia-reperfused challenged rats effectively and dose-dependently prevented the decrease in CAT activity, which may be correlated directly to the scavenging of radicals by Fisetin.

The level of glutathione peroxidase was decreased significantly ($p < 0.05$) in Control (IR injury) group as compared with the Sham group and this reduction was significantly ($p < 0.05$) reversed by different doses of Fisetin. The second line of defence consists of the non-enzymatic scavengers, viz. glutathione-S-transferase and reduced glutathione containing compounds, which scavenge residual free radicals escaping decomposition by the antioxidant enzymes. Reduced glutathione (GSH) is one of the major constituents of

erythrocytes and plays an important role in protecting against oxidative damage (27).

The level of reduced glutathione was depleted significantly ($p < 0.05$) in the control group, which may be due to its enhanced utilization for augmenting the activities of GPx and GST. Decreased concentration of mitochondrial GSH indicates a major mechanism of inducing an imbalance of mitochondrial function. In the present study, the GSH level was significantly ($p < 0.05$) elevated by Fisetin. Rats of the control group showed significant ($p < 0.05$) decrease in GST activity compared with sham group rats.

Histopathological examination revealed that treatment with Fisetin (10 and 20 mg/kg) as compared to the control group showed strong protection of myocardial cells from cellular tissue injury produced with ischemia-reperfusion.

Treatment with Fisetin increased the endogenous antioxidant enzyme levels in I/R injured rats. Epidemiological studies suggest an inverse correlation between the severity of oxidative stress-induced diseases and the levels of anti-oxidants. Therefore, one of the mechanisms of the Cardioprotection of Fisetin is associated with antioxidant effect.

The therapeutic efficacy of Fisetin may be due to its anti-oxidant, anti-lipid peroxidative, anti-apoptotic and anti-inflammatory property that could have prevented ischemia-reperfusion induced injury. Thus, we hypothesize that Fisetin deserves additional experimental and clinical research in the cardiovascular milieu.

Conclusion

Our study revealed that Fisetin showed significant Cardioprotection against ischemia-reperfusion induced myocardial infarction in rats by decreasing the infarct volume, CK-MB levels, LDH release and MDA levels, and by increasing the antioxidants levels, which further confirmed by histopathological reports.

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