Antiapoptotic and cardioprotective effects of a herbal combination in rats with experimental myocardial infarction

Ipseeta Mohanty*, Suresh Kumar Gupta, Dharamvir Singh Arya

Departments of Pharmacology, All India Institute of Medical Sciences, New Delhi, India

Submitted: 12 Dec. 2007; Accepted: 22 Dec. 2007

Abstract
The efficacy of the combination of herbal extracts of Ocimum sanctum, Withania somnifera and Curcuma longa (HCB) to limit myocardial injury in an open chest left anterior descending coronary artery (LAD) ischemia and reperfusion (I-R) experimental model was evaluated. Wistar albino rats were divided into four groups and orally fed saline once daily (sham, control IR) or HCB (HCB, HCB-IR) groups respectively for 1 month. On the 31st day the rats of the Control IR and HCB-IR groups LAD was occluded for 45 min, and reperfused for 1h. Hemodynamic parameters were recorded and subsequently sacrificed for biochemical, immunohistochemical and pathological studies. In the control IR group, significant ventricular dysfunction, cardiac necrosis, apoptosis; decline in antioxidant status and elevation in lipid peroxidation. HCB treatment significantly reduced the surrogate preload marker LVEDP and improved both inotropic and lusitropic function of the heart as compared to sham. HCB significantly increased GSH content and SOD, CAT, GSHPx (p<0.001) activity, decreased level of TBARS (p<0.01) as compared to control IR group. Most importantly, HCB decreased Bax (p<0.01), upregulated Bcl-2 (p<0.001) expression and attenuated TUNEL positivity (p<0.01). Cardioprotection by HCB treatment may be attributed to its favorable hemodynamic effects, myocardial adaptogenic, antioxidant and antiapoptotic properties.

Keywords: Herbs, Ischemia, Reperfusion, Apoptosis, Withania somnifera, Curcuma longa, Ocimum sanctum

INTRODUCTION
Myocardial ischemia initiated by occlusion or blockade of a major coronary artery leads to a complex series of cellular events that can result in myocardial cell death. While, as demonstrated by the thrombolytic therapy (i.e streptokinase, tissue plasminogen activator), prompt reperfusion of ischemic myocardium relieves or at least greatly reduces ischemia and the morbidity and mortality associated with an acute myocardial infarction (Hearse et al., 1992; Opie, 1989). Despite remarkable advances in medical therapy and revascularization procedures, free radical mediated injury is a potential threat to viable myocardium, and this may deny the patient the full benefit of reperfusion (Becker et al., 1987). In this context, there is a need to study the relationship of oxyradicals with left ventricular function along with their myocardial levels. Furthermore, oxidative stress is a major apoptotic i.e. programmed cell death, stimulus in myocardial ischemia and reperfusion among other cardiovascular diseases (Buttke et al., 1994). The progressive loss of cardiomyocytes by apoptosis in a heart that is already compromised poses an additional workload on the remaining viable myocytes that may be unbearable; leading to further deterioration of cardiac function and resulting in activation of pathological death signal pathways (Haunstetter et al.,1998; Maulik et al., 1998). It has been reported that these programmed cell death pathways can be inhibited by antioxidants (Galan, 2000). However, there are few studies addressing the inhibition of apoptosis and its direct effects on myocardial contractility. Modification of reperfusion can be achieved with the understanding of the above-mentioned
Cardioprotective effects of a herbal combination

factors; this will define potential benefits of adjuvant pharmacologic agents in modifying the process of reperfusion.

Nature has been a source of medicinal treatments for thousands of years and plant-derived products continue to play an essential role in the primary health care of about 80-85% of the world's population. Recently, the keen interest in medicinal plants for cardioprotection has increased because of their numerous possible cardioprotective mechanisms besides antioxidant activity (Ai et al., 2002). Hence, these herbal extracts traditionally used need to be evaluated scientifically with an aim to define the role of these agents in limiting the deleterious affects of myocardial ischemia and reperfusion injury by providing scientific data to validate their use as prophylactic approaches or as an adjunct to standard treatment (synthetic compounds employed in conventional treatment protocols) of myocardial ischemic reperfusion injury.

Curcuma longa (Cl), common Indian dietary pigment and spice has been shown to possess a wide range of therapeutic utilities in the traditional Indian medicine. It’s role in wound healing, urinary tract infections, liver ailments are well-documented (Dixit et al., 1998). The active component of turmeric identified as curcumin exhibits a variety of pharmacological effects including antioxidant, adaptogenic, anti-inflammatory and anti-infectious activities (Dikshit et al., 1995). Withania somnifera (Ws) most commonly known as Ashwagandha belongs to the natural order Solanaceae. The root of Ws contains withanolides and has been valuable drug in Ayurveda, the ancient Indian system of medicine. Although its therapeutic potential on account of its immunomodulatory, adaptogenic, antioxidant, hypoglycemic and anticancer activities are reported, very few studies assessing its cardioprotective potential are presently available (Archana et al., 1999; Dhuley, 2000). 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) (Gupta et al., 2002). The ancient systems of medicine including Ayurveda, Greek, Roman, Siddha and Unani, have mentioned its therapeutic applications in cardiovascular disorders, diabetes and asthma (Devi et al., 1999). However, only few studies are presently available that documents its cardioprotective potential.

Therefore, with the point of view that it might be interesting and possibly fruitful to study the effects of combination of herbal extracts of Ocimum sanctum, Withania somnifera and Curcuma longa (widely used in Ayurveda and Unani Systems of Medicine) in the setting of ischemic reperfusion injury, the present investigation was planned to unravel the cardioprotective potential of these time tested herbal drugs. In addition, to understand the underlying mechanism of their beneficial therapeutic effects, various hemodynamic, immunohistochemical and biochemical parameters were studied. The effect of the herbal extract combination on modulation of biochemical parameters: lipid peroxidation product thiobarbituric acid reactive substances (TBARS), endogenous antioxidant: glutathione (GSH), antioxidant enzymes {superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPxs)} and myocardial enzyme creatine phosphokinase (CPK) were evaluated. To correlate the biochemical derangement and altered cardiac performance during myocardial ischemia and reperfusion changes in the hemodynamic variables were also monitored in the present study. Alterations in mean arterial pressure (MAP), heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular peak positive (+) LVdP/dt (rate of pressure development) and negative (-) LVdP/dt (rate of pressure decline) were monitored and recorded at preset time points throughout the experimental period (1 hour 45 minutes). Cardioprotective action of this herbal combination was confirmed by assessing the severity of pathological changes. In addition, to understand the molecular mechanism by which oxidative stress causes cell death, the relative involvement of necrosis and apoptosis in cardiac ischemia and reperfusion was evaluated and anti-apoptotic activity of these herbs was investigated using immunohistochemical localization of Bax and Bcl-2 proteins and TUNEL staining.

MATERIALS AND METHODS

Experimental Animals

Adult male Wistar rats, 10 to 12 weeks old, weighing 150 to 200g were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conforms with the Indian National Science
Cardioprotective effects of a herbal combination

Academy Guidelines for the Use and Care of Experimental Animals in Research. Animals were obtained from the Central Animal Facility of All India Institute of Medical Sciences, New Delhi, India and were maintained under standard laboratory conditions in the department animal house.

Chemicals

All Chemicals were of analytical grade, purchased from Sigma Chemical Co., St Louis, USA. Hydro-alcoholic lyophilized extracts of Ocimum sanctum and Withania somnifera was procured from Dabur Research Foundation, and aqueous extract of Curcuma longa was obtained from Sanat Research Laboratories, India. The ABC staining kit and primary (Bax mouse monoclonal IgG2b and Bcl-2 mouse monoclonal IgGI) & secondary antibodies (Anti mouse IgG) were procured from Santa Cruz Biotechnology, USA. TUNEL assay kit was purchased from Roche Diagnostics, USA. Double distilled water was used in all biochemical assays.

Treatment protocol

The animals were randomly divided into four main groups comprising of thirteen animals each.

Group 1: Saline control group - Sham group

Rats were administered 0.9% normal saline for a month and then sacrificed on the 31st day. The animals were subjected to the entire surgical procedure and thread was passed beneath the coronary artery but the LAD coronary artery was not ligated.

Pilot Study

Ws at the doses of 25, 50 & 100 mg/kg; Cl at the doses of 25, 50, 100 & 200 mg/kg and Os at the doses of 25, 75 & 150 mg/kg were screened in the murine model of isoproterenol induced myocardial necrosis and the optimum dose exhibiting maximum cardioprotective effect was evaluated. Ws (50 mg/kg), Cl(100 mg/kg) and Os(75 mg/kg) doses respectively were found to be the most effective in functional recovery of the heart and favorable restoration of biochemical and histopathological alterations. Hence, these doses were selected for further evaluation singularly as well as in combination in the ischemia and reperfusion model of myocardial injury.

Group 2: Herbal combination control group-HCB

Hydro-alcoholic extract of the herbal combination of Withania somnifera (50 mg/kg) + Curcuma longa (100 mg/kg) + Ocimum sanctum (75 mg/kg) {HCB} was dissolved in normal saline and administered orally to healthy experimental rats once daily for 1 month.

Group 3: Ischemia and reperfusion group - Control IR

In this group, healthy experimental rats were administered 0.9% normal saline for 30 days; thereafter, on the 31st day, the experimental animals were subjected to 45 min LAD coronary artery ligation and 60 min reperfusion induced myocardial injury.

Group 4: Herbal drug combination treated group - HCB-IR

Hydro-alcoholic extract of HCB was administered orally to healthy experimental animals for 30 days and on 31st day, the rats were subjected to a protocol of 45 min LAD ligation and 60 min reperfusion.

Experimental protocol used in the present study

Surgical procedure: infarction protocol and hemodynamic studies

Rats of all the experimental groups were anesthetized intraperitoneally with pentobarbitone sodium (60 mg/kg). Atropine was co-administered with the anesthetic to keep the heart rate elevated especially during the surgery protocol and reduce broncho-tracheal secretions. The body temperature was monitored and maintained at 37°C throughout the experimental protocol. The neck was opened with a ventral midline incision, and a tracheostomy was performed and the rats were ventilated with room air from a positive pressure ventilator (Inco, India) using compressed air at a rate of 70 strokes/min and a tidal volume of 10ml/kg. The right carotid artery was cannulated and the cannula filled with heparinized saline was connected to the cardiac output monitor CARDIOSYS CO-101 (Experimetria, Hungary) via a pressure transducer for measurement of MAP and HR. The left jugular vein was cannulated with polyethylene tube for continuous infusion of 0.9% saline solution. A left
Cardioprotective effects of a herbal combination

thoractomy was performed at the fifth intercostal space and the pericardium was opened to expose the heart. The left anterior descending coronary artery (LAD) was ligated 4-5 mm from its origin by a 5-0 silk suture with atraumatic needle and ends of this ligature were passed through a small vinyl tube to form a snare. After the completion of the surgical procedure, the heart was returned to its normal position in the thorax. The thoracic cavity was covered with saline-soaked gauze to prevent the heart from drying. The animals were then allowed to stabilize for 15 min before LAD ligation. Myocardial ischemia was induced by one stage occlusion of the LAD by pressing the polyethylene tubing against the ventricular wall and then fixing it in place by clamping the vinyl tube with a hemostat. 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. The cannula was connected to a pressure transducer (Gould Statham P231D) and the whole system was filled with heparinized saline (heparin 50 units/ml). Left ventricular systolic and LVEDP was measured on a multichannel polygraph (Grass 7D, USA) from the left ventricular pressures curve at lower and higher sensitivity of the preamplifier respectively. The maximal rate of rise and fall of left ventricular pressure {peak (+) LVdP/dt and peak (-) LVdP/dt} were measured by the electronic differentiator from the signal output of the channel recording left ventricular pressure. A bolus of heparin (30 IU) was administered immediately before coronary artery occlusion for prophylaxis against thrombus formation around the snare. The animals then underwent 45 min of ischemia, confirmed visually in situ by the appearance of regional epicardial cynosis and ST-segment elevation. The myocardium was reperfused by releasing the snare gently for a period of 60 min. Successful reperfusion was confirmed by visualization of arterial blood flow through the artery, appearance of hyperemia over the surface of the previously ischemia cotic segment. At the end of reperfusion period, animals were sacrificed for biochemical, immunohistochemical and histological studies by an overdose of anesthesia.  

Biochemical studies

Hearts stored in liquid nitrogen were brought to room temperature and weighed. A ten-percent homogenate was prepared in 50mM phosphate buffer, pH 7.4 and an aliquot was used for the assay of MDA according to the method described by Okawa et al., 1979. The homogenate was centrifuged at 7000 rpm for 15 min and the supernatant was used for the estimation of the following biochemical parameters: GSH (Moron et al., 1979); GSHPx (Paglia et al., 1967), SOD(Mishra et al., 1967), CAT (Aebi, 1974) and protein (Lowry et al., 1951). CPK was estimated spectrophotometrically using a kit from Randox Laboratories, USA (Lamprecht. 1974).

Histopathological studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. Cross sections (5 μm thick) of the fixed myocardial tissues were cut. These sections were stained with hematoxylin and eosin (H&E). The investigators performing the histologic evaluation were blind to biochemical and hemodynamic results and to treatment allocation. The degree of necrosis was graded and scored.

Apoptotic studies

Immunostaining for the Localization of Bax and Bcl-2 proteins

The ImmunoCruz Staining Systems utilizes a horseradish peroxidase (HRP)-streptavidin complex for staining of formalin-fixed paraffin-embedded myocardial sections (Palojoki et al., 2001). Briefly, 4-6 micron thick fixed paraffin tissue sections were subjected to the following immunohistochemical procedure for the localization of Bax and Bcl-2 proteins using specific mouse monoclonal primary antibodies. Sections are first blocked, then incubated in primary antibody. Biotinylated secondary antibody is added followed by the addition of HRP-Straptavidin complex. The target protein (Bax/Bcl-2) was visualized by incubation in peroxidase substrate (H2O2) using DAB (3,3’ diaminobenzidine) as the chromogen.

Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL Assay)

The signal of terminal deoxynucleotidyl tranferase-mediated dUTP nick end labeling (TUNEL) assay was used to identify apoptotic cells using secondary reaction with antibodies and DAB chromogen. The slides were counterstained in hematoxylin and total cell counts and TUNEL positive cells in the
Cardioprotective effects of a herbal combination specimens were determined by means of a light microscope (Misao et al., 1996). The cells with clear nuclear labeling were defined as TUNEL positive cells. The apoptotic cells i.e. TUNEL positive cells were expressed as percentage of normal nuclei.

Statistical Analysis

All numerical data in text, figures and tables are expressed as the mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) or repeated measures ANOVA when data were compared at different time points within a study group and for time courses between study groups, followed by the Bonferroni post hoc test. Differences were considered statistically significant at p<0.05.

RESULTS

Effect of herbal drug combination (HCB) on

Hemodynamic variables

Figure 1: Time course of changes in MAP during myocardial I-R. The values are expressed as mean ± SD. Each value represents a mean of six readings. *p<0.05, **p<0.01 vs Control IR

The initial value of MAP in the HCB treated group was 129.6 ± 8.9 mm Hg and occlusion of the LAD coronary artery brought about a slight fall in the value of this variable (Fig. 1). HCB treatment did not significantly improve MAP and during the entire ischemic duration MAP remained more or less in the same range as compared to control IR values. Nevertheless on reperfusion there was a recovery in MAP and it reached statistically significant (p<0.05) values at the end of the reperfusion duration as compared to control IR values.

Figure 2: Time course of changes in HR during myocardial I-R. The values are expressed as mean ± SD. Each value represents a mean of six readings. *p<0.05, **p<0.01 vs Control IR

Initial value of HR in the HCB treated group was 334 ± 23.6 beats/min (Fig. 2). HCB combination failed to significantly restore HR during the ischemic duration and it remained more or less in the same range as compared to control IR value. After reperfusion the value of HR was significantly corrected at 45 and 60 min (p<0.05) of reperfusion compared to control IR group values at the similar time points.

Figure 3: Time course of changes in (+)LVdP/dt during myocardial I-R. The values are expressed as mean ± SD. Each value represents a mean of six readings. **p<0.01, ***p<0.001 vs Control IR

The baseline value of (+)LVdP/dt was 3141 ± 323 mm Hg/s (Fig. 3). Treatment with the HCB afforded no significant beneficial effect on contractility of the left ventricle during the initial 35 min of ischemia, however at 45 min of LAD occlusion, (+)LVdP/dt was significantly improved compared to control IR values. Thereafter, in the HCB group, during the entire period of reperfusion (+)LVdP/dt showed a steady improvement as it reached statistically significant (p<0.001) values at 60 min of reperfusion compared to control IR values.
Cardioprotective effects of a herbal combination

The initial baseline value of (-)LVdP/dt in the HCB treated group was 3106 ± 321 mm Hg/s (Fig. 4). The HCB treatment exerted a modest correction to the decreasing trend seen in the control IR group and the (-)LVdP/dt was nearly stable throughout the period of ischemia. However, (-)LVdP/dt was not significantly corrected at specific time points when compared to control IR value. Reperfusion of the ischemic myocardium resulted in a significant correction of (-)LVdP/dt at 5 min (p<0.01), 15, 30 min (p<0.05) and 60 min (p<0.01) as compared to control IR values at similar time points.

The LVEDP value in the HCB treated group before coronary artery occlusion was 3.4 ± 0.81 mm Hg (Fig. 5). Treatment with the HCB caused a significant correction of this hemodynamic variable after the initial 15 min of ischemia {15 min (p<0.01), 25 min (p<0.01), 35 min (p<0.05) and 45 min (p<0.001) and at 5 min (p<0.01) post reperfusion as compared to control IR values at the same time points.

Biochemical parameters

without I-R induced myocardial injury

The HCB intake per se resulted in enhanced myocardial antioxidant reserve. A significant increase in the basal antioxidant enzyme activities of SOD, CAT and GSHPx (p<0.01) was observed in reference to sham (Table 1). In addition, marked reduction in lipid peroxidation as evidenced by fall in basal TBARS level (p<0.05) was observed in this group in comparison to sham. However, no effect on basal GSH levels and CPK activity was seen on oral feeding of HCB for 1 month (Table 1).

following I-R induced myocardial injury

The HCB treatment resulted in a significant repletion of these biochemical markers compared to the control IR group. A marked restoration in the level of GSH (p<0.001), antioxidant enzymes {GSHPx, SOD & CAT (p<0.001, Table 1} and myocardial enzyme CPK (p<0.001) was observed as compared to control IR group. This combination also markedly reduced lipid peroxidation as evidenced by significant reduction in TBARS levels (p<0.001) as compared to control IR (Table 1).

Histopathological results

Microscopic histology revealed that the non-infarcted myocardium in the sham group is characterized by an organised pattern and shows normal architecture of the myocardium.
Cardioprotective effects of a herbal combination

Table 1: Biochemical parameters in the different groups without I-R

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HCB</th>
<th>Control-ir</th>
<th>HCB-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>1.86 ± 0.56</td>
<td>1.92 ± 0.35</td>
<td>0.60 ± 0.19</td>
<td>1.13 ± 0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>7.77 ± 0.95</td>
<td>18.30 ± 4.9</td>
<td>3.50 ± 1.07</td>
<td>8.85 ± 3.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
<tr>
<td>GSHPx</td>
<td>0.33 ± 0.04</td>
<td>0.64 ± 0.08</td>
<td>0.18 ± 0.05</td>
<td>0.39 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>21.60 ± 1.40</td>
<td>45.40 ± 7.98</td>
<td>14.76 ± 2.60</td>
<td>29.6 ± 2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
<tr>
<td>TBARS</td>
<td>63.09 ± 5.31</td>
<td>53.20 ± 5.30</td>
<td>94.50 ± 9.07</td>
<td>65.79 ± 6.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
<tr>
<td>CPK</td>
<td>162.20 ± 12.3</td>
<td>159.40 ± 19.32</td>
<td>9.8 ± 1.05</td>
<td>13.01 ± 2.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#**</td>
<td></td>
</tr>
</tbody>
</table>

GSH: Glutathione; SOD: Superoxide dismutase; GSHPx: Glutathione peroxidase; CAT: Catalase; TBARS: Thiobarbituric acid reactive substances; CPK: Creatine phosphokinase. The values are expressed as mean ± SD. Each value represents a mean of six readings. #p<0.05, ##p<0.01 vs Sham; *p<0.05,**p<0.01,***p<0.001 vs Control IR. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine. GSHPx activity was calculated from the extinction coefficient of NADPH. One unit of enzyme activity is defined as 1 nmol of NADPH utilized per min at 37°C. One unit of catalase activity represents 1 μmol of H2O2 decomposed / min. One unit of CPK transfers 1 μmol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30°C.

Contrastively, on histological evaluation, rat hearts, subjected to ischemia and reperfusion (Control IR) demonstrated marked edema, confluent areas of myonecrosis, myofiber loss and mild inflammation as compared to those in the sham group. In the HCB treated rats subjected to ischemia and reperfusion, occasional focal myofiber loss, necrosis, edema and inflammation was observed but it was significantly less as compared to control IR group.

Apoptotic results

Myocyte Bax protein expression

Slight brown Bax immunoreactivity (3.5% ± 0.4%) was observed in the myocytes of the sham group. Ischemia and reperfusion induced myocardial injury significantly increased the expression of Bax protein (p<0.001) compared with non-ischemic tissue from 3.50 ± 0.40 to 9.80 ± 0.50% (Fig. 6). Bax expression was significantly attenuated to 5.04 ± 0.35% in the HCB-IR (p<0.01) treated groups as compared to control IR (Fig 7); suggesting that inhibition of apoptosis by these drugs may in part be mediated by attenuation of expression of Bax protein.

Apoptotic results

Myocyte Bcl-2 protein expression

Bcl-2 protein was clearly expressed in the sham myocardium as indicated by slight positive Bcl-2 immunoreactivity in the myocytes. The basal expression of Bcl-2 was found to be 1.86 ± 0.17%. Coronary occlusion and reperfusion resulted in a slight reduction (non-significant) in
Cardioprotective effects of a herbal combination

Bcl-2 expression compared with non-ischemic tissue (Fig. 8). Treatment with HCB, was associated with greater Bcl-2 expression (p<0.01) compared to control IR group (Fig. 9). The expression of Bcl-2 in the HCB-IR was found to be 8.25 ± 0.72%.

**DISCUSSION**

It has been well documented that early reperfusion of viable but ischemic jeopardized myocardium is essential to prevent cardiac damage. However, reperfusion itself has been shown to enhance myocardial injury and leads to further complications such as diminished cardiac contractile function and metabolic derangements (Kwon et al., 2007; Hearse et al., 1992; Opie, 1989). Myocardial reperfusion may therefore be viewed as a ‘double-edged sword’, although it clearly exerts deleterious effects on the severely ischemic cells, when reperfusion is carried out relatively early in the course of ischemia its net effects are usually beneficial (Braunwald et al., 1985). Thus, given the enormous potential clinical importance of early reperfusion in limiting infarct size, preserving antioxidant status, left ventricular function, and thus ensuing a significant decrease in patient morbidity and mortality, the development and identification of safe and effective interventions to reduce myocardial ischemia and reperfusion induced injury and/or optimize the benefit/risk ratio remain fertile areas for clinical and experimental investigation.

The present investigation was undertaken to study the cardioprotective potential of the herbal combination of *Withania somnifera* (50 mg/kg) + *Curcuma longa* (100 mg/kg) + *Ocimum sanctum* (75 mg/kg) {HCB} and to elucidate the possible mechanisms of action on the basis of hemodynamic, biochemical and histopathological studies. In addition, the anti-apoptotic property of HCB was studied using a combination of techniques of TUNEL positivity and immunohistochemical localization of Bax and Bcl-2 proteins.

Post-ischemic reperfusion injury resulted in significant cardiac necrosis, apoptosis; depression of left ventricular dynamics, peripheral hemodynamics (mean blood pressure)
Cardioprotective effects of a herbal combination

and heart rate; and decline in antioxidant status and elevation in lipid peroxidation. In addition, consistent with the increase in TUNEL staining in the control IR group, ischemia and reperfusion slightly reduced Bcl-2 expression and significantly increased Bax expression (p<0.01) compared with that observed in the sham group, demonstrating the phenomenon of ischemia and reperfusion induced enhanced myocardial apoptotic cell death.

Figure 10: Representative photomicrographs of ventricular tissue stained for nick-end labeling (TUNEL) for DNA breaks of saline-treated group subjected to 45 min of ischemia and 1 h of reperfusion. TUNEL-positive nuclei are shown as brown nuclei indicated by arrowheads. Figures are representative of 6 separate experiments.

Figure 11: Representative photomicrographs of ventricular tissue stained for nick-end labeling (TUNEL) for DNA breaks. Relative to the control IR group the number of TUNEL positive cells were significantly decreased by treatment with HCB. TUNEL-positive nuclei are shown as brown nuclei indicated by arrowheads. Figures are representative of 6 separate experiments.

The HCB treatment was effective enough to significantly ameliorate myocardial ischemic injury following LAD coronary occlusion and reperfusion when compared to control IR group. HCB did not significantly affect MAP and HR during the ischemic period; however during the latter half of the reperfusion period, HCB significantly restored MAP and HR. However, the modest beneficial hemodynamic effects on HR and MAP exerted by HCB do not explain the marked cardioprotection observed during ischemic and reperfusion injury. In the present study, HCB exerted beneficial effects on left ventricular dynamics as evidenced by (a) the correction of the ischemia-reperfusion induced enhanced level of LVEDP and (b) by significant improvement in myocardial contractility and relaxation. It is well known that one of the major causes of myocardial infarction is an imbalance between oxidants and antioxidant defenses (Ferrari et al., 1998; Freeman et al., 1982). Hence, it is possible to prevent or ameliorate disease progression by favoring the balance towards lower oxidative stress. Potential antioxidant therapy should, therefore, include exogenous supplementation of natural antioxidants that affect augmentation of endogenous antioxidants (Rajak et al., 2004).

In the present study, chronic treatment with HCB augmented basal endogenous antioxidants and inhibited the increase in TBARS levels i.e enhanced the antioxidant reserve, favorably modulating the antioxidant defense mechanisms of the myocardium in the healthy experimental animals. However, a key question, which remains unanswered in the present study, is the mechanism by which HCB augments basal endogenous antioxidants. Although the precise mechanism of such an effect is not clear from the present protocol, several factors might be playing contributing roles. In this regard it has been reported earlier that both Ws and Os possess adaptogenic properties; hence, it is speculated that they may contribute to the myocardial adaptogenic activity observed in the HCB control group (Rege et al., 1999.). Subsequent to ischemia and reperfusion induced oxidative stress it was observed that the HCB group demonstrated significant antioxidant property, which might contribute to the observed cardioprotective effect of these interventions.

In addition, in the present study, HCB demonstrated significant anti-apoptotic potential as it upregulated the expression of anti-apoptotic protein, Bcl-2 and downregulated the expression of pro-apoptotic protein, Bax; in association with a reduction in the percentage of TUNEL positive
Cardioprotective effects of a herbal combination

cells in the ischemic-reperfused myocardium. The exact mechanism by which the herbal combination may reduce myocardial ischemia and reperfusion induced myocardial apoptosis is far from clear presently; and may not be answered fully by the present study. However it can be speculated that it may attenuate apoptosis via a number of mechanisms: Upregulation of Bcl-2 may result in formation of heterodimers with Bax, resulting in no/fewer free Bax protein available for homodimerization. If Bax homodimers predominate cell death will occur, but when Bcl-2 and Bax heterodimerization prevails cells can survive. Substantial evidence indicates that the mitochondria play a critical regulatory role in the signal transduction pathway leading to apoptosis (Palojoki et al., 2001). HCB may attenuate mitochondrial injury resulting from ischemia and reperfusion and preserve mitochondrial function. By this mechanism, it may prevent the formation of the permeability transition pore in the mitochondrial membrane, inhibit the release of pro-apoptotic molecules such as apaf-1 and apaf-2 (cytochrome c) from the mitochondria, and reduce myocardial apoptosis. HCB may also attenuate myocardial apoptosis through prevention of the dephosphorylation of Bad, a pro-apoptotic protein of Bcl-2 family, by calcineurin (a calcium/calmodulin dependent protein serine/threonine phosphate). Preventing the activation of calcineurin keeps Bad in its phosphorylated state and inhibits its translocation to the mitochondrial surface, preventing subsequent cytochrome c release. Moreover, free radicals have been demonstrated to directly activate calcium and magnesium dependent endonuclease (DNase I), thus resulting in DNA fragmentation and cell apoptosis (Cook et al., 1999). The herbal combination treatment through its antioxidant mechanism may prevent this DNAase activation and reduce myocardial apoptosis.

The protective effect of the HCB was supported by histopathologic examination and in concert with preserved myocardial CPK content. In order to elucidate, the additional mechanisms by which HCB may reduce myocardial reperfusion injury and the potential clinical implications of such actions, we need to further investigate the relationship of the detrimental effects of key oxidants and apoptotic signals with reperfusion injury. This will lead to a better understanding of basic physiological and pathological mechanisms relevant to myocardial ischemia and reperfusion injury and give new insight to novel therapeutic targets and strategies for its treatment. However, the present study provides a lead for further exploring other mechanisms contributing to the cardioprotective effect of HCB. Whether the conclusions drawn on the basis of the current data can be extrapolated to clinical setting, remains to be defined by well-controlled studies in-patients. Nonetheless, the results of the present study are rather encouraging, because they could unravel a new therapeutic approach for the prevention and/or treatment of ischemic heart disease.

CONCLUSION

Subsequent to I-R injury, pre-treatment with HCB reduced LVEDP, improved inotropic and lusitropic functions and restored the antioxidant defense capacity of the myocardium. Most importantly, HCB demonstrated significant anti-apoptotic effects and augmented endogenous antioxidants. Preserved myocardial CPK activity and histopathologic evaluation further confirm its cardioprotective effects.

Acknowledgement

The authors gratefully acknowledge the financial assistance from the Ministry of Environment and Forests, Government of India for conducting the study. The authors also thank Mr. Brij Mohan Sharma for his expert technical assistance during the course of the study.

References


Cook SA, Sugden PH et al. (1999) Regulation of bcl-2 family proteins during development and in response to
Cardioprotective effects of a herbal combination


Moron MS, Depierre JW et al. (1979) Level of glutathione, glutathione reductase and glutathione-s-transferase activity in rat lung and liver. Biochemical Biophysica Acta. 82: 67-78.


