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Cardioprotection from ischemia and reperfusion injury by Withania somnifera: A hemodynamic, biochemical and histopathological assessment

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Abstract

The efficacy of Withania somnifera (Ws) to limit myocardial injury after ischemia and reperfusion was explored and compared to that of Vit E, a reference standard known to reduce mortality and infarct size due to myocardial infarction. Wistar rats (150–200 g) were divided into six groups and received orally saline (sham, control group), Ws-50/kg (Ws control and treated group) and Vit E-100 mg/kg (Vit E control and treated group) respectively for 1 month. On the 31st day, rats of the control, Vit E and Ws treated groups were anesthetized and subjected to 45 min occlusion of the LAD coronary artery followed by 60 min reperfusion. Hemodynamic parameters: systolic, diastolic and mean arterial pressure (SAP, DAP, MAP), heart rate (HR), left ventricular end diastolic pressure (LVEDP), left ventricular peak (+)LVdP/dt and (–)LVdP/dt were monitored. Hearts were removed and processed for histopathological and biochemical studies: Myocardial enzyme viz, creatin phosphokinase (CPK), and antioxidant parameters: malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) were estimated. Postischemic reperfusion produced significant cardiac necrosis, depression of left ventricular functions (MAP, LVEDP, (+) and (–)LVdP/dt) and a significant fall in GSH ($p < 0.01$), SOD, CAT ($p < 0.05$), LDH and CPK ($p < 0.01$) as well as an increase in MDA level ($p < 0.05$) in the control group rats as compared to sham group. The changes in levels of protein and GPx was however, not significant. Ws and Vit E favorably modulated most of the hemodynamic, biochemical and histopathological parameters though no significant restoration in GSH, MAP (with Vit E) were observed. Ws on chronic administration markedly augmented antioxidants (GSH, GSHPx, SOD, CAT) while Vit E did not stimulate the synthesis of endogenous antioxidants compared to sham. Results indicate that Ws significantly reduced myocardial injury and emphasize the beneficial action of Ws as a cardioprotective agent. (Mol Cell Biochem **260**: 39–47, 2004)

Key words: myocardial infarction, Withania somnifera, Vitamin E, reperfusion, antioxidants, adaptogens

Introduction

Myocardial infarction (MI) is a therapeutic enigma with high morbidity and mortality rate. Extensive experimental studies have been performed to further our understanding of the pathophysiology of irreversible myocardial damage. Besides ischemia, restitution of coronary blood flow following ischemia has deleterious consequences on cardiac function and may lead to an extension of myocardial tissue injury [1]. During ischemia and reperfusion of the heart, oxygen free radicals (OFR) are thought to play an important role in the pathogenesis of MI [2]. Furthermore, there is much evidence

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that OFR are involved in reperfusion injury of the post-ischemic heart. In addition to tissue injury, OFR may result in depression in contractile function, arrhythmias, depletion of endogenous antioxidant network, increase in malondialdehyde (MDA) content resulting in membrane permeability changes [3]. Therapeutic intervention that could diminish free radicals production or improve impaired antioxidant defense mechanism hence may be one of the useful therapeutic modality for the treatment of MI.

A better understanding of the processes involved in the pathophysiology of MI has lead to the search for drugs, which could limit the extension of myocardial injury. Currently, there is an increasing realization that herbs can influence the course of heart diseases and its treatment [4]. In view of this, it is rational to identify and select inexpensive and safer approaches for the management of cardiovascular disease. The present study is an effort in this direction. We have evaluated the cardioprotective potential of *Withania somnifera* (Ws), known as Ashwagandha in Ayurveda and has been extensively used as rasayana and medhyarasayana. Although its therapeutic potential as immunomodulatory, adaptogenic, antioxidant, antidiabetogenic and anticancer agents are known, very few studies evaluating its cardioprotective potential are presently available [5–7].

The efficacy of Ws to limit myocardial injury after ischemia-reperfusion induced myocardial injury has been evaluated and compared to that of Vitamin E. The cardioprotective effects of Vitamin E are well documented [8]. The effect of Ws on modulation of biochemical parameters: lipid peroxidation product MDA, endogenous antioxidant: glutathione (GSH), antioxidant enzymes {superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx)} and myocardial enzymes {creatinine phosphokinase (CPK)} have been evaluated. In order to correlate biochemical and functional changes in the myocardium subjected to ischemia and reperfusion induced damage, different hemodynamic parameters have also been included in the present study. Alterations in mean arterial pressure (MAP), heart rate (HR), left ventricular end diastolic pressure (LVEDP), left ventricular (LV) peak positive (+)dP/dt (rate of pressure development) and negative (–)dP/dt (rate of pressure decline) were recorded. Protective action of Ws have also confirmed by assessing the ischemia and reperfusion induced myocardial injury histopathologically.

Materials and methods

Experimental animals

Wistar Albino rats of either sex, weighing 150–200 g, were used in the study. The study protocol was approved by the Institutional Ethics Committee and conducted according to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals. Rats were obtained from the Central Animal House facility of All India Institute of Medical Sciences, New Delhi, India. They were kept in standard laboratory conditions under natural light and dark cycle. The animals were fed normal diet (Hindustan Lever, India) and water *ad libitum*.

Test drugs

(1) *Withania somnifera* (Root); (2) Vitamin E. Hydro-alcoholic lyophilized extract of Ws was procured from Dabur Research Foundation, India and Vitamin E from Sigma Chemicals, USA.

All chemicals used in the study were procured from Sigma Chemicals, and were of analytical grade.

Experimental models of MI

A pilot study was conducted in the isoproterenol model of MI to select the optimum dose of Ws exhibiting cardioprotective effect.

Isoproterenol induced MI

Wistar albino rats of either sex (150–200 g) were divided into four main groups: sham, ISP control, Ws sham control and Ws treatment groups. Ws was administered to the rats at doses of 25, 50 and 100 mg/kg orally for 30 days. On days 29 and 30, the rats of the ISP control and Ws treatment groups were administered ISP (85 mg/kg), subcutaneously at an interval of 24 h. Before sacrificing the rats, on day 31, hemodynamic parameters of all the experimental groups were recorded. Hearts were removed and processed for histopathological and biochemical studies.

The optimum dose of Ws was selected on the basis of favorable modulation of hemodynamic, histopathological and biochemical parameters in the isoproterenol induced myocardial necrosis. The selected dose of Ws was further evaluated in the in vivo model of ischemia and reperfusion induced myocardial injury.

Ischemia-reperfusion (I-R) model in rats

Saline/Vit E/ Ws extract were administered orally to the rats for 30 days. Wistar rats were then anesthetized intraperitoneally with pentobarbitone sodium (60 mg/kg). A tracheostomy was then performed and the rats were ventilated with room air from a positive pressure ventilator (Inco, India) at a rate of 70 strokes/min and a tidal volume of 10 ml/kg. The right carotid artery was cannulated with an arterial catheter (BPR-10, Experimetria, Hungary) filled with heparinized saline (20 units/ml) for recording blood pressure and the left jugular vein was cannulated with polyethylene tube for saline

administration. A left thoracotomy was preformed at the fifth intercostal space, and the left anterior descending coronary artery (LAD) was ligated 8–10 mm from its origin and ends of this ligature were passed through a plastic tube to form a snare. After the completion of the surgical procedure, the heart was returned to its normal position in the thorax. The animals were then allowed to stabilize for 15 min before LAD ligation. Myocardial ischemia was produced by one stage occlusion of the LAD by pressing the polyethylene tubing against the ventricular wall. This was designated as time point 0. The animals then underwent 45 min of ischemia, confirmed by the appearance of epicardial cyanosis. Baseline hemodynamic parameters were measured before LAD occlusion and continued according to the experimental protocol throughout ischemia and reperfusion period. The myocardium was reperfused by releasing the snare gently for a period of 60 min. At the end of reperfusion period, animals were sacrificed for biochemical and histological studies.

Experimental groups

Sham groups

Saline control, Ws control, Vit E control.

The rats of the sham control groups were administered normal saline (0.9%)/Ws (50 mg/kg)/Vit E (100mg/kg) respectively for 30 days. The entire surgical procedure was performed except coronary artery ligation.

I-R groups

Saline treated, Ws treated, Vit E treated.

The rats of these groups were administered normal saline (0.9%)/Ws (50 mg/kg)/Vit E (100 mg/kg) respectively for 30 days and the experimental protocol of 40 min ligation and 60 min reperfusion was performed.

Experimental parameters studied

Hemodynamic studies

The right carotid artery was cannulated for the measurement of MAP and HR. The cannula was filled with heparinized saline and connected with CARDIOSYS CO-101 (Experimentria, Hungary) using a pressure transducer. A wide bore (1.5 mm) sterile metal cannula was inserted into the cavity of the left ventricle from the posterior apical region of the heart for recording LVEDP, (+)LVdP/dt and (–)LVdP/dt. The cannula was connected to a pressure transducer (Gould Statham P23ID, USA) through a pressure-recording catheter on Grass Polygraph 7D, USA.

Biochemical studies

Hearts stored in liquid nitrogen were brought to room temperature and weighed. A 10% homogenate was prepared in 50 mM phosphate buffer, pH 7.4 and an aliquot was used for the assay of MDA according to the method described by Okawa *et al*. [9]. The homogenate was centrifuged at 7000 rpm for 15 min and the supernatant thus obtained was used for the estimation of the following biochemical parameters: LDH [10], GSH [11], GSHPx [12], SOD [13], CAT [14] and protein [15]. CPK was estimated spectrophotometrically using a kit from Randox Laboratories, USA [16].

Histopathological studies

Myocardial tissue at the end of the experiment was immediately fixed in 10% buffered neutral formalin solution. The

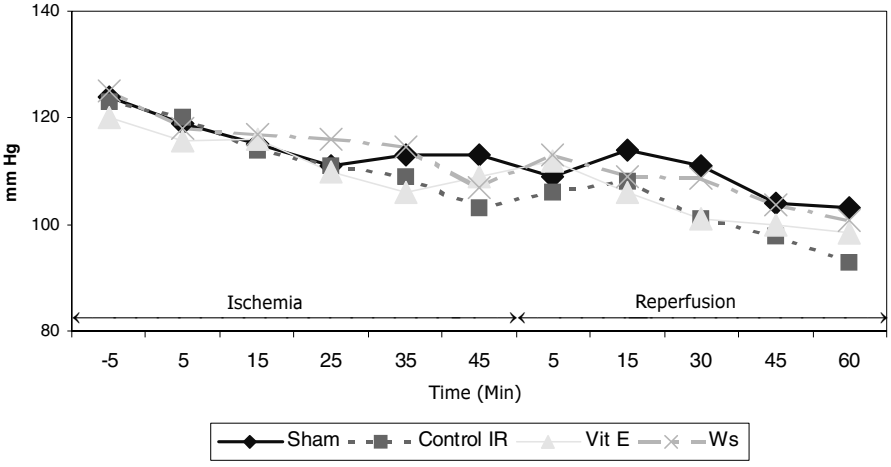

Fig. 1. Time course of change in MAP during ischemia-reperfusion injury in rats. Values are mean ± S.D. of 8 experiments.

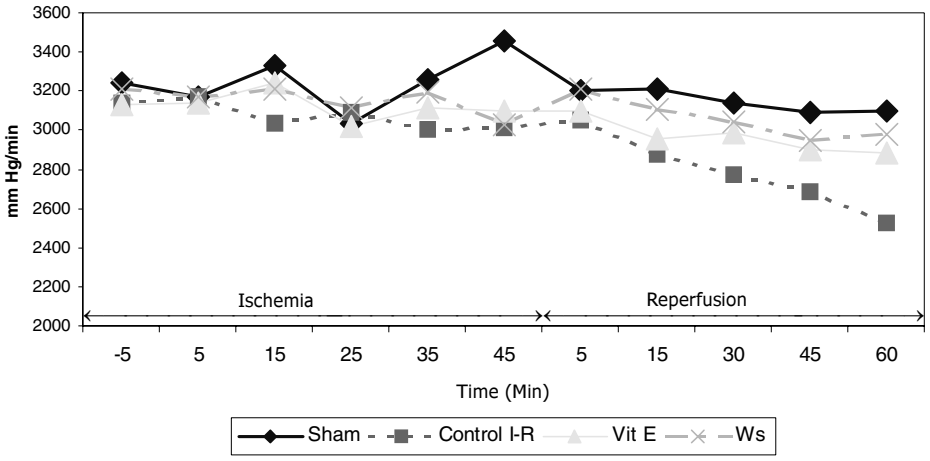

Fig. 2. Time course of change in HR during ischemia-reperfusion injury in rats. $^{\#}p < 0.05$ vs. sham. Values are mean ± S.D. of 8 experiments.

fixed tissues were embedded in paraffin and serial sections were cut. Each section was stained with hematoxylin and eosin. The sections were examined under light microscope (Nikon, Tokyo, Japan) and photomicrographs were taken.

Statistical analysis

Descriptive statistics such as mean and standard deviation were calculated for each and every variable for each group. In the ISP model, one-way analysis of variance (ANOVA) was applied for statistical analysis with *post hoc* analysis (Bonferroni Multiple Range Test) and p value < 0.05 has been considered as statistical significance level. Student's *t*-test was applied for statistical analysis in the ischemia and reperfusion model.

Results

Isoproterenol model of myocardial necrosis

A significant decrease in GSH ($p < 0.05$), activities of SOD, CAT, LDH and CPK ($p < 0.01$) as well as an increase in MDA

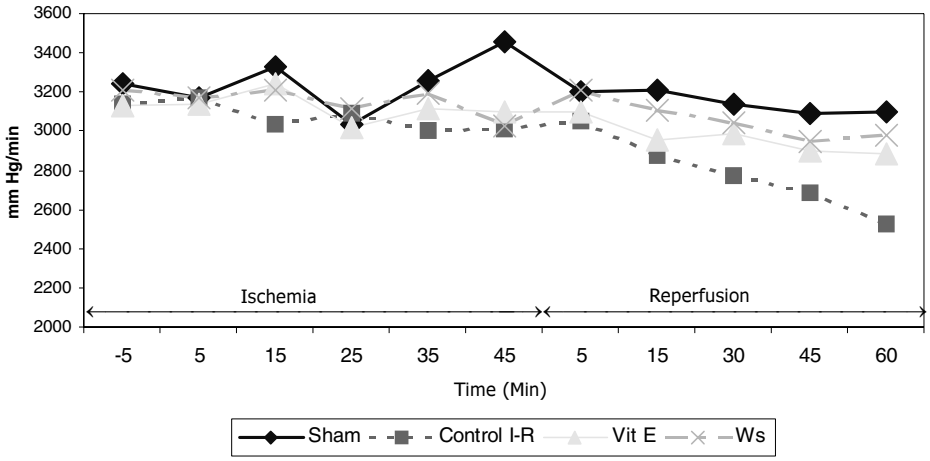

Fig. 3. Time course of change in (+)LVdP/dt during ischemia-reperfusion injury in rats. Values are mean ± S.D. of 8 experiments.

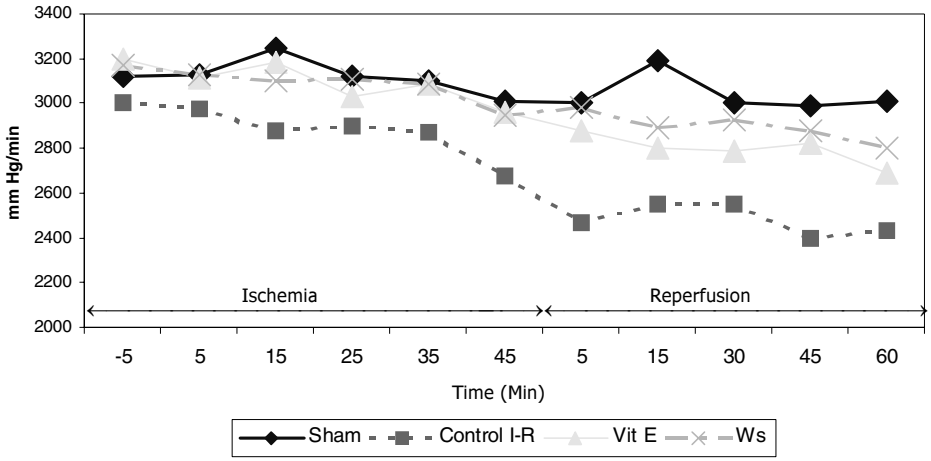

Fig 4. Time course of change in (-)LVdP/dt during ischemia-reperfusion injury in rats. $^{\#}p < 0.05$, $^{\#}p < 0.01$ vs. sham; $^{*}p < 0.05$,$^{**}p < 0.01$ vs. control I-R. Values are mean ± S.D. of 8 experiments.

level ($p < 0.01$) were observed in the control group as compared to sham group. However, the changes in levels of protein and GSHPx were not significant. A slight decrease in MAP, HR, (+)LVdP/dt, (–)LVdP/dt and marked elevation in LVEDP compared to sham group was also observed in the control group. Histopathological examination had further confirmed myocardial damage in this group. All the three doses of Ws significantly reversed myonecrosis, augmented endogenous antioxidants and restored most of the hemodynamic parameters except MAP. Among the various doses used, Ws at 50 mg/kg provided maximum cardioprotection in the study and therefore the same dose was subsequently used in the *in vivo* model of ischemia and reperfusion myocardial injury.

Ischemia reperfusion model of myocardial injury

Hemodynamic variables

In the control group, a continuous and significant fall in MAP was observed 25 min after coronary artery ligation and throughout the reperfusion period compared to sham group (Fig. 1). Similarly, the heart rate was significantly depressed throughout the experimental duration in the control group compared

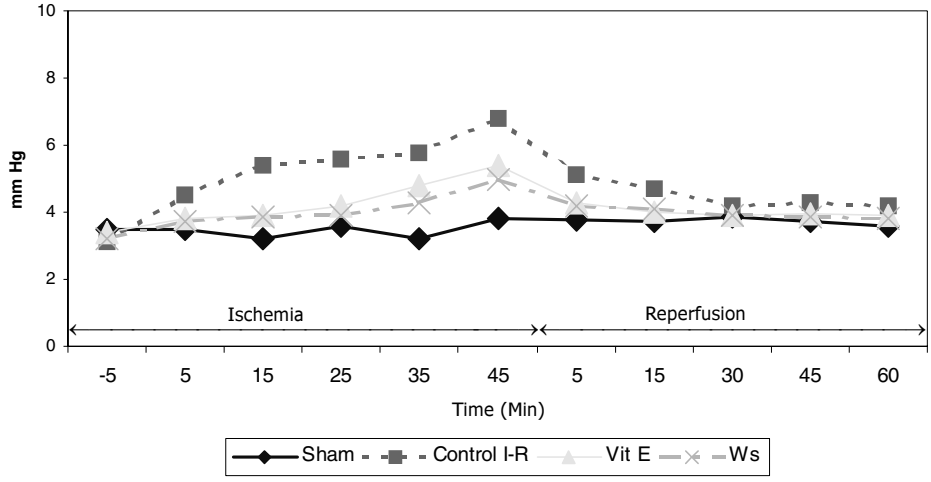

Fig 5. Time course of change in LVEDP during ischemia-reperfusion injury in rats. Values are mean ± S.D. of 8 experiments.

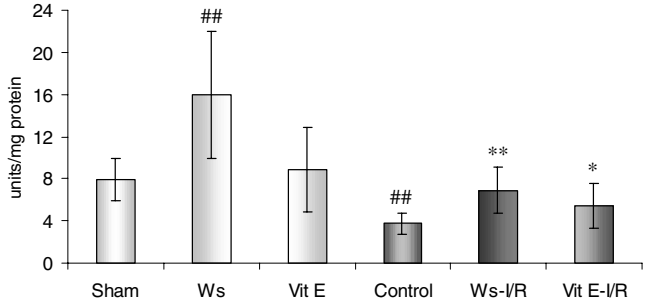

Fig 6. Changes in myocardial SOD activity after ischemia-reperfusion induced injury. $^{\#\#}p < 0.01$ vs. sham; $^{*}p < 0.05$,$^{**}p < 0.01$ vs. control. Values are mean ± S.D. of 8 experiments. One unit of SOD inhibits the rate of auto-oxidation of epinephrine by 50% at pH 7 at 25°C.

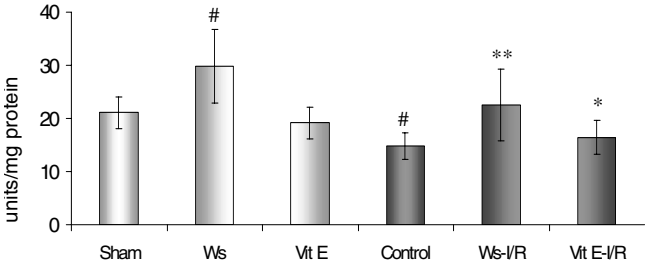

Fig 7. Changes in myocardial CAT activity after ischemia-reperfusion induced injury. $^{\#}p < 0.05$ vs. sham $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. Values are mean ± S.D. of 8 experiments. One unit of CAT activity represents amount of enzyme required to decompose 1 umol of H_2O_2/min.

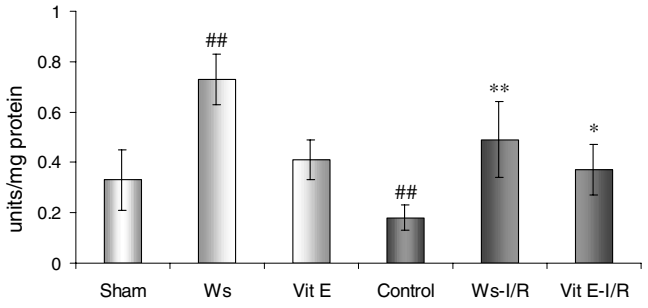

Fig 8. Changes in myocardial GSHPx activity after ischemia-reperfusion induced injury. $^{\#\#}p < 0.01$ vs. sham; $^{*}p < 0.05$,$^{**}p < 0.01$ vs. control. Values are mean ± S.D. of 8 experiments. One unit of GSHPx activity is defined as amount of enzyme required to utilize 1 nmol of NADPH/min at 25°C.

to sham (Fig. 2). The rats in the Vit E and Ws treated groups did not significantly restore MAP and HR as compared to control group at different time course of the experimental protocol.

A slight but non-significant fall in both (+)LVdP/dt and (–) LVdP/dt were recorded during ischemia. However it decreased significantly in the reperfusion period (Figs 3 and 4). Nonetheless, observed fall in diastolic function was more marked as compared to the decline in the systolic function. Both these drugs significantly improved these hemodynamic parameters as compared to control group.

A significant elevation in LVEDP marked the onset of ischemia and remained elevated throughout the ischemic period (Fig. 5). Although on reperfusion, there was a marked decline in LVEDP values, but it still remained slightly increased compared to the sham control group. A significant correction in LVEDP was seen in the Vit E and Ws treated groups compared to control group.

Biochemical parameters

Effect of Ws on antioxidant parameters. A significant decrease in GSH levels (Fig. 6) as well as in the activities of SOD, CAT (Table 1) and CPK (Fig. 8) and an increase in MDA level were observed in the control group as compared to sham group. However, Vit E and Ws treatment resulted in a significant repletion of these biochemical markers compared to the control group. A marked restoration in GSH content, antioxidant enzymes {GSHPx and SOD} and myocardial enzyme CPK was observed in both the treated group as compared to control group. Vit E and Ws also markedly reduced lipid peroxidation as evidenced by reduction in MDA levels as compared to control (Figs 6, 7, 8 and Table 1).

Myocardial adaptogenic property. In the present study chronic administration of Ws resulted in a concomitant increase ($p < 0.05$) in GSH, GSHPx, CAT along with SOD activity with Ws-50 mg/kg groups. However, no significant increase in the levels of any of the endogenous antioxidants was observed on treatment with Vit E. Thus, in the present study Vit E did not exhibit any adaptogenic property (Figs 6, 7, 8 and Table 1).

Histopathological assessment

On histopathological examination, control group showed myocardial membrane damage and infiltration of inflamma-

Table 1. Changes in myocardial GSH, MDA and CPK levels after ischemia-reperfusion induced injury in rats

	Sham	Control	Ws	Ws I/R	Vit E	Vit E I/R
CPK (units/mg protein)	162 ± 27.3	91.2 ± 8.89$^{\#}$	158 ± 23.3	118 ± 10.1**	170 ± 19.9	105 ± 14.8 **
MDA (nmol/g tissue)	63.1 ± 13.1	79.1 ± 7.36$^{\#}$	59.8 ± 10.1	62.5 ± 11.9*	53.2 ± 6.4	60.4 ± 12.2*
GSH (umol/g tissue)	1.86 ± 0.69	0.6 ± 0.01$^{\#\#}$	2.94 ± 0.43$^{\#\#}$	1.02 ± 0.11**	1.91 ± 0.26	0.79 ± 0.41

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Sham; $^{*}p < 0.05$,$^{**}p < 0.01$ vs. Control. Values are mean ± S.D. of 8 experiments. One unit of CPK will transfer 1 umol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30°C.

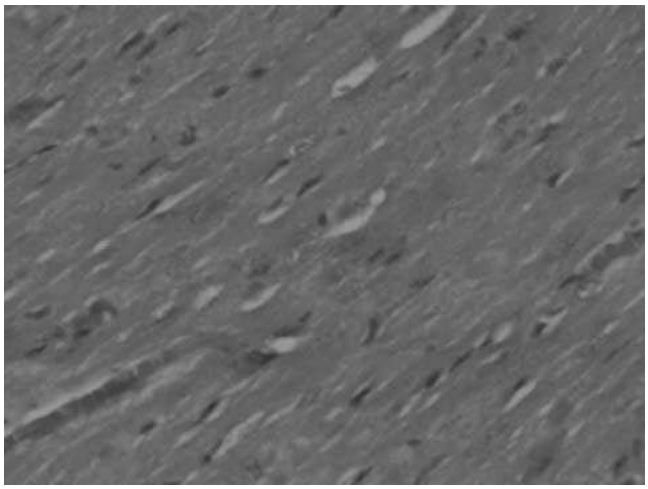

Plate 1. Photomicrograph showing normal architecture of rat heart of sham control group. Endocardium and pericardium are seen within normal limits with no inflammatory cells. (H&N; ×100).

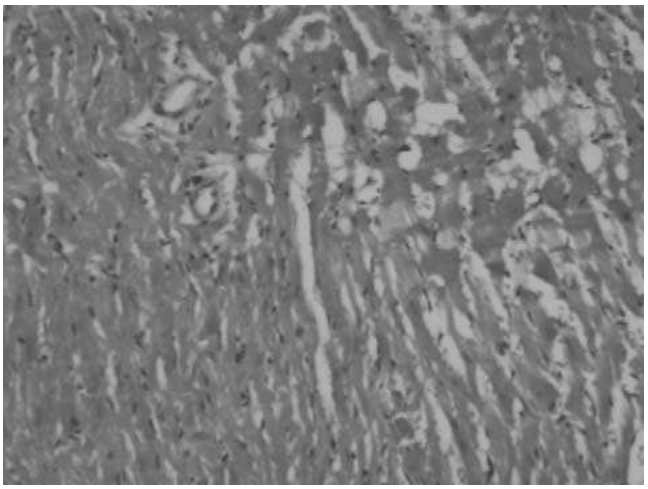

Plate 2. Photomicrograph of rat heart of the pilot study group subjected to ischemia reperfusion injury showing areas of focal myonecrosis, edema with fibroblastic proliferation. In subendocardium vacuolar changes and prominent edema along with chronic inflammatory cells are clearly visible (H&N; ×100).

tory cells as compared to those in sham control group. Significant myonecrosis with fibroblastic proliferation and presence of chronic inflammatory cells were observed in the control group (Plate 2) compared to that of sham control (Plate 1). Both Ws and Vit E showed a marked improvement in the degree of myonecrosis, reduced infiltration of inflammatory cells and lesser vacuolar changes as well as edema compared to the control (Plates 3 and 4).

Discussion

Myocardial ischemia, as a result of coronary occlusion results in cellular changes, which are initially reversible. However ischemia, of sufficient severity progresses to irreversible myocardial damage. Early reperfusion using techniques such as thrombolysis, angioplasty, and coronary bypass surgery has clearly shown to be the most effective means to prevent car-

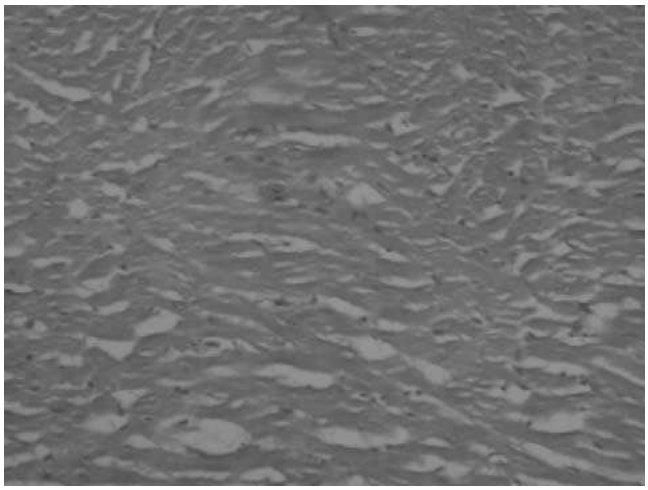

Plate 3. Photomicrograph of rat heart of the Vitamin E treated group administered 100 mg/kg showing decreased degree of necrosis, edema and less infiltration of inflammatory cells. Scattered areas of focal myonecrosis are visible (H&N; ×100).

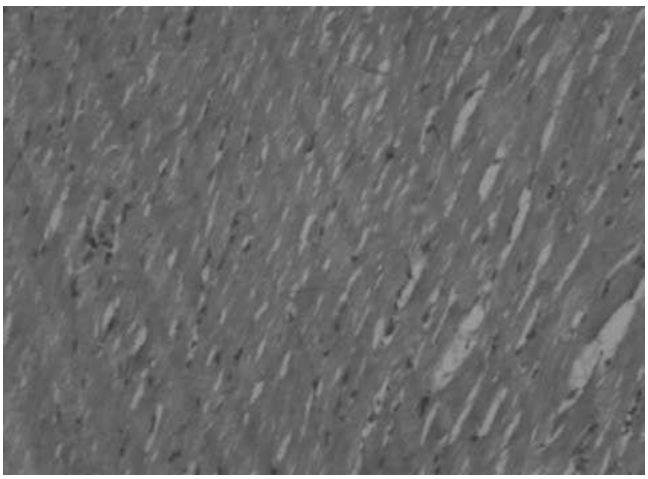

Plate 4. Photomicrograph of rat heart of the Withania somnifera treated group administered 50 mg/kg showing clear reversal of myocardial membrane damage in terms of decreased degree of necrosis and lesser infiltration of inflammatory cells (H&N; ×100).

diac cell death. Several experimental studies have provided compelling evidence that reperfusion, although essential for tissue and/or organ survival, is not without risk due to extension of cell damage as a result of reperfusion itself [17, 18]. Keeping this in view, studies on reperfusion injury are of prime importance for understanding the pathogenesis of post-ischemic dysfunction, and also to develop effective therapeutic strategies in patients that cannot be treated before ischemia.

Myocardial reperfusion generates free radicals that can damage cardiac cells and mimic the pathological features of ischemia-reperfusion injury [19]. Therefore, pharmacological intervention that scavenges free radicals may have cardioprotective potential. Free radicals are generated during reperfusion from different sources viz, the enzyme xanthine dehydrogenase/oxidase system, NADPH oxidase system located in polymorphonuclear leukocytes, leakage from mitochondrial electron transport chain, auto-oxidation of catecholamines, free metal ion catalyzed electron transfer reactions and during the metabolism of arachidonic acid [20]. The cytotoxic free radicals cause the loss of membrane integrity with disintegration of polyunsaturated fatty acids in the membrane bilayer and exert unfavourable effects on the heart structure and function. We have also observed an increase in the levels of MDA, a marker of lipid peroxidation in the heart tissue following I-R injury. On the contrary, Ws and Vit E treatment demonstrated decreased level of lipid peroxides and this could be imparted due to reduced formation of lipid peroxides from fatty acids.

In our study along with increased lipid peroxidation, I-R induced injury was found to reduce the content of GSH as well as antioxidant enzyme levels (SOD, CAT, GSHPx) in cardiac tissue and this observation concurs with earlier findings [21]. The fall in the activity of GSHPx in the I-R control group might be correlated to decrease availability of its substrate, reduced GSH. Due to depletion in antioxidant levels, the free radicals are not neutralized and myocardium shows enhanced susceptibility to lipid peroxidation. Besides alteration in the antioxidant parameters, change in CPK activity has been considered as one of the important markers of myocardial infarction [21]. In the present study, a significant myocardial depletion of CPK was observed. This may be due to increased myocardial permeability and dysfunction as a result of sequence of biochemical alteration such as increased calcium overload, degradation of phospholipid, reduction of creatine phosphate level and perhaps free radical generation and free fatty acid release. The observation that Vit E and Ws treatment significantly restores the marker enzymes activity of CPK compared to I-R control suggests the protective effect of the these drugs on the myocardium.

Besides these, hemodynamic parameters were incorporated into the experimental design for better understanding of the correlation between functional and biochemical changes in the myocardium subjected to I-R induced myocardial injury. Exposure of the hearts to an oxidation stress has also shown to depress left ventricular functions as well as blood pressure and the use of antioxidants have shown to reverse these hemodynamic alterations [21, 22].

In the present study, a significant fall in MAP, HR, (+) LVdP/dt, (–)LVdP/dt and a marked elevation in LVEDP were observed. The (–)LVdP/dt was significantly depressed indicating a diastolic dysfunction. Deteriorating myocardial contractile status following I-R induced injury might be responsible for the significant fall in MAP. In addition, absence of positive chronotropic effect in the face of a reduced MAP suggests impairment of conduction (A-V block) of the heart following I-R induced injury. Normally, a fall in MAP is expected to reflexively increase HR and myocardial contractility. However, none of these effects have been observed in the study due to I-R induced injury to inotropic and chronotropic function of the heart.

Ws and Vit E appeared to preserve left ventricular function as evidenced by significant improvement of inotropic and lusitropic state viz, (+)LVdP/dt and (–)LVdP/dt and correction of elevated LVEDP at various time intervals during the entire experimental protocol. However, these drugs at the doses used did not have significant affect on the MAP and HR during the ischemic period. Cardioprotection afforded by Ws cannot be explained by the hemodynamic variables as these drugs did not have significant effect on hemodynamic variables heart rate and MAP that determine myocardial O_2 demand.

A concept is now emerging of 'adaptogenic drugs', drugs that increase non-specific resistance of the users to a variety of stresses, first time reported by Brehman *et al.* in *Eleuthrococcus* and *Panax genseng* [23]. The definition of adaptogen is based on the following according to Brekhman: (1) Safety of the adaptogen's action on the organism; (2) a wide range of regulatory activity, but manifesting its action only against the actual challenge to the system; (3) act through a nonspecific mechanism to increase the non-specific resistance (NSR) to harmful influences of an extremely wide spectrum of physical, chemical and biological factors causing stress and has a normalizing action irrespective of the direction of forgoing pathological changes. Adaptogenic property of various herbs like *Ocimum sanctum*, *Bacopa monniera* and *Withania somnifera* has already been reported in various experimental studies [24, 25]. These herbs allow one to adapt to a variety of heightened stressful circumstances. Although the exact mechanism of such adaptation is not known, it has been proposed that these drugs may act by inducing a number of antioxidant enzymes (SOD, CAT, GSHPx) and antioxidants such as GSH, proteins like heat shock protein (HSP) in the heart. Present study demonstrated the adaptogenic property of Ws. On chronic treatment, Ws resulted in a concomitant increase in GSH, GSHPx, CAT along with SOD activity. Increase in antioxidant levels following chronic Ws use might considerably improve

its defense against oxidative stress. In contrast to Ws, Vit E in the present study did not exhibit any such adaptogenic activity. No significant increase in the levels of any of the endogenous antioxidants (GSH, GSHPx, CAT and SOD) was observed in the Vit E treated group. Importantly, nevertheless like Ws, it demonstrated cardioprotective effects in the present study.

Hemodynamic, biochemical and histopathological results in the ischemia and reperfusion model of MI emphasize the beneficial action of Ws as a cardioprotective agent. The study provides scientific rationale of the use of Ws in Ayurveda, the ancient Indian system of medicine as Maharasayana. However, further studies need to be carried out to ascertain whether these results can be reproduced in humans. In view of the safety, efficacy and traditional acceptability of Ws, well-controlled prospective clinical trials of Ws should be contemplated to establish its efficacy in the treatment of ischemic heart diseases.

Acknowledgements

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