RESEARCH ARTICLE

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Isolation and characterization of mitochondria and lysosome from isoproterenol induced cardiotoxic rats

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ABSTRACT: Mitochondrial and lysosomal membranes are prominent membranes of cardiac cells and are the factors that determine membrane function in myocardial ischemia. In this study, isolation of mitochondria and lysosome from heart tissue under the control, isoproterenol (ISO) (8.5mg/100g) induced cardiotoxic rats and oral pretreatment with *Z. armatum* fruit (200, 400mg/kg body weight) treated rats. Further characterization of marker enzymes was done. A decreased in the activity of all the mitochondrial and lysosomal marker enzymes in ISO administered cardiotoxic rats when compared to control rats which indicate ISO decreased the stability of the membrane. Pretreatment with hydroethanolic extract of *Z. armatum* fruit to ISO induced rats significantly reverted these biochemical alterations near to normal. The possible mechanism for the protection of heart mitochondria and lysosome against oxidative damage induced by ISO might be due to quenching of free radicals and enhancing the action of marker enzymes.

Keywords: ISO, lysosomal marker enzymes, membranes, *Z. armatum*.

1. Introduction

The heart 20-45% of the myocardial volume is taken up by mitochondria [1] and is highly dependent for its function on oxidative energy generated primarily by fatty acid oxidation, respiratory electron chain and oxidative phosphorylation. Defects in mitochondrial structure and function have been found in association with cardiovascular diseases such as hypertrophy cardiomyopathy, cardiac conduction defects and sudden death, ischemia as well as myocarditis. Mitochondrial energy production depends upon the mitochondrial enzymes activity. Discrete mitochondrial oxidative phosphorilation defects and respiratory chain deficiency are frequently accompanied by defective levels of specific respiratory enzyme activities.

ISO damages the heart by different mitochondrial mechanisms, mainly for an increase of mitochondrial ROS production including superoxide, hydroxyl radicals and hydrogen peroxide (H_2O_2). The main site for mitochondrial ROS generation are complex I and II activities of the respiratory chain, either excessive (or) diminished electron flex at these sites can stimulate the auto-oxidation of flavins and quinines producing superoxide radicals. The superoxide radicals can be converted to H_2O_2 in the presence of the enzymes superoxide dismutase which can further react to form the hydroxyl radicals. ISO also induce apoptosis, decrease in antioxidative capacity and

alteration of mitochondrial respiratory chain function through down regulation of specific mitochondrial respiratory enzyme activities [2]. All of these changes have an impact on mitochondrial function leading to inhibition and uncoupling of electron flow, activation of the mitochondrial permeability transition, and loss of intermembrane enzyme activities. Thus, mitochondria act as a major participant in these effects, and also that it might be the final arbiter of cell death in the apoptotic or necrotic changes that occur in myocardial infarct [3].

Lysosomes are intracellular organelles which maintain the internal pH between 5.0 to 5.5 through ATP-dependent proton pump. They play an important role in the secretion and transport process. The release of lysosomal enzymes into the cytoplasm stimulates the formation of inflammatory mediators such as oxygen radicals and prostaglandins which may result in the conversion of reversible myocardial ischemia to irreversible infarction [4-5]. In myocardial ischemia, there is increased activity of glycohydrolases and the lysosomal stability was decreased which results in the necrosis of myocardium [6].

One approach to ameliorate the damage due to myocardial injury is to stabilize the membrane of ischemic myocytes, including mitochondrial and lysosomal membrane and to protect the cells from autolytic and hydrolytic damage. Thus, mitochondria and lysosome play an important role in the pathology of MI. To achieve the greatest possible reduction in MI, treatment strategies should be aimed at correcting the levels of marker enzymes of heart membrane.

No specific research reports related to *Z. armatum* fruit on ISO treated MI was observed through a literature survey. Our previous stains true on hydroethanolic extract of *Z. armatum* fruit on serum marker enzymes, lipid lowering activity in serum and antioxidant properties in ISO induced MI rats. The current study focuses our research finding that supports scientifically the protection of heart tissue by regulation of mitochondrial and lysosomal enzymes. The results of the study could serve as a step for the development of a mechanism based therapeutic approach for the management of MI.

2. Materials and Methods

2. Procedure

2.1. Collection of plant material and preparation of extract

Z. armatum fruit was identified by ABS Botanical Garden, Salem, Tamilnadu. Extract was prepared by dried fruits using 50% ethanol for 5 days.

2.2. Animals

Male Wistar albino rats (Rattus norvegicus) weighing 150–180g were obtained from animal house of PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. The animals were kept in polypropylene cages under a 12:12 hr light and dark cycle at around 37°C. They were fed on a standard pellet diet (AVM Cattle and Poultry Feeds, Coimbatore) and water *ad libitum*. The clearance of the ethical committee for experimentation on animals was obtained before the start of the experiment (Proposal No: 158/PO/bc/99/CPCSEA) from PSG Institute of Medical Sciences and Research, Coimbatore.

2.3. Induction of MI

ISO hydrochloride was used to induce MI in rats. Animals were injected subcutaneously with freshly prepared ISO hydrochloride in sterile normal saline at a dose of 20mg/100g body weight.

2.4. Experimental design

Animals were divided into six groups of six rats in each group.

Group I: Control rats

Group II: Pretreated rats (*Z. armatum* fruit 400mg/kg body weight for 30 days).

Group III: ISO treated rats (ISO- 8.5mg/100g body weight at an interval of 24hr on 28th and 29th day).

Group IV: ISO + *Z. armatum* fruit (200mg/kg body weight for 30 days) treated rats.

Group V: ISO + *Z. armatum* fruit (400mg/kg body weight for 30 days) treated rats.

Group VI: ISO + standard drug verapamil (1mg/ kg body weight for 30 days) treated rats.

At the end of the experimental period i.e., 12 hr after the second dose of ISO injection, all the rats were scarified by cervical dislocation under mild chloroform anesthesia. The heart tissue was excised immediately and thoroughly washed with ice-cold physiological saline and

homogenized in 5.0ml of 0.1M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for various biochemical estimations.

2.5. Separation of heart mitochondrial fraction

Heart mitochondria were isolated by the method of Takasawa *et al*., (1984) [7] with slight modifications. The heart tissue was homogenised in a medium containing 250 mM sucrose, 0.5 mM EDTA, 50mM Tris HCl (pH 7.4). It was then resuspended in 20 ml of isolation medium containing 0.1% (w/v) defatted bovine serum albumin and transferred to a 50-mL glass homogenizer. The mixture was incubated for 1 min at 4°C and then rehomogenized. To isolate the mitochondria, the homogenate was subjected to differential centrifugation at 4°C. Mitochondrial fraction was resuspended in the same buffer (final concentration 0.2% v/v) in ice for 15 min and it was used for the determination of lipid peroxidation (LPO).

2.6. Effect of hydroethanolic extract of Z. armatum fruit on mitochondrial marker enzymes

The activities of MDH, SDH and ICDH in heart tissue homogenate were assayed by the method of Mehler *et al*., (1948),[8] Slater and Bonner, (1952) [9].

2.7. Separation of heart lysosomal fraction

The lysosomal fraction of the heart tissue was isolated by the method of Wattiaux (1977) [10]. Fresh heart tissues were homogenized in ice cold 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000rpm for 10 minutes. The pellet was removed and recentrifuged. The supernatants were pooled and centrifuged at 15000rpm for 20 minutes. The lysosomal pellet was suspended in 1.15% potassium chloride and used for the assay of enzymes.

2.8. Effect of hydroethanolic extract of Z. armatum fruit on lysosomal marker enzymes

The activities of acid phosphatase, β-NAG, Cathepsin-D in heart tissue homogenate was assayed by the method of Sapolsky *et al*.,(1973),[11] Marhun,(1976)[12] and King, (1965)[13] respectively.

2.8. Statistical analysis

The results were expressed as mean± standard deviation. Statistical analysis was carried between the experimental groups using one way analysis of variance (ANOVA) employing SPSS Version 16.0. Post hoc analysis was performed using Fisher's least significant difference (LSD) test and the level of significance was set as ($p<0.05$).

3. Results and Discussion

From table 1 it is evident that the activities of MDH, SDH and ICDH were decreased significantly ($p<0.05$) in the heart mitochondria of ISO induced rats (Group III) when compared to normal rats (Group I). Pretreatment with hydroethanolic extract of *Z. armatum* fruit (Group IV and V) significantly ($p<0.05$) increased the activity of the mitochondrial enzymes in ISO induced rats when compared to ISO alone treated rats (Group II). There was no significant difference between standard drug verapamil treated rats (Group VI) and hydroethanolic extract of *Z. armatum* fruit alone treated rats (Group II).

Figure 1 depicts the activities of ACP, β-NAG and cathepsin-D in the heart lysosomal fraction. The decreased activities of these enzymes upon ISO treatment were regained to near normal by the administration of hydroethanolic extract of *Z. armatum* fruit. When comparing standard drug treated rats (Group VI) and *Z. armatum* fruit alone treated rats (Group II) there was no significant difference.

Mitochondria play a central role in the energy-generating process within the cell [14]. The mitochondrial enzymes (ICDH, SDH and MDH) catalyzes the oxidation of several substrates through the tricarboxylic acid (TCA) cycle, yielding reducing equivalents, which are channeled through the respiratory chain for the synthesis of adenosine tri phosphate (ATP) [15]. The Krebs cycle results in the production of CO_2 and NADH the later is used in the second pathway of mitochondrial oxidative phosphorylation [16]. ATP synthesis and electron transport chain starts in the mitochondria, which is required for cardiac contraction and relaxation [17].

TCA cycle enzymes are affected by free radicals produced by ISO which result in the impaired production of ATP leads to decrease in intracellular pH and intracellular acidosis, which has a direct inhibitory effect on contractile function. AMP and other intermediates accumulate with subsequent mitochondrial swelling and progressive degeneration which result in the myocardial ischemia and necrotic cell death [18].

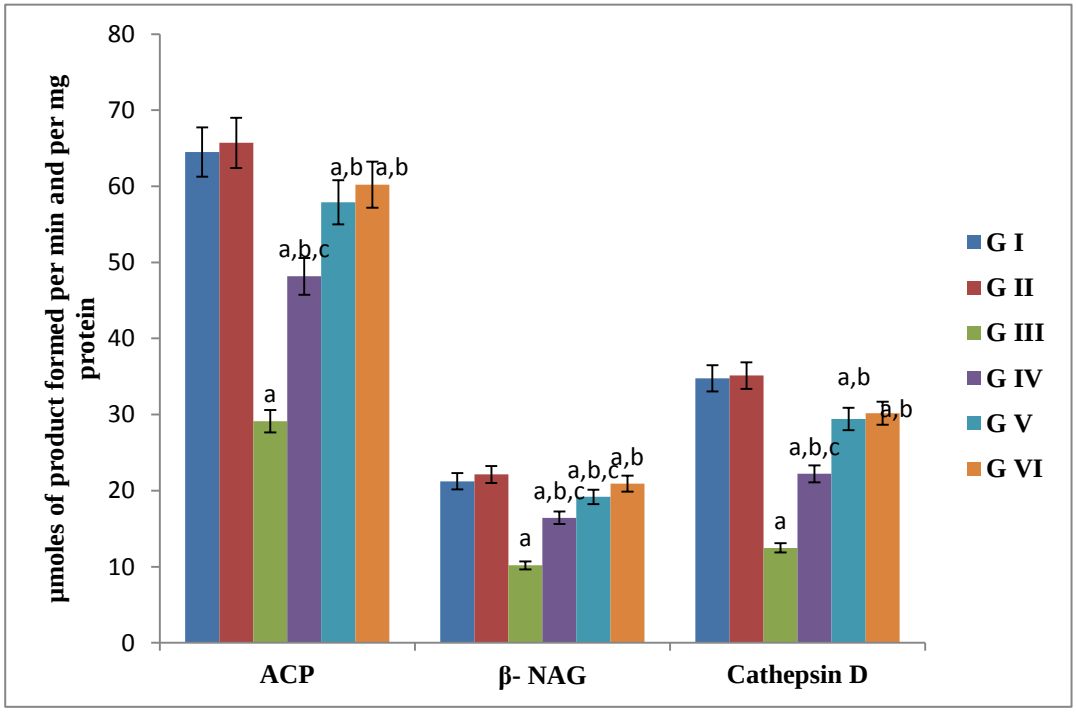

Figure 1. Effect of hydroethanolic extract of Z. armatum fruit on lysosomal marker enzymes

Values are mean + SD of six samples in each group. a,b,c- significant at 5% level ($p<0.05$). Group comparison: a- GI vs GII, GIII, GIV, GV, GVI: b- GIII vs GIV, GV, GVI: c- GVI vs GIV, GV.

Units of ACP – µmoles of phenol liberated/min/mg protein, β-NAG - µmoles of p-nitrophenol liberated/min/mg protein, Cathepsin D - µmoles of tyrosine liberated/hr/mg protein

Table 1: Effect of hydroethanolic extract of *Z. armatum* fruit on mitochondrial marker enzymes

	Heart mitochondria		
GROUPS	**MDH**	**SDH**	**IDH**
GROUP I	316.1 ± 10.13	240.06 ± 8.78	754.48 ± 15.77
GROUP II	311.8 ± 7.04	234.78 ± 7.41	756.72 ± 10.76
GROUP III	154.46 ± 6.73 [a]	144.75 ± 9.02 [a]	515.76 ± 14.23 [a]
GROUP IV	244.57 ± 9.78 [a,b,c]	188.16 ± 10.26 [a,b,c]	662.76 ± 11.21 [a,b.c]
GROUP V	278.74 ± 15.03 [a,b]	224.12 ± 18.7 [a,b]	728.16 ± 9.27 [a,b,c]
GROUP VI	287.81 ± 8.73 [a,b]	216.19 ± 17.7 [a,b]	694.18 ± 18.30 [a,b]

Values are mean ± SD of six samples in each group. a,b,c- significant at 5% level ($p<0.05$). Group comparison: a- GI vs GII, GIII, GIV, GV, GVI: b- GIII vs GIV, GV, GVI: c- GVI vs GIV, GV.
Units of MDH- nmoles of NADH oxidized /min/mg protein, SDH- nmoles of succinate oxisized /min/mg protein, IDH- nmoles of αKG formed/hr/mg protein.

ICDH is mainly expressed in the heart and skeletal mitochondria and is an NADP dependent which controls the mitochondrial redox balance and the subsequent oxidative damage [19]. Pre-treatment with hydroethanolic extract of *Z. armatum* fruit significantly increased the activity of ICDH which might be due to resistance of the heart enzyme against oxidative stress in heart mitochondria.

SDH is an integral membrane protein and it is tightly attached to the inner membrane and is directly linked to the electron transport, transferring electrons to the respiratory chain [20]. SDH is a site for metabolic control in TCA cycle [21] and contains many cysteine rich sulfur clusters and can be inhibited by agents that modify sulfhydryl groups. ISO administration is known to alter protein-bound sulfhydryl groups and have resulted in the inactivation of the enzyme. SDH is one of the important

enzymes that regulate the production of ATP in the mitochondria and it is sensitive to free radicals.

MDH is located in the outer membrane of the mitochondria and is affected by increased level of free radicals produced following ISO administration [22]. Inhibition of mitochondrial enzymes by ROS may affect the mitochondrial substrate oxidation, resulting in reduced oxidation of substrates, reduced rate of transfer of reducing equivalents of molecular oxygen and depletion of cellular energy [15].

Treatment with hydroethanolic extract of *Z. armatum* fruit showed significantly improved activities of TCA cycle enzymes in the heart mitochondrial fraction in ISO induced rats due to their free radical scavenging activities.

Lysosomal enzymes play a significant role in the breakdown of cell organelles, digest cell materials and modify the growth substances of connective tissue, thereby favouring growth or migration of both normal and malignant cells. They have also played a significant role in tumor invasion. Lysosomal enzymes like ACP, β-NAG and cathepsin-D are accomplished of degrading most of the cellular constituents. Cathepsin D is a lysosomal aspartic protease that is present in all animal cells. ACP is an important and critical enzyme in biological processes and is responsible for detoxification, metabolism and biosynthesis of energetic macromolecules for different essential function [23].

Lysosomes are organelles particularly vulnerable to oxidative stress since they exhibit the most important pool of reactive iron in the cell [24]. Oxidative stress can induce very fast lysosomal disruption creating intra lysosomal iron-mediated redox reactions [25]. It has also been suggested that abnormal release and activation of lysosomal enzymes during ischaemia and other potentially lethal events may contribute to the tissue damage.

In the present study administration of ISO resulted in declined activities of ACP, β-NAG and cathepsin-D in the lysosomal fraction. Pretreatment with hydroethanolic extract of *Z. armatum* fruit normalized the activities of ACP, β-NAG and cathepsin-D which might be due to the membrane stabilizing property of *Z. armatum* fruit in ISO induced myocardial infarcted rats.

4. Conclusion

Our results clearly confirmed the existence of cardiotoxicity due to ISO administration which was indicated by decreased activities of mitochondrial and lysosomal enzymes. *Z. armatum* fruit exerts cardioprotective activity which could be partly contributed by its membrane protective action. Therefore, it can be concluded that *Z. armatum* fruit can protect against cardiotoxicity by preserving the integrity of the membrane and restoring the activities of enzymes.

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Conflict of interest:

There are no conflicts of interest.

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Competing Interests:

The authors declare that they have no competing interests.