CARDIOPROTECTIVE EFFECT OF ESCULETIN ON CARDIAC MARKER ENZYMES AND MEMRANE BOUND ENZYMES IN ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION IN WISTAR RATS

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ABSTRACT

This study evaluates the cardioprotective effect of esculetin on isoproterenol (ISO)-induced myocardial infarction (MI) in rats. Rats were pretreated with esculetin (10 and 20 mg/kg) orally for a period of 21 days. After the treatment period ISO (85 mg/kg) was administered subcutaneously to rats at an interval of 24 h for 2 days. ISO-induced rats showed a significant increase in the activities of marker enzymes such as creatine kinase (CK), creatine kinase-MB (CK-MB), aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) in serum and there by subsequent decrease in the heart, and also ISO-induced rats showed a significant increase in heart weight. A significant decrease in the activity of sodium/potassium dependent adenosine triphosphatase (Na^+/K^+-ATPase) and increased in the activities of calcium and magnesium dependent adenosine triphosphatase (Ca^{2+} and Mg^{2+}-ATPase) were observed in the heart of ISO-induced rats. Pretreatment with esculetin positively altered the activities of marker enzymes and the biochemical parameters in ISO-induced rats. Thus, our study shows that esculetin posses cardioprotective effect in ISO-induced MI in rats. Results obtained from histopathological studies also supported that esculetin has preventive effect against ISO-induced myocardial infarction.

KEYWORDS: Myocardial infarction, isoproterenol, esculetin, cardiac markers, membrane bound enzymes, histopathology.

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INTRODUCTION

Myocardial infarction (MI) usually results from an abrupt reduction in coronary blood flow to a segment of the myocardium, which initiates continuum of progressively more severe cellular changes that unless interrupted by early reperfusion, inevitably culminate in cell death and tissue necrosis[1]. There is substantial evidence that ischemic tissue generates oxygen-derived free radicals i.e., oxygen molecule containing odd number of electrons, making them chemically reactive and often leading to chain reactions[2]. In this regard, animal experiments suggests that increases in free radical formation and subsequent oxidative stress associated with the occurrence of a relative deficit in the endogenous antioxidant reserve is one of the mechanism for the development of congestive heart failure[3]. Isoproterenol (ISO), a synthetic catecholamine and β-adrenergic agonist, administration causes severe stress on heart, results in infarct like necrosis[4]. ISO-induced

cardiac damage occurs due to generation of highly cytotoxic free radicals through its autoxidation[5]. The pathophysiological alterations following ISO-administration are comparable to human MI[6]. It is well known that administration of ISO influences the metabolic reactions occurring with in the cell.

Coumarins are low-molecular weight phenolics and have been widely used for the prevention and treatment of venous thromboembolism, MI and stroke[7]. In the recent years, coumarins received much attention for these diverse bioactivities and coumarins from natural sources have been also used as therapeutic agents in humans[8]. Esculetin (6,7 dihydroxy coumarin), posses radical scavenging activity against reactive oxygen species and inhibit the oxidative damage in rat liver[9]. Many scientific papers have also reported the pharmacological properties of esculetin such as anti-inflammatory effects, antioxidative, antiviral, antimutageneic activites,

ultraviolet absorption effect and inhibition of lipogenase activity[10].

In this study, the cardioprotective action of esculetin was investigated by studying the activities of cardiac marker enzymes such as creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and membrane bound enzymes of heart in ISO-induced MI in male albino wistar rats. In addition to the above biochemical parameters, histological alterations of the heart also studied.

MATERIALS AND METHODS

Experimental animals

All the experiments were carried out with male albino wistar rats weighing 140-160 g, obtained from the Venkateswara Enterprises, Bangalore, Karnataka, India. They were housed in polypropylene cages (47x34x20 cm) lined with husk, renewed every 24 h under a 12:12 h light/dark cycle at around 22˚C and had free access to tap water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The pellet diet consisted of 22.02% crude protein, 4.25% crude oil, 3.02% crude fibre, 7.5% ash, 1.38% sand silica, 0.8% calcium, 0.6% phosphorus, 2.46% glucose, 1.8% vitamins and 56.17% nitrogen free extract (carbohydrates). The diet provided metabolisable energy of 3, 600 kcal. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Drugs and chemicals

(±)Isoproterenol hydrochloride and esculetin were purchased from Sigma Chemical Company, St. Louis, MO, USA. Creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) kits were purchased from Agappe Diagnostics, Kerala, India. ATP was purchased from Hi-media, Mumbai. All other chemicals used in the study were of analytical grade.

Induction of experimental myocardial infarction

Isoproterenol (85 mg/kg) was dissolved in normal saline and injected subcutaneously to rats at an interval of 24 h for two days[11].

Experimental design

A total of 30 rats were divided into 5 groups of 6 rats each and used in this study. Group 1: Normal control rats; Group 2: Normal rats treated with esculetin (20 mg/kg); Group 3: ISO control rats (85 mg/kg); Groups 4&5: Rats pretreated with esculetin (10 and 20 mg/kg) and then subcutaneously injected with ISO. Esculetin was dissolved in 0.2% dimethyl sulfoxide (DMSO) and administered to rats orally using an intragastric tube

daily for a period of 21 days. At the end of the experimental period, after 12 h of second ISO-injection, all the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and sacrificed by cervical decapitation. Blood was collected, serum separated and used for various biochemical estimations. The heart tissue was excised immediately from the animals, washed off blood with ice-chilled physiological saline and used for further biochemical estimations. A known weight of the heart tissue was homogenized in appropriate buffer solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

Biochemical assays

Assay of cardiac marker enzymes

Creatine Kinase-MB and Creatine Kinase were assayed by Witt and Trendelenburg[12] method using a commercial kit (Product No. 11405002 and 11404002) obtained from Agappe Diagnostics, Kerala, India. Lactate dehydrogenase activity was assayed by Wei Bhaar[13] (Product No. 11407002). Aspartate amino transferase activity was assayed by Clim[14] (Product No. 11408001) and alanine transaminase activity was assayed by Thefeld et al.,[15] method using a commercial kit (Product No. 11409001) obtained from Agappe Diagnostics, Kerala, India.

Assay of membrane bound enzymes

The activity of Na^+/K^+-ATPase was assayed according to the procedure of Bonting (1970)[16]. The incubation mixture contained 1.0 ml of buffer, 0.2 ml of magnesium sulphate, 0.2 ml of potassium chloride, 0.2 ml of sodium chloride, 0.2 ml of EDTA, 0.2 ml of ATP and 0.2 ml of tissue homogenate. The contents were incubated at 37^oC for 15 min. 1.0 ml of ice-cold 10% TCA was added at the end of 15 min to arrest the reaction. The content of phosphorus liberated was estimated as described by Fiske and Subbarow (1925)[17]. 1.0 ml of the supernatant was made upto 4.0 ml with distilled water and 1.0 ml of 2.5% ammonium molybdate was added. This was incubated at room temperature for 10 min and 0.4 ml of ANSA was added. The colour developed was read spectrophotometrically at 640 nm after 20 min.

The activity of Ca^{2+}-ATPase was assayed according to the method of Hjerten and Pan (1983)[18]. The incubation mixture contained 0.1 ml of buffer, 0.1 ml of calcium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of tissue homogenate. The contents were incubated at 37^oC for 15 min. The reaction was then arrested by addition of 0.5 ml of ice-cold 10% TCA. The amount of phosphorus liberated was estimated by the method of Fiske and Subbarow (1925)[17].

The activity of Mg^{2+}-ATPase was assayed by the method of Ohnishi et al. (1982)[19]. The incubation mixture contained 0.1 ml of buffer, 0.1 ml of magnesium chloride, 0.1 ml of ATP, 0.1 ml of distilled water and 0.1 ml of tissue homogenate. The contents were incubated at 37°C for 15 min. The reaction was arrested by the addition of 0.5 ml of ice-cold 10% TCA. The content of phosphorous liberated was estimated by the method of Fiske and Subbarow (1925)[17].

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using statistical package for social sciences (SPSS), software package 9.05. Results were expressed as mean ± S.D for 6 rats in each group. *P* values <0.05 were considered as significant.

RESULTS

Effect of esculetin on cardiac marker enzymes

Tables 1,2 & 3 represent the effect of esculetin on heart weight and cardiac marker enzymes such as creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate tranaminase (AST) and alanine transaminase (ALT) in serum and heart of normal and ISO-induced rats. Rats induced with ISO, showed a significant increase in heart weight and the activities of these cardiac marker enzymes in serum with subsequent decrease in the heart, when compared with normal control rats. Pretreatment with esculetin (10 and 20 mg/kg) for a period of 21 days significantly decreased the heart weight and the activities of these marker enzymes in serum with significant increase in the heart of ISO-induced rats, when compared with ISO-alone induced rats.

Effect of esculetin on the activities of membrane bound enzymes

Table 4 depicts the effect of esculetin on the activities of sodium/potassium-dependent adenosine triphosphatase (Na^+/K^+-ATPase), calcium-dependent adenosine triphophatase (Ca^{2+}-ATPase) and magnesium-dependent adenosine triphophatase (Mg^{2+}-ATPase) in normal and ISO-induced rats. The activity of Na^+/K^+-ATPase was decreased significantly and the activities of Ca^{2+} and Mg^{2+}-ATPases were increased significantly in the heart of ISO-induced rats. Pretreatment with esculetin to ISO-induced rats significantly minimized the alterations in the activities of these membrane bound enzymes in the myocardium.

Effect of esculetin on the histopathology of heart

The effect of esculetin on the histological changes in myocardial tissues in normal and ISO-induced rats are shown in Figs 1-5. Histopathological findings of the ISO-induced myocardium (Fig.3) showed infarcted zone with oedema and inflammatory cells of the myocardium also showed separation of muscles fibres. Pretreatment with esculetin at a dose of 10 mg/kg (Fig.4) showed mild oedema and necrosis without inflammatory cells. Esculetin pretreatment at a dose of 20 mg/kg (Fig.5) showed mild oedema and the myocardical fibres were within normal limits. Eesculetin 20 mg/kg to normal rats did not have any pathological changes in the myocardium (Fig. 2). Fig. 1 shows the normal architecture of the rat myocardium.

For all the parameters studied esculetin treatment to normal rats for a period of 21days didn't show any significant effect. Esculetin at a dose of 20 mg/kg showed better effect than 10 mg/kg.

DISCUSSION

Isoproterenol (ISO), a synthetic catecholamine and β-adrenergic agonist, is reported to produce myocardial infarction (MI) in large doses[20]. ISO is well known cardiotoxic agent due to its ability to destruct myocardial cells. In this study, significant increase was observed in the heart weight and significant decrease in cardiac markers such as creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in the heart of ISO-induced rats, which is in consistent with earlier report[21,22]. The observed increase in the body weight in ISO-induced rats could be due to accumulation of water content in the oedematous intramuscular area in addition with necrosis of cardiac muscle fibres. Decreased activities of theses cardiac marker enzymes in the cardiac tissue could be due to the leakage from damaged cardiac tissue into the circulation as a result of necrosis induced by ISO. Significant increase was noticed in the activities of these cardiac markers enzymes in serum of ISO-induced rats, which might be due to enhanced susceptibility of myocardial cell membrane to ISO-mediated peroxidative damage, resulting in increased release into the systemic circulation[23].

In the present study, the prior administration of esculetin at a dose of 10 and 20 mg/kg, for a period of 21 days, significantly prevented the ISO-induced elevation in the activities of these marker enzymes in serum, and significantly increased the activities of these markers in the heart, which indicates the protective activity of esculetin. The protective effect could be due to reducing the cardiac damage thereby restricting the leakage of these enzymes in to the circulation. Pretreatment with esculetin significantly decreased the heart weight in ISO-induced rats; this could be due to reduction in the process of necrosis offered by esculetin[24].

We have observed decreased activities of sodium/potassium-dependent adenosine triphosphatase (Na^+/K^+-ATPase) and increased activity Mg^{2+}-ATPase and Ca^{2+} ATPase in ISO-induced rats. In this study altered activities of membrane associated enzymes such as ATPases indicate an alteration in membrane is under pathological conditions. Inactivation of Na^+/K^+-ATPase could be due to enhanced lipid peroxidation by ISO. The inhibition of Na^+/K^+-ATPase can activate the Na^+, Ca^{2+} exchange mechanism in the myocardium, which plays an important role in regulating cellular calcium levels[25]. Ca^{2+}-ATPase, a calcium transport protein is responsible for the maintenance of intracellular calcium levels in a variety of cell types[26].

Enhanced Ca^{2+}ATPase are due to adenylate cyclase activation by ISO. Calcium overloaded in the myocardial cells during ischemia activate the Ca^{2+} dependent ATPase, which depleting high energy phosphate there by inhibiting Na^+ and K^+ transport and inactivation of Na^+/K^+-ATPase[27,28]. Pretreatment with esculetin increased the activity of Na^+/K^+-ATPase and decreased the activity of Ca^{2+} and Mg^{2+}-ATPase in ISO-induced rats. This could be due to the ability of esculetin to protect the 'SH' groups from the oxidative damage through the inhibition of peroxidation of membrane lipids. This effect might be due to the free radical scavenging and membrane stabilizing action of esculetin. The histopathological findings of the ISO-induced myocardium showed infarcted zone with oedema with inflammatory cells. Normal rats treated with esculetin did not show any pathological alteration in the heart. This indicates that esculetin does not possess any adverse effects under normal conditions. Pretreatment with esculetin significantly decreased the histopathological alteration, this could be due to free radical scavenging, antioxidant and membrane stabilization properties of esculetin.

CONCLUSION

In conclusion, oral pretreatment with esculetin for the period of 21 days significantly minimized the alterations in heart weight, cardiac marker enzymes, membrane bound enzymes and histopathological alterations. Thus, our study demonstrates the cardioprotective role of esculetin in ISO-induced oxidative stress in rats.

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Table 1: Effect of esculetin on the activity of serum creatine kinase-MB (CK-MB) and heart weight of normal and isoproterenol (ISO)-induced myocardial infarction (MI) in rats.

Groups	CK-MB (IU/L)	Heart weight (mg)
Normal control	82.5 ± 6.2[a]	495.5 ± 15.5 [a]
Normal + Esculetin (20 mg/kg)	80.3 ± 5.8[a]	510.7 ± 18.3[a]
ISO (85 mg/kg) control	210.5 ± 12.7[b]	947.9 ± 25.0[b]
Esculetin (10 mg/kg) + ISO	127.0 ± 10.1[c]	721.0 ± 12.7[c]
Esculetin (20 mg/kg) + ISO	103.5 ± 8.3[d]	593.8 ± 10.8[d]

Each value is mean ± S.D. for 6 rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Table 2: Effect of esculetin on the activities of creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in serum of normal and isoproterenol (ISO)-induced myocardial infarction (MI) in rats.

Groups	CK (IU/L)	LDH (IU/L)	AST (IU/L)	ALT(IU/L)
Normal control	197.9 ± 6.25 [a]	62.7 ± 3.31[a]	18.62 ± 1.02[a]	13.9 ± 0.67[a]
Normal + Esculetin (20 mg/kg)	192.7 ± 5.30[a]	61.7 ± 3.31[a]	18.43 ± 1.13[a]	14.1 ± 0.52[a]
ISO (85 mg/kg) control	332.9 ± 18.12[b]	107. ± 4.02[b]	36.85 ± 2.82[b]	23.9 ± 1.25[b]
Esculetin (10 mg/kg) + ISO	253.0 ± 14.04[c]	87.8 ± 2.6[c]	28.85 ± 1.44[c]	19.8 ± 0.84[c]
Esculetin (20 mg/kg) + ISO	222.8 ± 10.41[d]	72. ± 3.31[d]	23.51 ± 0.83[d]	16.9 ± 0.54[d]

Each value is mean ± S.D. for 6 rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Table 3: Effect of esculetin on the activities of creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in the heart of normal and isoproterenol (ISO)-induced myocardial infarction (MI) in rats.

Groups	CK	LDH	AST	ALT
Normal control	13.3 ± 0.71[a]	92.0 ± 4.47[a]	32.1 ± 2.05[a]	25.1 ± 1.37[a]
Normal + Esculetin (20 mg/kg)	13.5 ± 0.67 [a]	91.7 ± 3.82[a]	32.4 ± 2.65[a]	25.4 ± 1.42[a]
ISO (85 mg/kg) control	6.81 ± 0.33[b]	64.9 ± 3.04[b]	23.1 ± 1.41[b]	16.0 ± 0.81[b]
Esculetin (10 mg/kg) + ISO	9.82 ± 0.46[c]	77.5 ± 3.31 [c]	28.5 ± 1.73[c]	19.1 ± 1.27[c]
Esculetin (20 mg/kg) + ISO	11.72 ± 0.79[d]	86.3 ± 3.50[d]	30.6 ± 2.44[d]	22.3 ± 1.13[d]

CK activity: μmol of phosphorus liberated/min/mg protein. LDH, AST and ALT activities: nmol of pyruvate liberated/min/mg protein.
Each value is mean ± S.D. for 6 rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Table 4: Effect of esculetin on the activities of sodium/potassium dependent adenosine triphosphatase (Na$^+$/K$^+$-ATPase), calcium dependent adenosine triphosphatase (Ca^{2+}-ATPase) and magnesium dependent adenosine triphosphatase (Mg2+ ATPase) in the heart of normal and isoproterenol (ISO)-induced myocardial infarction (MI) in rats.

| Groups	Na$^+$/K$^+$-ATPase Units*/mg protein	Ca^{2+}-ATPase Units*/mg protein	Mg^{2+}-ATPase Units*/mg protein
Normal control	0.57 ± 0.03[a]	1.43 ± 0.05[a]	6.16 ± 0.43[a]
Normal + Esculetin (20 mg/kg)	0.59 ± 0.04[a]	1.45 ± 0.07[a]	6.10 ± 0.38[a]
ISO (85 mg/kg) control	0.31 ± 0.02[b]	2.54 ± 0.10[b]	9.90 ± 0.35[b]
Esculetin (10 mg/kg) + ISO	0.42 ± 0.03[c]	2.08 ± 0.13[c]	7.07 ± 0.56[c]
Esculetin (20 mg/kg) + ISO	0.51 ± 0.04[d]	1.77 ± 0.09[d]	6.75 ± 0.42[d]

*Activity expressed as units: µmol of phosphorus liberated/min/mg protein.
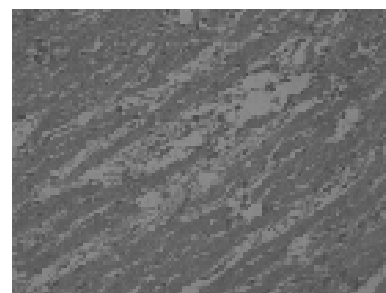Each value is mean ± S.D. for 6 rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
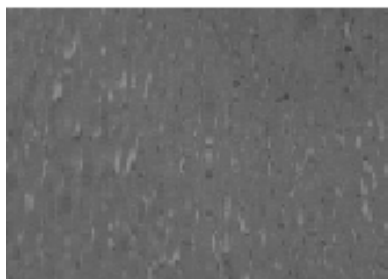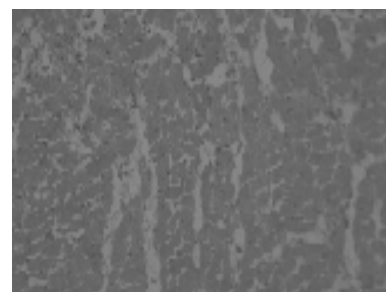

**Fig. 1. Normal architecture of rat heart showing the intact myocytes with normal striations**
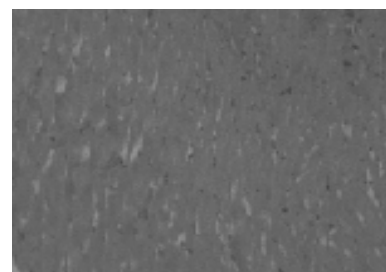
Fig.2. Normal with esculetin (20 mg/kg) treated rat heart showing myocytes within normal limits

Fig. 3. ISO treated rat heart showing severe disturbance of myocardial fibres with scattered inflammatory cells and oedema

Fig. 4. Esculetin (10 mg/kg) and ISO-induced rat heart showing mild disturbance of myocardial fibres with a few inflammatory cells
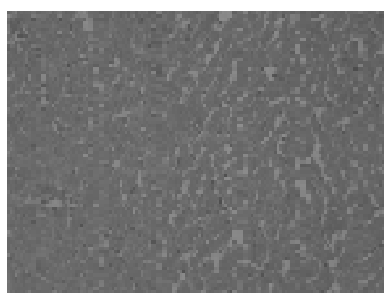
Fig. 5. Esculetin (20 mg/kg) and ISO-induced rat heart showing normal pattern of myocardial tissue with a few inflammatory cells.

Histopathological Study