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CARDIO-PROTECTIVE EFFECT OF SILYMARIN AND ITS COMBINATION WITH ALLOPURINOL IN ISCHEMIA REPERFUSION INJURY USING ISOLATED PERFUSED RAT HEARTS

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ABSTRACT

Ischemia- reperfusion injury is one of the major cause of cardiovascular mortality and may lead to myocardial infarction, cardiac arrhythmias, and contractile dysfunction. The present study was designed to investigate the cardioprotective effect of Silymarin in ischemia reperfusion injury. Myocardial ischemia reperfusion injury was produced by mounting isolated rat hearts on Langendorff's apparatus and global ischemia was produced for 30 min followed by reperfusion for 120 min. The animals were divided into six groups. In control group only global ischemia followed by reperfusion for 120 min was given. The standard groups received Allopurinol (50mg/L) and Silymarin (10mg/L) in vitro respectively. In treated groups, respective interventions Silymarin in different doses (2.5 mg/L, 5 mg/L and 10 mg/L) in combination with Allopurinol (50mg/L) were given. Myocardial infarct size was estimated macroscopically using TTC staining. The magnitude of cardiac injury was measured by lactate dehydrogenase and creatine kinase concentration in the coronary effluent. The increase in infarct size and the release of LDH and CK are documented to be an index of I/R induced myocardial injury. The results revealed that the Silymarin (10mg/L) significantly reduced ischemia-reperfusion induced myocardial injury in vitro. However, the combination of Silymarin and Allopurinol (10mg/L+ 50mg/L) prevented marked ischemia-reperfusion injury when compared with other combination assessed in terms of infarct size, release of LDH and CK level. Thus, it may be concluded that administration of a combination of Allopurinol and Silymarin may provide better cardioprotection by preventing ischemia and reperfusion injury in rat models.

KEYWORDS: Allopurinol, Ischemia and reperfusion injury, Myocardial infarct size, Silymarin

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1. INTRODUCTION

Myocardial ischemia is a condition in coronary artery disease during which the heart tissue is deprived of an adequate O_2 supply, this may trigger myocardial damage with pathological consequences in cardiovascular disease. The main cause of myocardial ischemia is narrowing of coronary arteries, usually due to atherosclerotic plaques as well as arterial hypertension, left ventricular hypertrophy, diabetes and insulin resistance, aging and stress or any of these factors combined. It is well recognized that atherosclerosis itself may cause ischemia/reperfusion (I/R) injury of downstream myocardial tissues due to transient or permanent arterial vessel occlusion [1, 2]. Episodes of coronary syndromes are triggered by alterations in coronary atheromatous plaque. These alterations consist of endothelial dysfunction, platelet aggregation, and spasm leading to plaque erosion, rupture, haemorrhage, and thrombosis. ROS generation and intracellular calcium overload as a direct result of reperfusion are pivotal aspects of this pathology. Myocardial reperfusion is the restoration of oxygenated blood supply to ischemic heart. Timely reperfusion facilitate cardiomyocyte salvage and decreases cardiac dysfunction. However, reperfusion of ischemic area after a prolong period produces a marked damage in myocardium rather than restoration of normal cardiac function, a phenomenon known as ischemia reperfusion (I/R) injury[3, 4].Oxidative stress is one of the mechanisms implicated in the pathogenesis of I/R injury[1]. Myocardial IR injury is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to the ischemic tissue, resulting in widespread lipid and protein oxidative modifications, mitochondrial injury, and cell death. The role of oxygen free radicals (OFR) in the development of reperfusion injury led to a widespread interest in the use of antioxidant therapy to attenuate ischemia reperfusion injury[5]. Silymarin is a unique flavonoid complex containing silybin, isosilybin, dehydrosilybin, silychristin and silydianin derived from plant *Silybum marianum*[6]. There is evidence for their role in reducing tumour growth, preventing liver toxicity, and protecting a number of organs including heart against ischemic damage[7]. The ability of Silymarin and its isolated components to protect cardiomyocytes (rat) against doxorubicin-induced oxidative stress is mainly due to their cell membrane stabilizing effect and radical scavenging potency[8,9]. Furthermore, along with beneficial effects on vasodilator capacity, direct myocardial effects have been observed for Allopurinol. Allopurinol is used primarily to treat hyperuricaemia. It is a potent xanthine oxidase inhibitor. Xanthine oxidase is an enzyme involved in the production of uric acid. Moreover, xanthine oxidase enzyme has also been reported to involve in the generation of free radicals[10]. Furthermore, as xanthine oxidase inhibitor, Allopurinol also limits the formation of reactive oxygen species[11]. Recent studies in both CHF patients and models of experimental heart failure showed that xanthine oxidase inhibition increased contractile capacity due to a calcium (Ca^{2+}) sensitising mechanism and improved myocardial efficiency by reducing myocardial oxygen consumption[12]. These investigations suggest that effective restoration of myocardial function with Allopurinol in the presence of other antioxidants could lead to attenuation or prevention of myocardial I/R injury in rats. Therefore, depending upon the studies of Silymarin and Allopurinol, the present study has been designed to investigate the effect of Silymarin and synergistic effect of Silymarin with Allopurinol in reducing ischemia reperfusion injury using isolated perfused rat heart preparation.

2. MATERIALS AND METHODS

2.1. Chemicals and Test samples

Allopurinol gift sample was procured from Kwality Pharmaceutical Pvt. Ltd., (Amritsar, India). Silymarin gift sample was procured from Herbo Nutra (Delhi, India). All chemicals used in the study were purchased from Loba cheme Pvt. Ltd, Rankem, HiMedia Pvt. Ltd., Laboratories, Mumbai, India, CDH Pvt. Ltd. Laboratories, Mumbai, India and S.D. fine Chemicals Ltd., India.

2.2. Approvals

The adult Albino wister rats were obtained from National Institute of Pharmaceutical Education and Research, Mohali. The animals were kept for 7 days for acclimatization in the Institutional animal house facility room, Central Animal house, Lovely Professional University, Phagwara (Reg.No. 954/PO/ac/06/CPCSEA). The animals were housed in the group of 5 animals each in clean acrylic cages. The animals were kept under natural day and night cycle with temperature 21±2°C. The experimental protocol was approved by Institutional Animal Ehics Committee (IAEC) with 954/ac/06/CPCSEA/06/25/1/14.

2.3. Grouping of Animals

Rats were divided into 6 groups, each consisting of 5 animals. In Group 1, the isolated rat hearts, after 10 min of stabilization, were perfused with K-H solution only. Group 2 and 3 were treated with Allopurinol (50mg/L) and Silymarin (10mg/L) respectively. Groups 4, 5 and 6 were treated with combinations of Silimarin (2.5mg/L, 5mg/L and 10mg/L) with Allopurinol (50mg/L) respectively. After administering the above treatments, the rats were subjected to 30 min of global ischemia followed by 120 min of reperfusion with K-H solution containing Silymarin, Allopurinol and their combinations.

2.4. Isolated Perfused Rat Heart

Rats were heparinised (500 IU, i.p.) about 20 min before sacrificing the animals by cervical dislocation. Heart was rapidly excised and immediately mounted on Langendorff's apparatus. Isolated heart was retrogradely perfused at constant pressure of 80 mm Hg with Kreb's Henseleit (KH) buffer (Nacl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4.7H_2O$ 1.2 mM; $NaHCO_3$ 25 mM; KH_2PO_4 1.2 mM; $C_6H_{12}O_6$ 11m M), pH 7.4, maintained at 37^0C and bubbled with 95% O_2. Flow rate was maintained at 7- 9 ml/min using Hoffman's screw. The heart was enclosed in a double walled jacket, the temperature of which was maintained by circulating water heated to 37^0C. Global ischaemia was produced for 30 min by blocking the inflow of Kreb's Henseleit solution followed by reperfusion for 120 min. Coronary effluent was collected immediately, 5 min and 30 min after reperfusion for estimation of lactate dehydrogenase (LDH) and creatine kinase (CK) [13].

2.5. Size Assessment of Infarct

Heart was removed from Langendorff's apparatus. Both the auricles and the root of aorta was excised and ventricles were kept overnight at 0^0C. Frozen ventricles were sliced into uniform sections of 2-3 mm thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37^0C in 0.2 M Tris buffer (pH 7.4) for 20 min. TTC is converted to red formazone pigment by NADH and dehydrogenase enzyme and therefore, the viable cells stained deep red. The infarcted cells have lost the enzyme and cofactor and thus remained unstained or dull yellow. The ventricular slices were placed between two glass plates. A transparent plastic grid with 100 squares in 1 cm^2 was placed above it. Average area of ventricular slice was calculated by counting the number of squares on either side. Similarly, numbers of square falling over non-stained dull yellow area were counted. Infarct size was expressed as percentage of average ventricular area. Whole of ventricular slices were weighed. Infarcted dull yellow part was dissected out and weighed. Infarct size was expressed as a percentage total ventricular weight[13].

2.6. Estimation of Lactate Dehydrogenase

LDH was estimated in samples of coronary effluent collected after stabilization, immediately and 30 min after reperfusion using 2, 4-DNPH method[14].

2.7. Estimation of Creatine Phosphokinase (CK)

CK was measured in samples of coronary effluent after stabilization and 5 min after reperfusion using modified method[15].

2.8. Statistical Analysis

The results were expressed as mean± SEM for 5 animals per group. The data obtained from various groups was statistically analysed using one way ANOVA followed by ‘dunnett’s test'. A *p* value of less than 0.05 was considered to present a statistically significant difference.

3. RESULTS

3.1. Effect of Silymarin, allopurinol and its combination on ischemia and reperfusion induced myocardial infarct size

Global ischemia for 30 min followed by reperfusion for 120 min significantly increased infarct size measured by volume and weight method (Figure 5.1). Allopurinal (50mg/L) and Silymarin (10mg/L) significantly reduced the ischemia and reperfusion induced increase in myocardial infarct size when compared with control group. However, Silymarin (10mg/L) significantly reduced the myocardial infarct size as compared to Allopurinol (50mg/L). Silymarin (10mg/L)+ Allopurinol (50mg/L) in combination significantly reduced the ischemia and reperfusion induced increase in myocardial infarct size (Figure 5.1).

3.2. Effect of Silymarin, allopurinol and its combination on ischemia and reperfusion induced LDH release

LDH was estimated in coronary effluent after stabilization of isolated rat heart (Basal), immediate (0 min) and 30 min after reperfusion. Global ischemia followed by 120 min increased LDH release immediately and 30 min after reperfusion. Allopurinal (50mg/L) and Silymarin (10mg/L) significantly reduced the ischemia and reperfusion induced increase in LDH release immediately and 30 min after reperfusion. However, Silymarin (10mg/L) significantly reduced the LDH release immediately and 30 min after reperfusion as compared to Allopurinol (50mg/L). The combination of Silymarin (10mg/L)+ Allopurinol (50mg/L) significantly reduced the LDH release immediately and 30 min after reperfusion (Figure 5.2).

3.3. Effect of Silymarin, allopurinol and its combination on ischemia and reperfusion induced CK release

CK was estimated in coronary effluent after stabilization of isolated rat heart (Basal), and 5 min after reperfusion. Global ischemia followed by 120 min increased CK level immediately and 5 min after reperfusion injury (Figure 3). Allopurinal (50mg/L) and Silymarin (10mg/L) significantly reduced the ischemia and reperfusion induced increase in CK release after 5 min of reperfusion. However, Silymarin (10mg/L) significantly reduced the CK release after 5 min of reperfusion as compared to Allopurinol (50mg/L). The combination of Silymarin (10mg/L)+ Allopurinol (50mg/L) significantly reduced the CK release after 5 min of reperfusion.

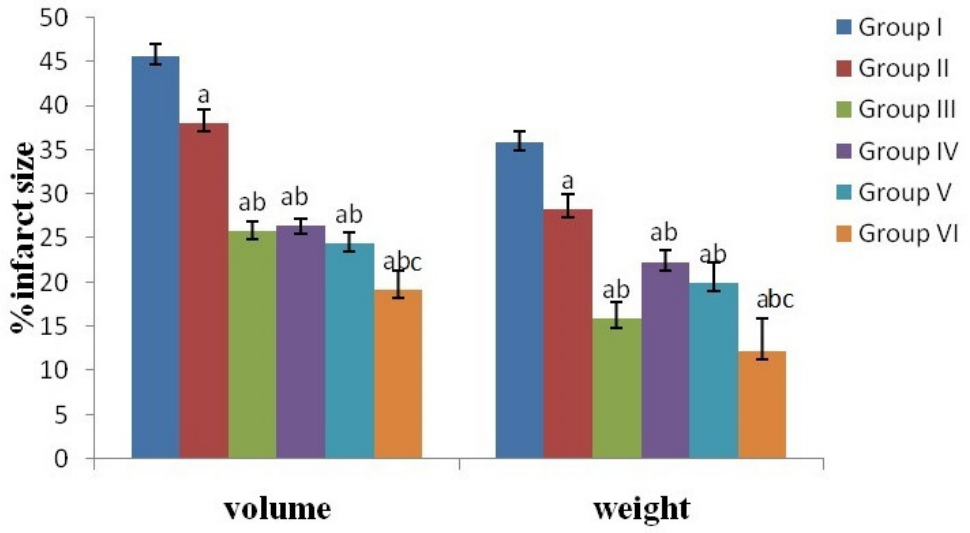

Figure 1

Effect of Silymarin, Allopurinal and its combination on ischemia and reperfusion induced myocardial infarct size measured by volume and weight method. Values are mean±SEM. (a=p<0.05 vs I), (b=p<0.05 vs II), (c=p<0.05 vs III)

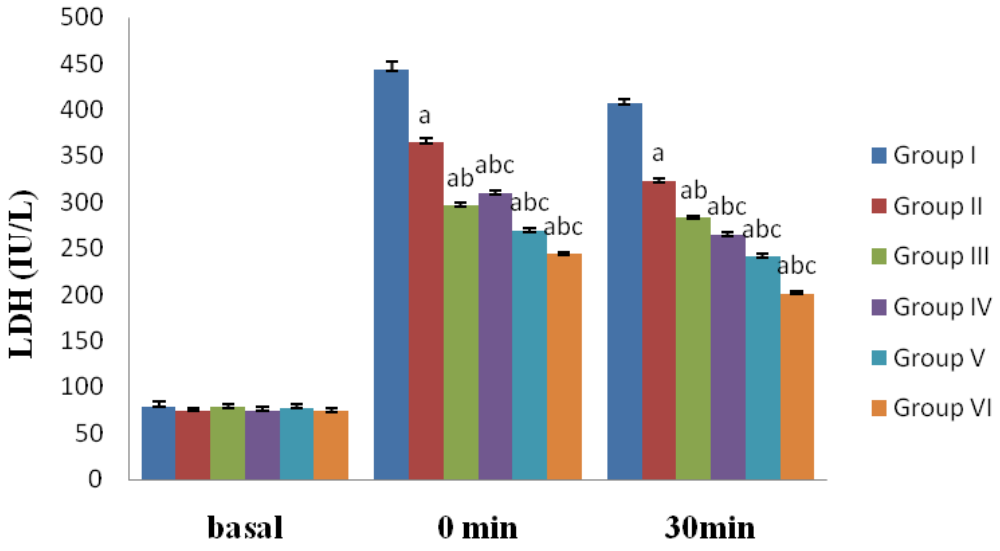

Figure 2

Effect of Silymarin, Allopurinol and its combination on ischemia and reperfusion induced LDH release. Values are mean±SEM. (a=p<0.05 vs I), (b=p<0.05 vs II), (c=p<0.05 vs III)

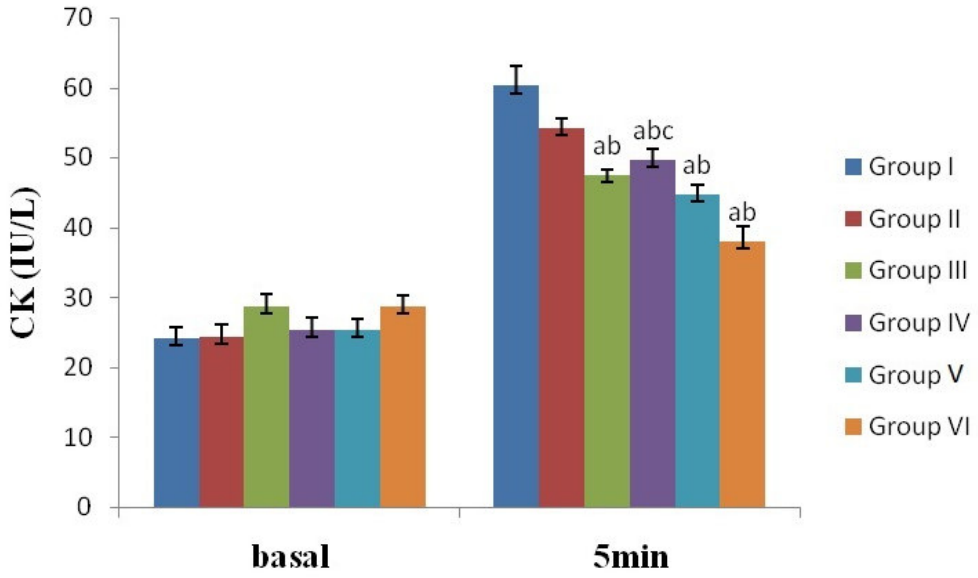

Figure 3

Effect of Silymarin, Allopurinol and its combination on ischemia and reperfusion induced CK release. Values are mean±SEM, n=5 (a=p<0.05 vs I), (b=p<0.05 vs II), (c=p<0.05 vs III)

4. DISCUSSION

It is well recognized that ischemia followed by reperfusion in myocardium leads to ischemia reperfusion injury and it represents an important clinical problem in many cardiovascular diseases. Ischemia reperfusion injury is the result of the interaction between the substances that accumulate during ischemia and those delivered in reperfusion. The cornerstone of these events is oxidative stress, an imbalance between oxygen free radicals and endogenous scavenging system. The focus of present study is the prevention or amelioration of myocardial damage due to reperfusion by employing antioxidant agents. Langendorff technique including isolated perfused mammalian heart preparation is a

tool for studying heart physiology and is regarded one of the most widely adopted models for basic and pre-clinical drug research. The isolated rat heart preparation perfused retrogately on langendorff apparatus has been used in the present study[16]. Global ischemia for 30 min leads to cardiac dysfunction such as myocardial ischemia. Reperfusion of previously ischemic myocardium is often followed by detrimental changes in coronary arteries and cardiac tissue known as I/R injury[17, 18]. The increase in infarct size and release of CK and LDH are documented to be an index of I/R induced myocardial infarction[19]. The peak release of LDH was observed immediately after reperfusion which is in accordance with the earlier reports[19, 20, 21]. The initial release of LDH observed immediately after reperfusion may be due to ischemic injury and delayed release of LDH, observed after 30 min of reperfusion may be due to reperfusion injury[13, 19]. Similarly CK was observed to increase in cardiac injury[21]. The infarct size has been assessed macroscopically because a good correlation has been reported between macroscopic and microscopic assessment of infarct size[13]. The NADH and dehydrogenase enzyme present in viable myocardium convert triphenyltetrazolium chloride (TTC) to red fromazone pigment and stained it deep red in colour[22]. However infracted cells lost dehydrogenase enzyme and cofactor NADH and thus remained unstained or dull yellow[23]. Moreover the reperfusion of 120 min employed in present study is sufficient to washout the NADH and dehydrogenase enzyme from infracted cells[24]. Generation of free radicals have been reported to play an important role in I/R induced myocardial injury[25, 26]. Many antioxidant agents have been suggested to attenuate the myocardial injury caused by I/R by preventing the generation of free radicals[27, 28]. In the present study an attempt has been made to examine the effect of Silymarin, and its combination with Allopurinol on I/R induced myocardial injury using in vitro rat heart. Both drugs have been reported to exhibit free radical scavenging activity. Global ischemia for 30 minutes followed by reperfusion for 120 minutes results in oxidative stress associated with increase in formation of various reactive oxygen species which may be responsible for the myocardial injury[29]. Therefore, a marked increase in infarct size, release of LDH and CK was observed in the control group due to ischemia-reperfusion injury. In the present study, it has been observed that the administration of Silymarin (10mg/L), a free radical scavenger, significantly attenuated the I/R induced myocardial injury. Furthermore, Allopurinol (50mg/L), a xanthine oxidase inhibitor, has been observed to prevent the generation of free radicals and plays an important role in the protection of ischemic myocardium. The findings of various parameters in the present study such as decrease in infarct size, LDH and CK due to administration of Allopurinol are consistent with previous reports[30]. Dosages of Allopurinol and Silymarin were chosen on the basis of earlier studies. Besides, in many studies, the antioxidant activity of Allopurinol and Silymarin has been reported, however in vitro *Silymarin* activity in I/R injury and synergistic effect of Silymarin and Allopurinol in I/R injury in rat model has not been reported. Therefore, taking this into consideration, the present study has been designed. It has been observed that I/R injury results in an increase in infarct size, release of LDH and CK and this increase was observed markedly in control group. It has been investigated in this study that Silymarin (10mg/L) has provided a significant cardioprotection in vitro as compared to Allopurinol (50mg/L). Furthermore, the combination of Silymarin and Allopurinol (10mg/L+50 mg/L) has significantly reduced the I/R injury assessed in terms of myocardial infarct size, release of LDH and CK, however, results with the combination of Silymarin and Allopurinol (5mg/L+50 mg/L and 2.5mg/L+50 mg/L) were not significant.

5. CONCLUSION

On the basis of above discussion, it may be concluded that Silymarin and its combination with Allopurinol may provide better cardioprotective effect by inhibition of I/R injury.

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