

Myocardial salvaging effects of *Ocimum sanctum* in experimental model of myocardial necrosis: a haemodynamic, biochemical and histoarchitectural assessment

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The present study envisages evaluation of the cardioprotective efficacy of *Ocimum sanctum* (Os) (Tulsi) in the experimental model of isoproterenol (ISP)-induced myocardial necrosis. Wistar male albino mature rats were randomly divided into four main groups: sham, ISP-control, Os control and Os-treated. Os-control and Os-treated groups were further divided into three subgroups and administered with 25, 75 and 150 mg/kg Os orally, once daily for a month. On the 29th and 30th day, animals of the ISP-control and Os-treated groups received ISP (85 mg/kg) subcutaneously at an interval of 24 h. On the 31st day, 24 h after the second dose of ISP, the haemodynamic variables were recorded and the animals were sacrificed for biochemical and histopathological studies. Significant ventricular dysfunction, myocardial necrosis and depletion of endogenous antioxidants were observed in the ISP-control group compared to sham. Os pre-treatment augmented the basal endogenous antioxidants and restored the antioxidant status of the heart. The myocardial salvaging beneficial effects also translated into functional recovery of the myocardium. Histopathological studies further confirm its myocardial salvaging effects. Our study emphasizes the cardioprotective effect of Os against ISP-induced myocardial necrosis. The present study has demonstrated the cardioprotective effects of Os, which likely result from improved ventricular function, augmentation of endogenous antioxidants and suppression of oxidative stress.

Keywords: Antioxidants, isoproterenol, medicinal herbs, myocardial infarction, *Ocimum sanctum*.

HERBAL medicine is increasingly gaining greater acceptance from the public and medical profession due to greater advances in the understanding of the mechanisms by which herbs positively influence health and quality of life¹. Leading the way in the new understanding is the discovery of herbs as potent free-radical scavengers; antioxidants². Evidence for a role of deleterious effects of free radicals in the pathophysiology of ischaemic heart disease (IHD)

is clear and indisputable³. IHD is perhaps one of the human conditions in which the role of oxidative stress has been extensively investigated. The free radicals and consequent expression of oxidative damage have been demonstrated during post-ischaemic reperfusion injury in humans. The protective role of antioxidants has been validated in several experimental studies addressing the pathophysiology of acute ischaemia⁴. The multitude of free radicals generated during oxidative stress associated with isoproterenol (ISP)-induced myocardial necrosis can damage every major cellular component, including carbohydrate, membrane lipids, protein and DNA⁵. The pathophysiological consequences of such uncontrolled injury are widespread tissue damage and associated contractile dysfunction, arrhythmias, depletion of endogenous antioxidant network and enhanced lipid peroxidation resulting in increased myocardial malondialdehyde (MDA) content⁶.

The most active principles having antioxidant property found in botanical products are not only vitamins but also chemicals like phenols, polyphenols and flavanoids. *Ocimum sanctum* (Os, commonly known as Tulsi in India) is a local herb containing potent antioxidants, flavanoids (orientin, vicenin) and phenolic compounds (eugenol, cirsilineol, apigenin). The ancient systems of medicine, including Ayurveda, Greek, Roman, Siddha and Unani, have mentioned its therapeutic applications in cardiovascular disorders, diabetes and asthma^{7,8}. However, its potential as a cardioprotective agent has not been extensively studied. Besides its antioxidant properties, Os interacts by various other mechanisms in a complex way to elicit its therapeutic effects.

Our study is an effort in the same direction. The ISP-induced myocardial necrosis model was used to evaluate the cardioprotective potential of Os and to understand the molecular mechanism of its therapeutic effects by evaluating various biochemical and haemodynamic parameters. Results were further confirmed by histopathological studies.

Hydro-alcoholic extract of Os leaves was a generous gift from Dabur Research Foundation, India. The plant was identified and authenticated by routine pharmacognostical studies, including organoleptic tests, and macroscopic and microscopic observations. The voucher specimen (O-02) has been retained in our laboratory for further reference.

All chemicals were procured from standard companies and were of analytical grade. Isoproterenol was obtained from Sigma, USA. Double-distilled water was used for all biochemical measurements.

Wistar male albino mature rats, weighing 150 to 200 g, 10 to 12 weeks old were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) and conforms to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research. Animals were obtained from the Central Animal House Facility of All India Institute of Medical Sciences, New Delhi, India and were housed in polyacrylic cages (38 × 23 × 10 cm), with not

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more than four animals per cage. They were housed in an air-conditioned room; kept in standard laboratory conditions under natural light and dark cycles (approximately 12 h light/12 h dark) and maintained at humidity $50 \pm 5\%$ and an ambient temperature of $25 \pm 2^\circ\text{C}$. All experiments were performed between 9.00 and 16.00 h. The animals were allowed free access to standard pellet diet (Ashirwad Industries Ltd, Chandigarh, India) and tap water *ad libitum*. The commercial pellet diet contains 24% protein, 5% fat, 4% fibre, 55% carbohydrates, 0.6% calcium, 0.3% phosphorus, 10% moisture and 9% ash w/w. The animals were allowed to acclimatize for one week before the experiments.

The animals were randomly allocated into four main groups comprising six animals each. Haemodynamically unstable rats were excluded from the present study.

Group 1 – Saline control group – sham control: Rats were administered 0.9% normal saline once daily for a month and in addition received normal saline (0.5 ml, sc) on 29th and 30th day at an interval of 24 h.

Group 2 – ISP-control group – ISP-control: The animals were orally fed 0.9% normal saline once daily for a month and in addition received ISP (85 mg/kg, sc) on the 29th and 30th day at an interval of 24 h.

Group 3 – Os-control group: This was divided into three subgroups – Group 3a – 25 mg/kg Os (Os-25). Group 3b – 75 mg/kg Os (Os-75), and Group 3c – 150 mg/kg Os (Os-150).

Hydro-alcoholic extract of Os was dissolved in normal saline and orally fed once daily for a month.

Group 4 – ISP-challenged, Os-treated group: This was divided into three subgroups – Group 4a – ISP + 25 mg/kg Os (IOs-25); Group 4b – ISP + 75 mg/kg (Os IOs-75), and Group 4c – ISP + 150 mg/kg Os (IOs-150).

Animals were orally fed Os (25, 75, 150 mg/kg) once daily for one month and in addition received ISP (85 mg/kg, sc) on the 29th and 30th day.

On the 31st day, 24 h after second dose ISP administration, animals were sacrificed under overdose of anaesthesia after recording haemodynamic parameters. The hearts were excised and immediately processed for histopathological studies. For biochemical analysis, the hearts were stored in liquid nitrogen.

All animals were anaesthetized intraperitoneally with pentobarbitone sodium (60 mg/kg). Atropine (4 mg/kg) was administered along with the anaesthetic to maintain the heart rate, especially during surgery and to reduce tracheo-bronchial secretions. Body temperature was monitored and maintained at 37°C during the experimental protocol. The neck was opened with a ventral midline incision to perform tracheostomy and animals were ventilated with room air from a positive pressure ventilator (Inco, Ambala,

India) using compressed air at a rate of 90 strokes/min and a tidal volume of 10 ml/kg. Ventilator setting and PO_2 were adjusted as needed to maintain the arterial blood gas parameters within the physiological range. The left jugular vein was cannulated with polyethylene tube for continuous infusion of 0.9% saline solution. The right carotid artery was cannulated and the cannula was filled with heparinized saline and connected with CARDIOSYS CO-101 (Experimentria, Hungary) using a pressure transducer for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The left thoractomy was performed at the fifth intercostal space and the heart was exposed. A sterile metal cannula (1.5 mm bore) was introduced into the cavity of the left ventricle from the posterior apical region of the heart for measuring ventricular dynamics, left ventricular end diastolic pressure (LVEDP), (+) LVdP/dt (rate of pressure development) and (–) LVdP/dt (rate of pressure decline). The cannula was connected to a pressure transducer (Gould Statham P23ID, USA) through a pressure-recording catheter on the Polygraph (Grass 7D, USA). The thoracic cavity was then covered with saline-soaked gauze to prevent the heart from drying. Animals were allowed to stabilize for 10 min before measuring the basal haemodynamic parameters.

Hearts stored in liquid nitrogen were brought to room temperature and weighed. A 10% homogenate was prepared in phosphate buffer (50 mM, pH 7.4) and an aliquot was used for the assay of MDA according to the method described by Ohkawa *et al.*⁹. The homogenate was centrifuged at 7000 rpm for 15 min and the supernatant was used for estimation of the following biochemical parameters: glutathione (GSH)¹⁰, glutathione peroxidase (GSHPx)¹¹, superoxide dismutase (SOD)¹², catalase (CAT)¹³ and protein¹⁴. Myocardial injury marker, creatine kinase-MB (CK-MB) isoenzyme was estimated spectrophotometrically using a kit from Randox Laboratories, USA¹⁵.

Myocardial tissue at the end of the experiment was immediately fixed in 10% buffered neutral formalin solution. The fixed tissues were embedded in paraffin and serial sections were cut. Each section was stained with hematoxylin and eosin (H&E). The sections were examined under a light microscope (Nikon, Japan) and photomicrographs were taken.

Descriptive statistics such as mean and standard deviation was calculated for all variables for each group. One-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) for the evaluation of haemodynamic variables and Student's *t* test was used for biochemical analysis. A *P* value < 0.05 has been considered as statistical significance level.

We observed no significant change in the basal GSH and MDA level as compared to sham in the Os-control group (Table 1). However, Os at 75 and 150 mg/kg doses significantly augmented basal endogenous antioxidant enzyme activities of CAT and SOD ($P < 0.05$) compared to sham

Table 1. Biochemical values in different experimental groups

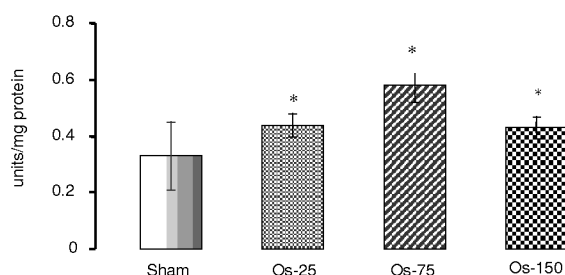
| Biochemical parameter | Sham | Os (25) | Os (75) | Os (150) |
|---------------------------------|--------------------|--------------------|--------------------|--------------------|
| GSH ($\mu\text{mol/g}$ tissue) | 1.86 \pm 0.69 | 1.84 \pm 0.17 | 1.99 \pm 0.20 | 2.09 \pm 0.32 |
| MDA ($\mu\text{mol/g}$ tissue) | 63.00 \pm 13.90 | 62.41 \pm 13.90 | 59.70 \pm 5.54 | 60.94 \pm 14.10 |
| CAT (units/mg protein) | 21.10 \pm 3.10 | 26.01 \pm 5.62 | 34.87 \pm 8.00* | 30.30 \pm 9.63* |
| SOD (units/mg protein) | 7.94 \pm 2.90 | 7.81 \pm 1.80 | 12.53 \pm 2.95* | 14.53 \pm 4.90* |
| CK-MB (units/mg protein) | 162.40 \pm 27.30 | 154.00 \pm 16.45 | 165.80 \pm 12.60 | 177.67 \pm 12.60 |

GSH, Reduced glutathione; SOD, Superoxide dismutase; CAT, Catalase; CK-MB, Creatine phosphokinase-MB; MDA, Malonaldehyde. Values are expressed as mean \pm SD. Each value represents a mean of six readings. * $P < 0.05$ vs sham. One unit of CK-MB transfers 1 μmol of phosphate from phosphocreatine to ADP per min at pH 7.4 and 30°C. One unit of SOD inhibits the rate of auto-oxidation of adrenaline by 50% and pH 7 at 25°C. One unit of CAT activity represents amount of enzyme required to decompose 1 μmol of $\text{H}_2\text{O}_2/\text{min}$.

Table 2. Haemodynamic parameters in different experimental groups

| Haemodynamic parameter | Sham | Os (25 mg/kg) | Os (75 mg/kg) | Os (150 mg/kg) |
|------------------------|----------------------|----------------------|----------------------|----------------------|
| MAP (mmHg) | 127.20 \pm 21.20 | 111.00 \pm 10.80 | 122.00 \pm 10.90 | 129.00 \pm 17.70 |
| HR (beats/min) | 350.30 \pm 41.50 | 326.00 \pm 17.00 | 361.00 \pm 21.40 | 325.50 \pm 19.40 |
| (+) LVdP/dt (mmHg/s) | 3150.00 \pm 113.60 | 2996.60 \pm 437.50 | 3072.90 \pm 257.50 | 2934.80 \pm 250.50 |
| (-) LVdP/dt (mmHg/s) | 3087.00 \pm 72.17 | 2964.25 \pm 268.40 | 2923.62 \pm 289.30 | 2897.58 \pm 317.10 |
| LVEDP (mmHg) | 4.10 \pm 0.05 | 3.62 \pm 65.00 | 3.73 \pm 0.33 | 3.94 \pm 0.37 |

MAP, Mean arterial blood pressure, HR, Heart rate, (+) LVdP/dt, Rate of change in left ventricular peak positive pressure; (-) LVdP/dt, Rate of change in left ventricular peak negative pressure and LVEDP, Left ventricular end-diastolic pressure. Values are expressed as mean \pm SD. Each value represents a mean of six readings.



* $P < 0.05$ vs Sham. Values are mean \pm SD of six experiments. One unit of GSHPx activity is defined as the amount of enzyme required to utilize 1 nmol of NADPH/min at 25°C.

Figure 1. Myocardial glutathione peroxidase activity in different experimental groups.

(Table 1). In addition, Os also significantly increased basal GSHPx activity ($P < 0.05$) at all doses studied in reference to sham (Figure 1).

Oral feeding of Os per se did not adversely modulate any of the haemodynamic parameters: MAP, HR, LVEDP, (+) and (-) LVdP/dt as compared to sham (Table 2).

On histopathological examination, there was no evidence of any cellular injury in any of the Os (25, 75 and 150 mg/kg)-control groups.

ISP-induced myocardial necrosis produced significant depletion of antioxidant enzymes such as CAT ($P < 0.05$) and SOD ($P < 0.05$) compared to sham control (Table 3). Chronic Os 75 and 150 mg/kg treatment significantly restored the activities of antioxidant enzymes CAT, SOD

and GSHPx ($P < 0.05$) compared to ISP-control. Os only at 25 mg/kg dose, significantly restored GSHPx activity and failed to significantly increase the activities of other antioxidants CAT and SOD as compared to ISP-control. Significant decline in myocardial GSH ($P < 0.05$) was observed in ISP-control group and Os treatment failed to restore its levels compared to ISP-control (Table 3).

MDA, the myocardial lipid peroxidation marker was significantly elevated ($P < 0.05$) in the ISP-control group in comparison to sham (Table 3). MDA levels generally correlated inversely with myocardial CK-MB isoenzyme activity. A fall in myocardial CK-MB isoenzyme activity was observed in the ISP-control group compared to sham (Figure 2). Os treatment at all the doses studied significantly decreased MDA ($P < 0.05$) levels in reference to ISP-control. However, Os only at 75 mg/kg dose restored CK-MB isoenzyme activity of the myocardium (Table 3).

A significant fall in MAP ($P < 0.05$) and HR ($P < 0.05$) was observed in ISP-control group compared to sham (Table 4). In addition, ISP administration resulted in left ventricular dysfunction, as indicated by a significant fall in both (+) and (-) LVdP/dt ($P < 0.05$) and a rise in LVEDP ($P < 0.05$) compared to sham (Table 4). Os (25, 75 and 150 mg/kg) significantly improved HR ($P < 0.05$) as compared to sham. However, only the 75 mg/kg dose significantly restored MAP ($P < 0.05$) in comparison to ISP-control (Table 4). In addition, Os at all doses, failed to improve both myocardial contractility (+LVdP/dt) and relaxation (-LVdP/dt) significantly compared to ISP-control. Nonetheless, it markedly reduced LVEDP

property, which enhances myocardial tolerance subsequent to stress, a phenomenon known as adaptation¹⁹. Although the exact mechanism of the adaptation is not fully understood, it may work through the induction of antioxidant enzymes such as SOD, CAT, GSHPx and antioxidants such as GSH and proteins like heat shock protein¹⁹. We have also observed a concomitant increase in the activities of CAT, GSHPx and SOD in Os-treated animals. Augmentation of endogenous antioxidants may enhance the myocardial antioxidant reserve and strengthen myocardial defence mechanisms operating in the myocardium. Therefore myocardial adaptation seems to be one of the likely mechanisms contributing to its cardioprotective activity.

Our data are consistent with the idea that the generation of highly cytotoxic free radicals through the auto-oxidation of catecholamines is one of the important causative factors for ISP-induced myocardial necrosis²⁰. ISP-induced myocardial necrosis resulted in reduced GSH content as well as antioxidant enzymes (SOD, CAT and GSHPx) in cardiac tissue^{21,22}. The fall in the activity of GSHPx in the ISP group might be correlated to decreased availability of its substrate, reduced GSH. Moreover, due to impairment in both enzymatic and non-enzymatic antioxidant defence mechanisms, it is quite likely that the free radicals are not effectively neutralized and hence myocardium shows enhanced susceptibility to lipid peroxidation. Although the antioxidant enzyme levels were significantly preserved by Os treatment, it failed to prevent the loss of GSH content of the myocardium significantly. We observed enhanced lipid peroxidation, as indicated by elevated MDA level in the ISP-control group and Os treatment significantly decreased its levels by preventing the formation of lipid peroxides from fatty acids.

In our heart model, the myocardial dysfunction was observed following ISP administration, indicating its myocardial deleterious effects. A significant fall in MAP, HR, $\{(+)\text{LVdP/dt}\}$, $\{(-)\text{LVdP/dt}\}$ and an elevated LVEDP was observed, which might be due to ISP-induced myocardial necrosis. The fall in MAP normally increases HR and myocardial contractility due to reflex sympathetic action. However, none of these effects was observed in the present study, suggesting impairment in the cardiac reflexes following ISP-induced myocardial injury. As outlined above, administration of Os (75 mg/kg) significantly increased both HR and MAP, an indirect measure of cardiac oxygen consumption. In addition, the 25 and 150 mg/kg doses also restored HR significantly compared to ISP-control. Therefore, it is likely that the myocardial salvaging effect demonstrated by Os (75 mg/kg) in the study, might partly be the due to the reduction in myocardial oxygen demand.

The $(-)\text{LVdP/dt}$ was more markedly depressed indicating a more diastolic ventricular dysfunction per se compared to systolic function. Os at all the doses studied significantly reduced surrogate preload marker LVEDP compared to ISP-control. The major consequence of the

reduction in LVEDP is to increase blood flow through the sub-endocardial region of the ventricular muscle that bears the maximum brunt of the ischaemia. There is disproportionate reduction in blood flow to the subendocardial regions of the heart, which is subjected to the greatest extra-vascular compression during systole. However, it failed to significantly improve both inotropic and lusitropic functions of the heart.

In summary, our study demonstrated that Os (75 mg/kg) significantly reduced ISP-induced myocardial injury. Histopathological examination further confirmed its cardioprotective effects. Most importantly, we have demonstrated that chronic treatment with Os decreased myocardial necrosis, oedema and inflammation, and improved cardiac functions by different mechanisms. Decreased myocardial necrosis (as evidenced by reduced CK-MB release and histopathological changes), reduction in myocardial oxygen demand and preload as well as augmentation of endogenous antioxidants, i.e. myocardial adaptation and restored antioxidant status, all contribute to its cardioprotective effects. In this context, it is important to mention that Os at 25 and 150 mg/kg doses failed to demonstrate any significant myocardial salvaging effects.

The additional mechanisms by which Os may reduce myocardial injury and potential clinical implications require further investigation. However, the present investigation provides a lead for exploring other mechanisms contributing to its cardioprotective effect. Whether the conclusions dealing with our experimental data can be extrapolated to the clinical scenario or not, remains to be defined by well-controlled studies in humans. Nonetheless, the results of our study are rather encouraging, because they could disclose a new therapeutic approach in the treatment of ischaemic heart disease.

The present results clearly emphasize the beneficial action of Os (75 mg/kg) as a cardioprotective agent. Our results are consistent with earlier reports that generation of ROS in myocardium subjected to ISP-induced injury and subsequent oxidative stress induced cardiac deterioration. Although the precise mechanism of its cardioprotective effects in ISP-induced myocardial injury is not fully understood, we have proposed some of the likely mechanisms for its myocardial salvaging effects. Favourable modulation of the haemodynamics, which may decrease myocardial oxygen demand and increase blood flow to the subendocardial region of the myocardium, augmentation of basal endogenous antioxidants and restored endogenous antioxidant network are some of its proposed cardioprotective mechanisms. Preserved myocardial CK-MB activity and histopathological study further confirm its myocardial salvaging effects.

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