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Protective effects of *Centella asiatica* against isoproterenol-induced myocardial infarction in rats: biochemical, mitochondrial and histological findings

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Abstract

Myocardial infarction (MI) induced by isoproterenol (ISO) is a standardized model to study the beneficial effects of different drugs. The protective effect of *Centella asiatica* on ISO-induced cardiotoxicity and the antioxidant activity involved in the protection were investigated in rats. Wistar albino rats were used in the present study. Animals were randomly divided into six groups comprising of six animals in each group. MI was induced in rats with ISO (85 mg/kg, sc) twice at an interval of 24 hrs. ISO produced significant alterations in the creatine kinase, biochemical parameters and moderate necrosis in the heart. The effect of *Centella asiatica* oral treatment for 21 days at two doses (100 mg and 200 mg/kg, body weight) was evaluated against ISO -induced cardiac necrosis. Levels of marker enzymes (LDH and CK) were assessed in serum while antioxidant parameters viz., reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehde (MDA) were assayed in heart homogenate. Significant myocardial necrosis, depletion of endogenous antioxidants and increase in serum levels of marker enzymes were observed in ISO-treated animals as compared with the normal control animals. *Centella asiatica* showed a significant cardioprotective activity by lowering the levels of serum marker enzymes and lipid peroxidation as well as elevated the levels of antioxidant enzymes. The findings of the present study demonstrated that the cardioprotective effects of *Centella asiatica* on ISO-induced oxidative damage may be due to an augmentation of the endogenous antioxidants and inhibition of lipid peroxidation of membrane.

Keywords: Antioxidant, *Centella asiatica*, Creatine kinase, Isoproterenol, Myocardial infarction.

Introduction

Globally, cardiovascular diseases (CVD) constitute a leading cause of mortality. Developing countries like India are also struggling to manage the impact of CVD along with the growing burden of obesity. CVDs will account for one third of the deaths by the year 2020. Current projections suggest that India will have the largest CVD burden in the world by the year 2020.[1]

Myocardial infarction (MI) is an acute condition of the myocardium necrosis that occurs as a result of imbalance between coronary blood supply and myocardial demand.[2] It is one of the serious disorders among ischemic heart diseases invariably followed by several biochemical alterations such as hyperlipidemia, lipid peroxidation, free radical damage, thrombosis, etc., leading to qualitative and quantitative alteration of myocardium.[3]

Isoproterenol (ISO), a synthetic catecholamine and β adrenergic agonist is documented to produce MI in large doses due to the generation of highly cytotoxic free radicals and lipid peroxides through its auto-oxidation.[4] ISO induced myocardial injury involves membrane permeability alterations, which brings about the loss of functions and cause irreversible damage to the myocardial membrane.[5,6] MI induced by ISO in rats has been shown to be accompanied by hyperglycemia, hyperlipidemia and increase in serum CK-NAC, Alanine aminotransferace, Lactate dehydrogenase activities.[7] Animal experiments have suggested that increases in free radical formation and subsequent oxidative stress associated with the occurrence of a relative deficit in the endogenous antioxidant enzymes may be one of the mechanisms for the development of MI.[8]

The Indian system of medicine has treated diseases with its herbals for ages. Recently, several Indian medicinal plants have beneficial effects in different diseases like atherosclerosis, ischemia, cancer, diabetes and liver dysfunction due to their antioxidant activity. Examples are of *Centella asiatica, Ocimum sanctum, Curcuma longa, Terminalia arjuna, Withania somnifera* and *Bacopa monniere*.[9]

Centella asiatica, is a small, herbaceous, annual plant of the family Apiaceae. *Centella asiatica* has been

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valued as a tonic and is used in memory[10], cognition impairment[11], depression, anxiety[12], cardio- protective[13] and antioxidant[14].

In the present study the cardioprotective activity of *Centella asiatica* extract was evaluated in ISO-induced cardiac damage and mitochondrial dysfunction by assessing the mitochondrial complexes in rats as well as attempts to understand the molecular mechanism of its therapeutic effect with reference to biochemical markers and antioxidant enzymes, lipid peroxidation.

Materials and Methods

Plant Material

Centella asiatica extract was obtained as a gift sample from Sanath Products Limited, Vikas Marg, Delhi, India (Ref. No.: 023023). The preparation of *Centella asiatica* extract was freshly prepared every day by dissolving in distilled water before the oral administration via intra gastric tube once daily.

Drugs and chemicals

ISO and enalapril were purchased from Sigma- Aldrich Chemicals Pvt. Ltd. USA. Thiobarbituric acid (TBA), reduced glutathione, and dithiobisnitrobenzoic acid (DTNB) were obtained from Himedia Laboratories, Mumbai, India.

Animals

Thirty six male Wistar albino rats (150-250 g, b. wt.) were used in the present study. They were housed in clean polypropylene cages under standard conditions of humidity (50±5%), temperature (25±2°C) and light (12 h light/12 h dark cycle) and fed with standard rat chow diet (Amrut Laboratory Animal Feed, Nava Maharashtra Chakan Oil Mills, Pune, India) and water *ad libitum*. The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) having a Registration number and date of registration: 1099/c/07/CPCSEA, dated 27/07/2007.

Treatment schedule

Animals were randomly divided into six groups comprising of six animals in each group and treated in the following way: Group I: Normal control (normal saline 2 ml/kg, p.o.) daily for 21 days. Group II: ISO control (normal saline 2 ml/kg, p.o.) daily for 21 days and in addition received ISO (85 mg/kg, s.c.) on 20th and 21st day. Group III – *Centella asiatica* (100 mg/kg, p.o.) daily for 21 days and in addition received ISO (85 ml/kg, s.c.) on the 20th and 21st day. Group IV – *Centella asiatica* (200 mg/kg, p.o.) daily for 21 days and in addition received ISO (85 mg/kg, s.c.) on 20th and 21st day. Group V – Enalapril (10 mg/kg, p.o.) daily for 21 days and in addition received ISO (85 mg/kg, s.c.) on 20th and 21st day. Group VI – *Perse* group i.e. *Centella asiatica* (200 mg/kg p.o.) daily for 21 days.

Biochemical estimation in serum

Blood was collected from the retro-orbital plexus under light ether anesthesia and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation at 3000 rpm at 30 °C for 15 min and used for the estimation of marker enzymes viz., LDH and CK.

Estimation of lactate dehydrogenase (LDH) and CK (Creatinine kinase) Serum

The concentrations of LDH (Coral Clinical System, Goa, India) and CK (Erba Mannheim, Germany) in serum were measured with commercial kits. The assay is carried out as per the procedure given by the manufacturer. The results were expressed as IU/L for LDH and CK.

Tissue preparation

The hearts were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1M phosphate buffer (pH 7.4). The post nuclear fraction was obtained by centrifugation of the homogenate at 12000×g for 20min at 4 °C. An aliquot was used for the estimation of biochemical estimations.

Biochemical tests

Measurement of lipid peroxidation (LPO)

The quantitative estimation of LPO was done by determining the concentration of Thiobarbituric Acid Reactive Substances (TBARS) in the heart using the method of Ohkawa *et al.*[15] The amount of malondialdehyde (MDA) formed was quantified by reaction with TBA and used as an index of lipid peroxidation. The results were expressed as nmol of MDA/g of wet tissue using a molar extinction coefficient of the chromophore (1.56×10^{-5}/M/cm) and 1,1,3,3-tetraethoxypropane as standard.Catalase estimation

Catalase activity was assayed by the method of Luck[16], where in the breakdown of hydrogen peroxides (H_2O_2) is measured at 240 nm. Briefly, assay mixture consisted of 3 ml of H_2O_2 phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and change in absorbance was recorded at 240 nm UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). The results were expressed as micromole H_2O_2 decomposed per milligram of protein/min.

Glutathione estimation

Reduced glutathione was estimated according to the method of Ellman.[17] The absorbance was read at 412 nm and the results were expressed as μmol of the GSH/ milligram of protein.

Superoxide dismutase activity (SOD)

Superoxide dismutase activity was assayed according to the method of Kono[18] wherein the reduction of nitrazobluetetrazolium (NBT) was inhibited by the superoxide dismutase, is measured at 560 nm using a UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). Briefly, the reaction was initiated by the addition of the hydroxylamine hydrochloride to the mixture containing nitrazobluetetrazolium (NBT) and sample. The results were expressed as unit/mg protein.

Isolation of mitochondria

The heart was removed and stored at −70°C until analysis could be completed. Heart tissues were excised washed thoroughly with ice cold saline to remove the blood. They were gently blotted between the folds of a filter paper and weighed in an analytical balance. The 10% of the heart homogenate was prepared in 50 mmol/L phosphate buffer (pH 7.0) containing 0.25 mol/L (w/v) sucrose and the mitochondrial pellets were prepared from the heart homogenate according to the method as described in our previous publication. The isolated mitochondria were suspended in 50 mmol/L phosphate buffer (pH 7.0). The mitochondrial fraction was frozen and thawed 3–5 times to release the enzymes (except complex IV, which was extracted with 0.5% Tween 80 in phosphate buffer, v/v).[19]

COMPLEX-I (NADH Dehydrogenase activity)

The method involves the catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome-C. The reaction mixture contained 0.2 M glycyl glycine buffer *p*H 8.5, 6 mM NADH in 2 mM glycyl glycine buffer and 10.5 mM cytochrome-C. The reaction was initiated by the addition of a requisite amount of solubilised mitochondrial sample. The absorbance change at 550 nm was

followed for 2 min by using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).[20]

COMPLEX-II (Succinate Dehydrogenase)

The method involves the oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of the mitochondrial sample and the absorbance change at 420 nm was followed for 2 min by using a UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).[20]

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium-bromide) assay

The MTT assay is based on the reduction of (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess activity of the mitochondrial respiratory chain in isolated mitochondria. Briefly, 100 μl mitochondrial samples were incubated with 10 μl MTT for 3 hrs at 37 °C. The blue formazan crystals were solubilized with dimethylsulfoxide and measured by an ELISA reader with a 580 nm filter using Bio-rad microplate absorbance reader for 96-well plate.[21]

Mitochondrial complex-IV (Cytochrome oxidase) assay

Cytochrome oxidase activity was assayed in brain mitochondria as mentioned, the assay mixture contained 0.3 mM reduced cytochrome-C in 75 mM phosphate buffer. The reaction was initiated by the addition of the solubilised mitochondrial sample and absorbance change at 550 nm was followed for 2 min using a UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).[22]

Histopathology of Heart Tissue

Animals were sacrificed on the day of withdrawal of blood; hearts were removed, washed immediately with saline and then fixed in 10% buffered formalin. The hearts stored in 10% buffered formalin were embedded in paraffin, sections cut at 5 mm and stained with hematoxylin and eosin. These sections were then examined under a light microscope for histoarchitectural changes.

Statistical analysis

The results were expressed as the mean ± standard error of means (SEM). The results were analyzed using one-way ANOVA followed by *post-hoc* analysis using Tukey's Multiple Comparison Test. The p value < 0.05 was considered to be statistically significant.

Results

Effect of *Centella asiatica* treatment on LDH in serum

In the present study, ISO treatment produced significantly (p< 0.05) increase in LDH levels as compared to NC group. Treatment with CA (200 mg/kg) to ISO challenged animals decreased significantly (p<0.05) the levels of LDH elevated by ISO. Enalapril treatment showed significant decrease in LDH levels as compared to ISO control group. While there was no significant change in the LDH levels in CA *per se* group (Figure 1).
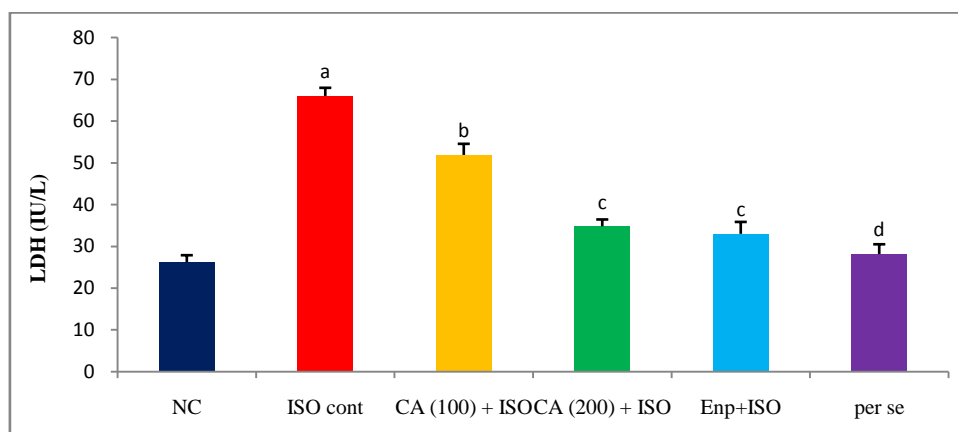
Values are expressed Mean ± S.E.M. (ANOVA followed by Turkey test).[a]p<0.05 as compared to normal control group, [b]p>0.05 non-significant as compared to isoproterenol control group, [c]p<0.05 as compared to isoproterenol control group, [d]p>0.05 non-significant as compared to normal control group
NC- normal control group, **ISO control-** Isoproterenol control group, **CA (100 mg/kg)-** *Centella asiatica* (100 mg/kg), **CA (200 mg/kg)-** *Centella asiatica* (200 mg/kg), **Enp-** Enalapril (10 mg/kg), **per se-** *Centella asiatica* (200 mg/kg) treatment alone

Figure 1: Effect of *Centella asiatica* extract treatment on LDH levels

Effect of *Centella asiatica* treatment on creatine kinase of serum

In the present study, ISO treatment caused a significant increase in CK levels as compared to NC group. Treatment with CA (200 mg/kg) and Enalapril showed significant decrease in elevated CK levels as compared to ISO control group. While there was no significant change in the CK in CA *per se* group (Figure 2).

**Effect of *Centella asiatica* treatment on oxidative stress & antioxidant enzymes in heart**

The myocardial lipid peroxidation marker, i.e. MDA level was significantly elevated (p<0.05) in the ISO control group in comparison with the NC group. Animals treated with CA (100 mg/kg

and 200 mg/kg) significantly (p<0.05) decreased the MDA levels elevated by ISO.

In the present study significant decline in myocardial GSH (p<0.05) was observed in ISO control group as compared to the normal control group. Treatment with CA (200 mg/kg) and Enalapril significantly (p<0.05) prevented the ISO-induced decreased GSH levels. While the treatment with CA (100 mg/kg) non-significantly elevated the decreased GSH levels.

ISO-induced myocardial necrosis produced a significant (p<0.05) depletion in the activities of antioxidant enzymes such as SOD and catalase levels as compared to NC animals. Treatment with CA (200 mg/kg) to myocardial necrotic rats significantly restored the activities of SOD and catalase. But CA (100 mg/kg) treatment non-significantly

increased the levels of antioxidant enzymes. CA (200 mg/kg) *per se pre* treatment to rats did not produce significant changes in the levels of antioxidant enzymes as compared to the NC rats (Table 1).
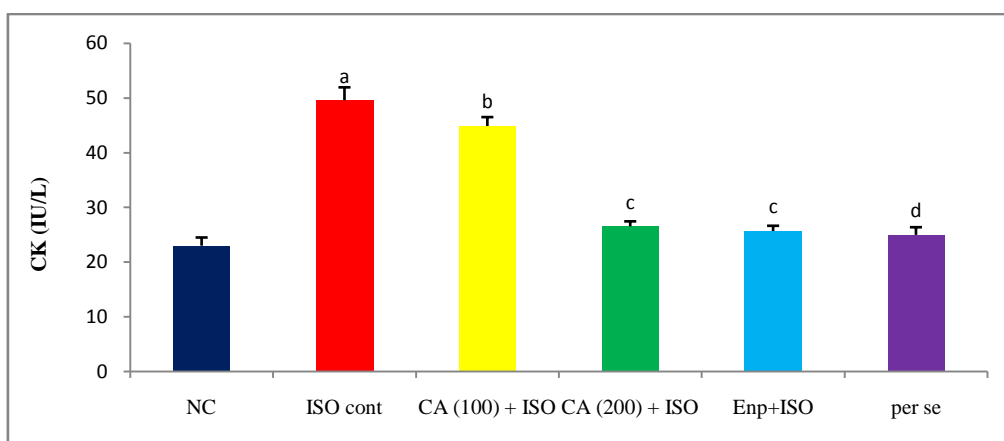
Values are expressed Mean ± S.E.M. (ANOVA followed by Turkey test).[a]P<0.05 as compared to normal control group, [b]p>0.05 non-significant as compared to isoproterenol control group, [c]p<0.05 as compared to isoproterenol control group, [d]p>0.05 non-significant as compared to normal control group

NC- normal control group, **ISO control-** Isoproterenol control group, **CA (100 mg/kg)-** *Centella asiatica* (100 mg/kg), **CA (200 mg/kg)-** *Centella asiatica* (200 mg/kg), **Enp-** Enalapril (10 mg/kg), **per se-** *Centella asiatica* (200 mg/kg) treatment alone

Figure 2: Effect of *Centella asiatica* extract treatment on creatine kinase (CK) levels

Table 1: Effect of *Centella asiatica* extract on heart TBARS, SOD, CAT & GSH in ISO treated rats

Treatment groups	TBARS (nmoles MDA/mg protein)	SOD levels (unit/mg protein)	Catalase (μ mole of H_2O_2/min/mg protein)	GSH (μ mole of GSH/mg protein)
Normal Control	0.812±0.04	7.49±0.05	21.42±1.09	12.66±0.29
ISO Control (85 mg/kg, s.c.)	3.22±0. 12[a]	2.02±0.23[a]	10.12±0.64[a]	3.00±0.11[a]
CA (100 mg/kg) + ISO (85 mg/kg, s.c.)	2.46±0.04[b]	5.39±0.027[b]	15.56±0.42 [b]	6.23±0.13[b]
CA (200 mg/kg) + ISO (85 mg/kg, s.c.)	1.21±0.04[b,c]	6.81±0.39[b,c]	17.79±0.87 [b,c]	7.93±0.06[b,c]
Enalapril (10 mg/kg, p.o.)+ISO (85 mg/kg, s.c)	1.09±0.077[b]	6.95±0.15[b]	19.75±0.78[b]	8.49±0.10[b]
Centella asiatica (200 mg/kg) *per se*	0.871±0.03[d]	7.38±0.66[d]	21.79±0.67[d]	11.73±0.15[d]

Values are expressed Mean ± S.E.M. (ANOVA followed by Turkey test).[a]p<0.05 as compared to normal control group, [b]p<0.05 as compared to isoproterenol control group, [c]p>0.05 non-significant as compared to CA (100 mg/kg), [d]p>0.05 non-significant as compared to normal control group
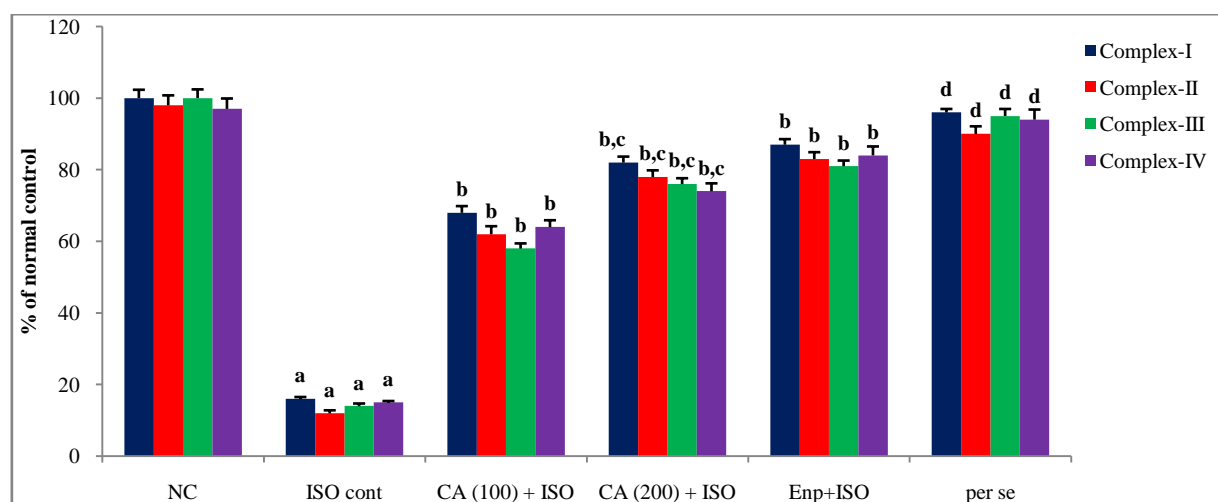
ISO control- Isoproterenol control group, **CA (100 mg/kg)-** *Centella asiatica* (100 mg/kg), **CA (200 mg/kg)-** *Centella asiatica* (200 mg/kg)

Effect of *Centella asiatica* treatment on mitochondrial enzyme complex levels in heart

In the present study, ISO significantly impaired mitochondrial enzyme complex (I, II and IV) and MTT (mitochondrial redox) activity in heart as compared to NC group. CA (100 mg/kg & 200 mg/kg) significantly cause restoration of mitochondrial enzyme complex as compared to ISO control group. Enalapril pre-treatment significantly restored mitochondrial complex enzyme activity (I, II and IV) and MTT activities as compared to the ISO control group (p < 0.05). However, CA *per se* pre-treatment did not produce significant effects on mitochondrial complex activities as compared to NC group (Figure 3).

Effect *Centella asiatica* treatment on histopathological studies

Histopathological examination of the myocardium of NC animals showed the clear integrity of myocardial cell membrane & no inflammation. Rats treated with 85 mg/kg ISO showed moderate to marked myocardial necrosis with moderate infiltration of lymphocytes and macrophages. Hearts of CA (100 mg/kg) treatment to ISO administered rats showed mild to moderate multifocal myocardial necrosis with mild to moderate infiltration of lymphocytes and macrophages. CA (200 mg/kg) treatment to ISO administered rats, exhibited mild necrosis and less infiltration of inflammatory cells. Treatments of CA (200 mg/kg) *per se* showed the heart sections to have similar histoarchitecture to that of normal rats (Figure 4).

Values are expressed Mean ± S.E.M. (ANOVA followed by Turkey test).[a]$p<0.05$ as compared to normal control group, [b]$p<0.05$ as compared to isoproterenol control group, [c]$p>0.05$ non-significant as compared to *Centella asiatica* (100 mg/kg) , [d]$p>0.05$ non-significant as compared to normal control group

NC- normal control group, ISO control- Isoproterenol control group, CA (100 mg/kg)- *Centella asiatica* (100 mg/kg), CA (200 mg/kg)- *Centella asiatica* (200 mg/kg), Enp- Enalapril (10 mg/kg), per se- *Centella asiatica* (200 mg/kg) treatment alone

Figure 3: Effect of *Centella asiatica* extract treatment on mitochondrial enzyme complex I, II, III and IV levels in heart
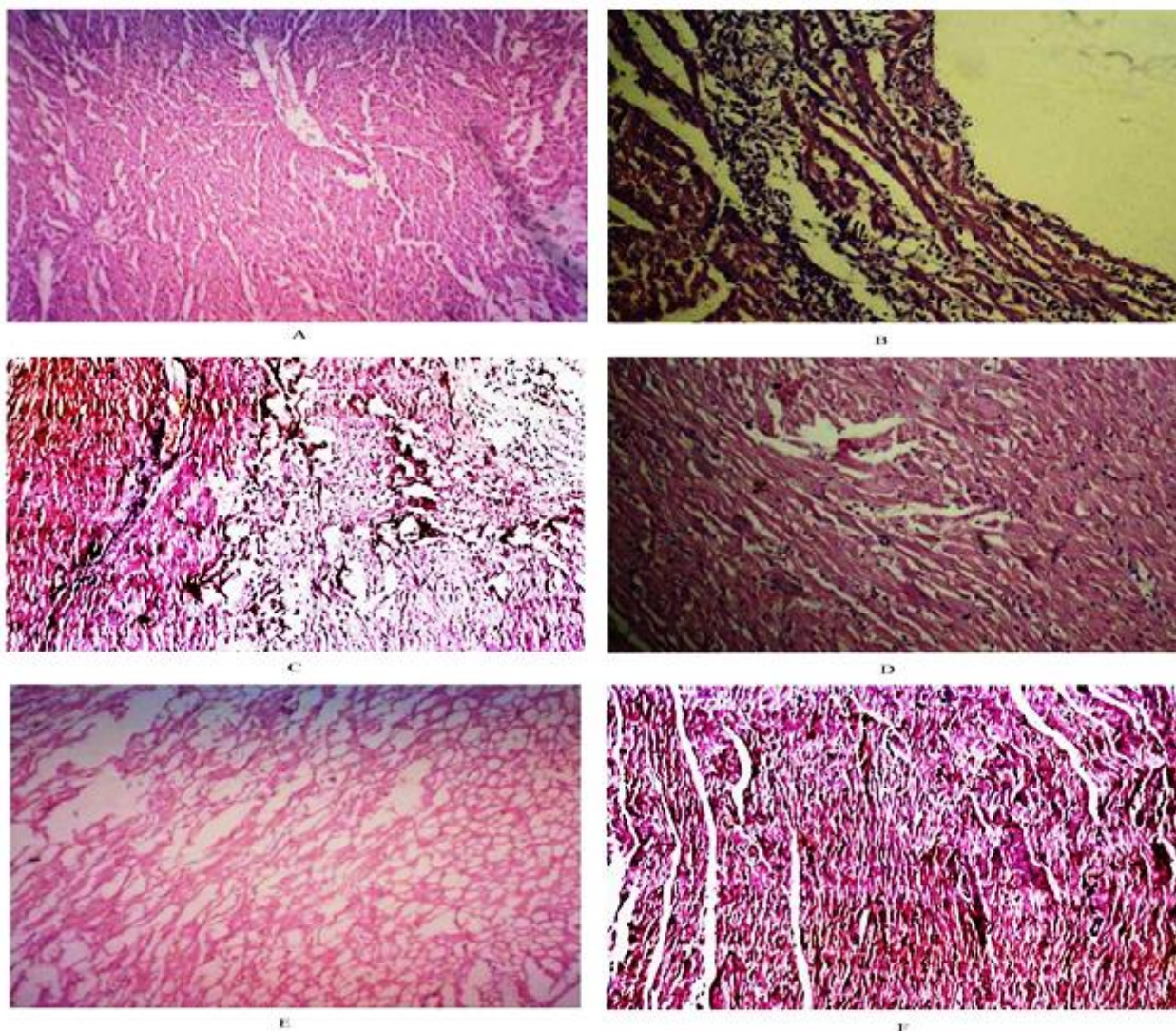
A. Myocardium of normal control group showed clear integrity of myocardial cell membrane & no inflammation. **B.** ISO (85 mg/kg) treated group showed marked myocardial necrosis with moderate infiltration of lymphocytes and macrophages. **C.** *Centella asiatica* (100 mg/kg) treatment to ISO administered rats showed mild to moderate myocardial necrosis with moderate infiltration of lymphocytes and macrophages. **D.** *Centella asiatica* (200 mg/kg) treatment to ISO administered rats, exhibited mild necrosis and less infiltration of inflammatory cells. **E.** Enalapril (10 mg/kg) treatment to ISO administered rats, exhibited mild necrosis and focal infiltration of inflammatory cells. **F.** *Per se* treatments of *Centella asiatica* (200 mg/kg) showed the similar histoarchitecture to that of normal rats.

Figure 4: Effect of *Centella asiatica* extract treatment histopathological changes

Discussion

CVD is a global health issue. The economic burden rests on both its mortality rate and its risk factors. It was predicted that CVD will be ranked 1st from 1990 to 2020 [23]. Isoproterenol at 85mg/kg s.c., 2 doses at 24hr interval, has been used to develop an acute model of MI.[24]

In the present study, oxidative cardiac injury was induced by ISO in rats confirmed by biochemical estimations and microscopic examination. ISO-induced cardiotoxicity provide good insight into this pathology which clearly indicates the involvement of oxidative stress.[9]

In the present study, development of oxidative cardiac injury due to ISO was confirmed by the myocardial cell damage, the alteration in oxidative stress markers as the significant increase in TBARS, and the significant decrease in the activities of GSH, catalase and superoxide dismutase in the heart tissue. The changes in the antioxidant parameters are in accordance with published results.[25]

The increased levels of MDA indicate excessive formation of free radicals by auto-oxidation of ISO and activation of the lipid peroxidative process, resulting in irreversible damage to heart in animals subjected to ISO stress.[9] CA (200 mg/kg) +ISO treatment significantly decreased the MDA levels by preventing formation of lipid peroxides from fatty acids.

Reduced glutathione is one of the most abundant non-enzymatic antioxidant bio-molecules present in the body.[26]

In the present study, SOD activity was decreased significantly in the ISO control animals may be due to an excessive formation of superoxide anions. A decrease in SOD activity results in the decreased removal of superoxide anions, which can be harmful to the myocardium.[27] The activity of H_2O_2 scavenging by catalase was decreased significantly in ISO treated animals. The decline in these enzyme levels may be explained by the fact that excessive superoxide anions may inactivate SOD, thus, resulting in an inactivation of the H_2O_2 scavenging enzymes.[28] Oral administration of CA to ISO treated rats prevented the decrease in SOD, catalase and GSH levels, which may be correlated directly to the scavenging of radicals by *Centella asiatica* resulting in the protection of these enzymes.

Mitochondria are the main intracellular sites of ROS generation and are also targets for oxidative damage in the heart. Previously finding suggested that a consistent decrease in the mitochondrial complexes-I, II, IV and MTT levels in cardiovascular disease in rats. However, the present study suggested that there was a consistent decrease in the mitochondrial complexes in ISO treated hearts. *Centella asiatica* pre-treatment restores mitochondrial complexes.

In the present study, ISO treated rats there was an increase in the activities of the marker enzymes LDH and CK in the serum. The plasma LDH and CK enzyme activities are important measures for both early and late phases of cardiac injury. The release of the cardiac specific isoenzymes LDH1 and LDH2-into the circulation might be due to the necrosis induced by ISO.[29] These finding confirm the onset of myocardial necrosis and leaking out of the marker enzymes from heart to blood.[9] *Centella asiatica* treatment prevents the increase of marker enzymes in serum indicating the cardioprotective activity of *Centella asiatica.*

The histopathological findings, ISO-induced MI of the heart show the evidence of necrosis, which comply with previous literature of ISO.[30] *Centella asiatica* (200 mg/kg) treatment showed less necrosis due to antioxidant effect, but this needs further molecular level establishment.

Other important factors in the present study were the timing and the period of administration of the antioxidant agents. All of these drugs viz. *Centella asiatica* were found to have positive results in studies in which they were administered before the initiation of MI. Although the present experimental study was designed to resemble the actual clinical conditions of acute myocardial infarction, all such experimental models have important differences compared with the setting of myocardial infarction in humans.[31]

Conclusion

In summary, the present study demonstrated that *Centella asiatica* has a cardioprotective effect against ISO-induced MI in rats. The mechanism of the cardioprotective effect may involve prevention of lipid peroxidation and preservation of antioxidant enzymes (glutathione, SOD and Catalase) as well as scavenging of free radicals. *Centella asiatica* reduced the deleterious effects in the setting of myocardial infarction through potent antioxidant actions.These findings indicate that Centella asiatica could be a useful intervention in the management of cardiovasculardisease.

Acknowledgement

Conflict of interest

Authors declare no conflict of interest.

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