Full Length Research Paper

Tribulus teresstris modulates heat shock protein and key anti-apoptotic proteins in the Langendorff model of myocardial ischemia and reperfusion injury

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The cardioprotective effects of *Tribulus teresstris* (Tt), a medicinal herb, used in Indian system of medicine was evaluated in the Langendorff model of myocardial ischemia and reperfusion (I-R) injury. Tt (1, 2.5, 5, 10 mg/kg) was orally fed to healthy experimental rats once a day for 21 days followed by global ischemia and reperfusion injury. Biochemical parameters: lipid peroxidation product thiobarbituric acid reactive substances (TBARS), endogenous antioxidant: glutathione, antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GSHPx)) and myocardial enzyme creatine phosphokinase (CPK) were evaluated. To correlate the biochemical derangement and altered cardiac performance during I-R, changes in the hemodynamic variables heart rate (HR) and coronary perfusion pressure (CPP) was measured. Myocardial apoptotic was quantified using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method. The expression of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, enzyme: Caspase 3 and heat shock protein (HSP) in cardiac myocytes was detected by immunohistochemistry. As compared with sham group, the CPP, TBARS levels, myocardial apoptosis, expression of Caspase 3, Bax, heat shock protein (HSP 72) proteins were increased significantly in I-R control group. Tt pre-treatment significantly restored the antioxidant network of the myocardium, reduced myocardial apoptosis, Bax, HSP 72 protein expression. These beneficial effects also translated into favourable hemodynamic effects. Histopathological studies and myocardial CPK content further confirmed the cardioprotective effects of Tt (2.5 mg/kg) in the experimental model of I-R injury.

Key words: Apoptosis, ischemia and reperfusion, medicinal herbs, *Tribulus teresstris*, antioxidants.

INTRODUCTION

Experimental and clinical data demonstrate that reperfusion to the previously ischemic myocardium may contribute to additional tissue injury and profound structural, biochemical and functional abnormalities (Becker and Ambrosio, 1987; Braunwald and Kloner, 1985). Involvement of oxygen free radicals has been proposed to explain the myocardial injury observed after I-R (Ferrari, 1982). However, the molecular mechanisms by which myocytes die during I-R are not clearly understood. Previously necrosis was regarded as the only mode of myocardial cell death but recent studies suggest the involvement of both apoptosis and necrosis in cardiomyocyte demise. The progressive loss of cardiomyocytes by apoptosis in a heart that is already compromised leads to further deterioration of cardiac function. Apoptosis is a genetically regulated process hence, a better understanding of the cellular mechanisms that control apoptosis, could lead to defining novel and effective therapeutic strategies to limit the amount of tissue damage during I-R injury (Haunstetter and Izumo, 1998; Narula et al., 1999).

Apoptotic processes are regulated by several proteins including, Bax and Bcl2 which both play important roles. Bcl2 and Bax expression is a critical intracellular

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checkpoint of apoptosis within a distal common cell death pathway executed by proteolytic enzymes - caspases. The ratio Bcl2/Bax is important in determining the susceptibility to apoptosis and whether the cells survives or dies (Buttke and Sandstrom, 1998). Recently, advances in the understanding of the molecular events in apoptosis have led to the realization that caspases 3, the main effector caspase of the apoptotic cascade; is by far the most specific indicator of this cell suicide mechanism (Haunstetter and Izumo, 1998; Shobu, 1998).

Heat shock proteins HSP27, HSP70 and HSP90 are molecular chaperones whose expression is increased after many different types of stress. They have a protective function helping the cell to cope with lethal conditions. The cytoprotective function of HSPs is largely explained by their anti-apoptotic function. HSPs have been shown to interact with different key apoptotic proteins. At the pre-mitochondrial level, HSP70 binds to and blocks c-Jun N-terminal Kinase (JNK1) activity. At the mitochondrial level, HSP70 inhibits Bax translocation and insertion into the outer mitochondrial membrane. As a consequence, HSP70 prevents mitochondrial membrane permeabilization and release of cytochrome c and apoptotic inducing factor (AIF). At the post-mitochondrial level HSP70 has been demonstrated to bind directly to Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome. As a result, HSPs can block essentially all apoptotic pathways, most of them involving the activation of cystein proteases called caspases (Lanneau and Thonel, 2007).

These programmed cell death pathways can be inhibited by antioxidants (Marczin, 2003). However, there are a very few studies addressing the inhibition of apoptosis by medicinal plants (known to possess antioxidant property) and its direct effects on cardiac performance, endogenous antioxidants, expression of key regulatory proteins like HSP, Bax, Bcl-2, and caspase 3 (Singal, 1993). Recently, the keen interest in medicinal plants for cardioprotection has also increased because of their numerous possible cardioprotective mechanisms. A concept is now emerging of 'adaptogenic drugs' drugs that increase non-specific resistance of the users to a variety of stresses (Rajak et al., 2004). Thus, a major opportunity exists in using our natural resources for identifying and selecting efficacious, inexpensive and safer approaches for the cardioprotection against I-R hearts.

As few systematically designed studies are currently available, these medicinal plants need to be investigated scientifically. In the present investigation, modification of the condition of reperfusion has been achieved with Tt, used in Ayurvedic and Unani systems of India as an aphrodisiac, diuretic and nervine (Guo et al., 2007; Kavitha and Jagadeesan, 2006). In the present study the therapeutic potential of Tt in the Langendorff model of I-R injury has been evaluated. In addition, to elucidate the potential clinical implications of such actions, the relationship of the detrimental effects of key oxidants and

apoptotic signals with I-R injury has been studied.

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats, 10 to 12 weeks old, weighing 150 to 200 g were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conformed to the Indian National Science Academy Guidelines for the use and care of experimental animals in research. Animals were obtained from the Animal Facility of Mahatma Gandhi Mission Medical College, Navi Mumbai, India. Rats were housed in polyacrylic cages (38x23x10 cm) with not more than four animals per cage. They were housed in an air-conditioned room and were kept under natural light and dark cycles (approximately 14 h light/10 h dark) and maintained at humidity $60 \pm 5\%$ and an ambient temperature of $25 \pm 2°C$. All experiments were performed between 9.0 and 16.0 h. The animals were allowed free excess to standard diet and tap water ad libitum and allowed to acclimatize for one week before the experiments. Commercial pellet diet contained 24% protein, 5% fat, 4% fiber, 55% carbohydrates, 0.6% calcium, 0.3% phosphorous, 10% moisture and 9% ash w/w.

Chemicals

All chemicals were of analytical grade, purchased from Sigma Chemical Co., St Louis, USA. Hydro-alcoholic lyophilized extracts of *Tribulus teresstris* was procured from Dabur Research Foundation, India. Double distilled water was used in all biochemical assays.

Dose selection studies: Isoproterenol (ISP) model of myocardial necrosis

The ISP (85 mg/kg) model of myocardial necrosis was used for the evaluation of therapeutic intervention of herbal extracts on the extent of jeopardized myocardium and evolution of infarction in ISP administered rats and select the optimum cardioprotective dose of the herbal extracts for further studies in the langendorff model of I-R injury (Mohanty et al., 2007). According to the experimental protocol, normal saline (NS)/Tt (1, 2.5, 5 and 10 mg/kg doses) was administered orally, using an intragastric tube, to the rats for three weeks. Infarct-like myocardial lesions were developed by (ISP injection (85 mg/kg, subcutaneous) for two consecutive days, that is, on 20th and 21st day of the experiment. On the 22nd day, the rats of all the experimental groups were sacrificed. The hearts were rapidly removed and processed for biochemical estimations and histological evaluation.

Myocardial I-R model: Langendorff heart preparation

The rats of all the experimental groups were orally fed NS/Tt (2.5 mg/kg) for 21 days. On 22nd day, the rats were anesthetized.
Diaphragm was cut and thoractomy was performed and the pericardium was opened to expose the heart. The heart was gently elevated by cradling it gently in the finger tips to avoid contusion injury. The aorta, vena cava and pulmonary arteries were incised. Immediately after excision, the heart was placed in to a beaker containing cold Kreb's Hensleit buffer. The heart was then set on the langendorff apparatus (Inco, India) by cannualating the aorta attached to a reservoir containing oxygentated Kreb's Hensleit solution. The solution was then delivered in a retrograde direction down the aorta at constant flow delivered by an infusion pump.

After 10 min stabilization period, global myocardial I-R was induced by completely stopping perfusion flow rate for 20 min followed by 30 min reperfusion at 37°C.

Hemodynamic parameters HR, CPP were recorded at preset time points of the study protocol that is, before ligation, 5, 10, 20 min post ischemia and 10, 20 and 30 min post reperfusion. At the end of reperfusion period, hearts were snap frozen in liquid nitrogen for biochemical studies or in formalin for histopathological/immunohistochemical studies.

Experimental groups and treatment protocol

The animals were assigned to the following experimental groups:

Group 1 – Saline control group (Sham): Rats were administered 0.9% NS per orally using a feeding cannula for 21 days and then sacrificed on the 22nd day. There were twelve animals in this group. **Group 2 – *Tribulus teresstris* (Tt) control group:** This group comprised of twelve rats. Hydro-alcoholic extract of Tt (2.5 mg/kg) was dissolved w/v in 0.9% normal saline administered orally to healthy experimental rats once daily for 21 days at the dose of 2.5 mg/kg (Tt-2.5)

Group 3 – Langendorff group - control IR: The rats were administered NS for 21 days and on 22nd day, sacrificed and heart mounted on langendorff apparatus. After 10 min of stabilization period, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. There were twelve animals in this group.

Group 4- Tt treated groups – Tt-IR: Tt (2.5 mg/kg) was dissolved in 0.9% NS and administered for 21 days. On 22nd day, rats were sacrificed and heart mounted on langendorff apparatus. After 10 min of stabilization period, global occlusion was undertaken for 20 min followed by 30 min of reperfusion. There were twelve animals in this group.

EXPERIMENTAL PARAMETERS STUDIED

Biochemical studies

A ten-percent homogenate of myocardial tissue was prepared in 50 mM phosphate buffer, pH 7.4 and an aliquot was used for the assay of TBARS according to the method described by Ohkawa et al. (1979). The homogenate was centrifuged at 7000 rpm for 15 min and the supernatant was used for the estimation of the GSH (Moron, 1979)' GSHPx (Paglia and Valentine, 1967), SOD (Misra and Fridovich, 1976), CAT (Aebi, 1974) and protein (Lowry et al., 1951). CPK was estimated spectrophotometrically using a kit from Randox Laboratories, USA (Lamprecht, 1994).

Cardiac parameters

The time-course of changes in HR, CPP were monitored and recorded at preset time points that is, before ligation, 5, 10, 20 min post ischemia and 10, 20 and 30 min post reperfusion during the study protocol in different experimental groups.

DETERMINATION OF MYOCARDIAL APOPTOSIS

Immunostaining for the localization of Caspase 3, Bax and Bcl-2 proteins

A monoclonal mouse anti-human Caspase 3, Bcl-2 and Bax proteins as the primary antibody were used for active Caspase 3,

Bcl-2/Bax immunohistochemical staining. The ImmunoCruz staining systems utilizes a horseradish peroxidase (HRP) - streptavidin complex for staining of formalin-fixed paraffin-embedded myocardial sections. Indirect immunoperoxidase staining was performed as described by Misao et al. (1996). Briefly, 4-6 micron thick fixed paraffin tissue sections of different experimental groups were first blocked then incubated in primary antibody. Biotinylated secondary antibody is added followed by the addition of HRP-Straptavidin complex. The target protein (Caspase 3, Bax/Bcl-2) was visualized by incubation in peroxidase substrate (H_2O_2) using DAB (3, 3' diaminobenzidine) as the chromogen.

TUNEL assay

Myocardial apoptosis was quantitatively analyzed by detection of DNA fragmentation using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique (Misao, 1996). Briefly, the enzyme terminal deoxynucleotidyl tranferase was used to incorporate residues of digoxigenin nucleotide into 3' OH ends of DNA with the aid of terminal deoxynucleotidyl transferase to the ends of DNA fragments. The signal of TUNEL assay was used to identify apoptotic cells using secondary reaction with antibodies or other detection system. Total cell counts and TUNEL positive cells in the specimens were determined by means of a light microscope. The cells with clear nuclear labeling were defined as TUNEL positive cells. The apoptotic cells that is, TUNEL positive cells were expressed as percentage of normal nuclei.

Immunostaining for the localization of HSP proteins

Immunohistochemistry using the rabbit anti-cleaved heat shock protein antibody was performed on deparaffinized tissue sections using a routine avidin–biotin–immunoperoxidase technique. Before incubation with the primary antibody (1:200 dilution), tissue sections were subjected to heat-induced epitope retrieval by incubation in a pH 8.0 0.01 M EDTA solution for 10 min in a vegetable steamer, followed by 20 min cool-down and treatment with 3% hydrogen peroxide before antibody application. Bound antibodies were detected using HRP-Straptavidin complex. The target protein (HSP) was visualized by incubation in peroxidase substrate complex and DAB (3, 3' diaminobenzidine) as the chromogen. Counterstaining was performed with Meyer's hematoxylin.

Histopathological studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. The tissues were carefully embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. Cross sections (5 μm thick) of the fixed myocardial tissues were cut. These sections were stained with hematoxylin and eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the myocardium. The degree of necrosis, edema and inflammation was graded and scored.

Statistical analysis

All numerical data in text, figures and tables are expressed as the

Table 1. Biochemical parameters in the different groups.

	Sham (N=9)	Tt-2.5 (N=9)	Control IR (N=9)	Tt-IR (N=9)
GSH (umol/g tissue)	4.03±0.3	4.63±0.4	2.60 ± 0.19 ##	3.92 ± 0.11**
TBARS (nmol/g tissue)	27.73±5.7	28.33±1.3	80.48±3.4	48.33±1.3*
GSHPx (IU/mg protein)	0.33±0.1	0.41±0.1	0.18 ± 0.05 #	0.21±0.1
SOD (IU/mg protein)	7.94±2.9	28.04±1.2**	3.50 ± 1.07 #	28.04±1.2
CAT (IU/mg protein)	21.1±3.1	54.8± 8.0*	14.76 ± 2.60 #	54.8± 8.0
CPK (IU/mg protein)	6.7±1.4	6.8±0.4	1.7±0.8	4.7±0.4*

*$p<0.05$, **$p<0.01$ vs sham. GSH (Glutathione); TBARS (Thiobarbituric acid reactive substances) and Glutathione peroxidases (GSHPx), Superoxide dismutase (SOD), Catalase(CAT) and CPK (Creatinine phosphokinase).. One unit of catalase activity represents 1 μmol of H_2O_2 decomposed / min. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine. One unit of enzyme activity is defined as 1 nmol of NADPH utilized per min at 37°C. One unit of CPK is defined as the amount of enzyme that will transfer 1 μmol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30°C. N= no of rats included in the study group.

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

Pilot study

In our laboratory, Tt at 1, 2.5,5, 10 mg/kg doses was screened in the ISP model of myocardial necrosis (Data submitted for publication). Except the lowest dose 1 mg/kg, the other doses significantly prevented leakage of GSH following ISP induced myocardial injury. TBARS levels generally correlated inversely with myocardial CPK activity. Tt (2.5 and 5 mg/kg) treatment significantly decreased TBARS levels and restored CPK activity ($p<0.05$) of the myocardium in reference to the group administered ISP. Tt (2.5 and 10 mg/kg) treatment significantly prevented myonecrosis, infiltration of inflammatory cells, edema and vacuolar changes as compared to the ISP induced myocardial injury. In the Tt (1 and 5 mg/kg) doses of the study protocol the degree of edema and necrosis was nearly comparable to that of the group administered ISP with similar morphological changes. Tt - 2.5 mg/kg demonstrated significant myocardial salvaging effects and hence was selected as the optimum cardioprotective dose for further studies in the I-R model of myocardial injury (Mohanty et al., 2008).

Effect of *Tribulus teresstris*

Biochemical parameters

Without I-R induced myocardial injury: Augmentation of myocardial endogenous antioxidant reserve [SOD, CAT ($p<0.05$)] following oral administration of Tt (2.5 mg/kg) to healthy controls (study group of animals without any experimental challenge to the myocardium; viz ISP administration or inducing ischemia and reperfusion injury) significantly improved defense against oxidative stress as compared to the sham group (oral administration of saline for 21 days to healthy experimental animals).

Following I-R induced myocardial injury

In the control IR group following I-R a significant depletion in the antioxidant enzyme activities of SOD, GSHPx and CAT, GSH; myocardial CPK activity and increase in TBARS level was observed in comparison to sham (Table 1). Tt-IR (2.5 mg/kg) treatment significantly prevented leakage of myocardial GSH ($p<0.01$) and CPK ($p<0.05$) in reference to control IR group. In addition, Tt also markedly reduced lipid peroxidation ($p<0.05$) as evidenced by reduction in TBARS levels as compared to control IR group. Nonetheless, it failed to significantly restore the antioxidant enzymes GSHPx, SOD and CAT as compared to control IR group.

Cardiac parameters following I-R induced myocardial injury

Heart rate (HR)

Initial value of HR in the control IR group was 343 ± 22.6 beats/min. Following LAD occlusion, at 5 min a significant ($p<0.05$) fall in the value of this variable was recorded as compared to sham. Subsequently, the HR remained depressed during ischemic duration as compared to sham. On reperfusion, a steady decline in HR, which was statistically significant at 20 and 30 min was observed as compared to baseline sham values of this variable (Figure 1). In the Tt (2.5 mg/kg) treated group, there was no significant correction in HR during the period of global ischemia as compared to control IR group. The HR
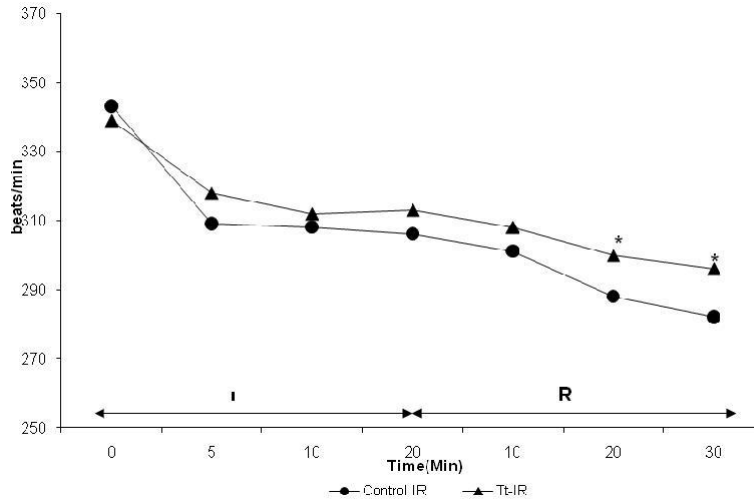
Figure 1. Time course of changes in heart rate in the different experimental groups. *p<0.05 Vs Control IR.
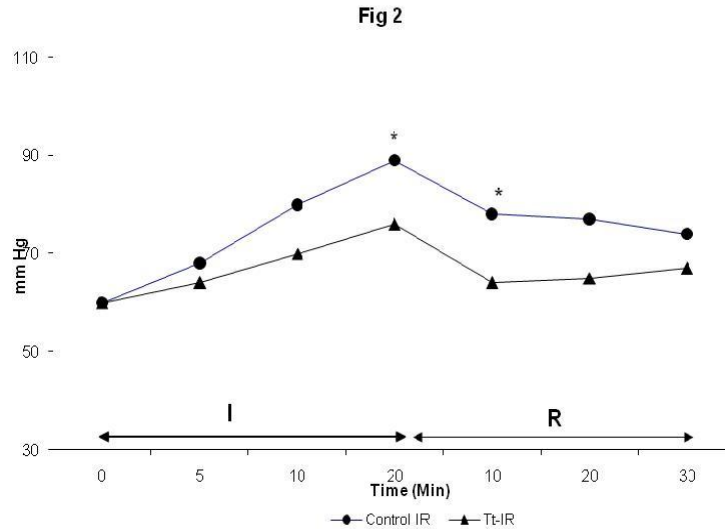

Figure 2. Time course of change in coronary perfusion pressure in the different experimental group. *p<0.05 Vs Control IR.

values of the animals in the Tt group were comparable to the control IR values throughout the entire ischemic period. However, Tt significantly (p<0.05) restored HR at 20 and 30 min of reperfusion as compared to control IR values at the same time points.

Coronary perfusion pressure (CPP)

20 min of ischemia resulted in significant increase in coronary perfusion pressure in the control IR group as compared with sham group baseline values. There was a transient decrease in CPP after 10 min of reperfusion. However, CPP did not significantly differ from sham at 20 and 30 min post reperfusion (Figure 2). Although there

was a steady and continuous rise in CPP in the Tt-IR (2.5 mg/kg) group during the experimental duration, the value of CPP was lower throughout the global ischemia and reperfusion period as compared to the control IR group. At 20 min post ischemia and 10 min post reperfusion, Tt (2.5 mg/kg) significantly reduced CPP in comparison to control IR values at same time points (Figure 2).

Myocardial apoptotic parameters following I-R induced myocardial injury

Myocyte Bax protein expression

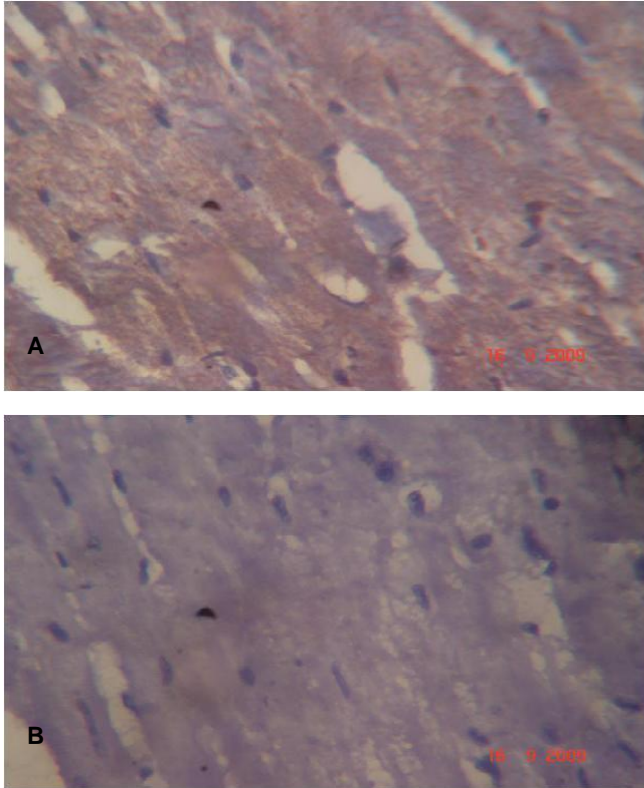Slight brown Bax immunoreactivity (3.5 + 0.4%) was

Figure 2. Immunohistochemical findings of Bax protein: A). I-R induced myocardial injury significantly increased the expression of Bax protein compared with non-ischemic tissue; B). Tt (2.5 mg/kg) treatment significantly attenuated the expression of Bax protein versus the control IR group. Figures are representative of 6 separate experiments.

Figure 3. Immunohistochemical findings of Bcl-2 proteins: A. Global I-R resulted in a slight reduction (non significant) in Bcl-2 expression in the control IR group compared to non-ischemic tissue; B. Upregulation in the expression of Bcl-2 as indicated by dark brown positive immunoreactivity is evident in the Tt treated group. Figures are representative of 6 separate experiments.

observed in the myocytes of the sham group. I-R induced myocardial injury significantly increased the expression of Bax protein (p<0.01) compared with non-ischemic tissue from 3.50 + 0.40 to 9.80 + 0.50% (Figure 2A). Bax expression was significantly attenuated to 4.04 ± 0.35% in the Tt-IR (p<0.05) group as compared to control IR (Figure 2B).

Myocyte Bcl-2 protein expression

Bcl-2 protein was clearly expressed in the sham group 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%. Global I-R resulted in a reduction (p<0.05) in Bcl-2 expression compared with non-ischemic tissue (Figure 3A). Interestingly, treatment with Tt (2.5 mg/kg) was associated with increased Bcl-2 expression (p<0.01) as compared to control IR group (Figure 3B).

Myocyte Caspase 3 protein expression

Slight positive immunoreactivity against active Caspase 3 was observed in the myocytes of the sham group. In the control IR group significant immunostaining of subset of
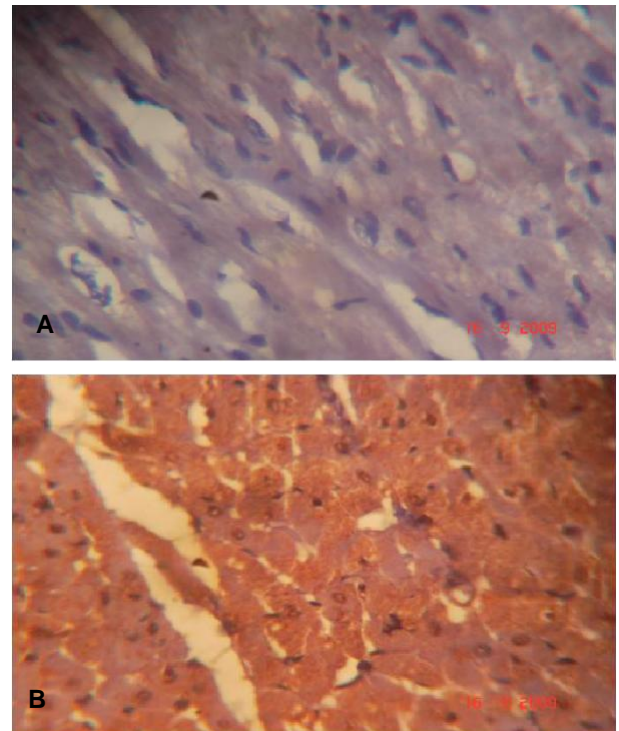
apoptotic cells within germinal center, as well as polymorphonuclear leukocytes within capillary was present (Figure 4A). However, Caspase 3 expression was significantly attenuated in the Bm-IR (p<0.05) treated group as compared to control IR (Figure 4B).

TUNEL positivity

TUNEL positivity was expressed as percentage of total normal nuclei. Slight TUNEL positive staining was detected in the sham group (0.2 ± 0.01%). However, the number of TUNEL positive cells expressed as percentage of total normal nuclei was significantly increased subsequent to I-R induced myocardial injury in the control IR group (3.0 ± 0.2%, p<0.001) compared to sham non-ischemic myocardium as indicated by increased intensity of TUNEL staining (Figure 5A). The TUNEL positivity was significantly attenuated to 0.4 ± 0.03%, in the Tt-IR (p<0.05) group as compared to control IR (Figure 5B).

Heat shock protein expression following I-R induced myocardial injury

HSP72 immunoreactivity in myocytes was granular and localized in the cytoplasm HSP72 immunostaining in the
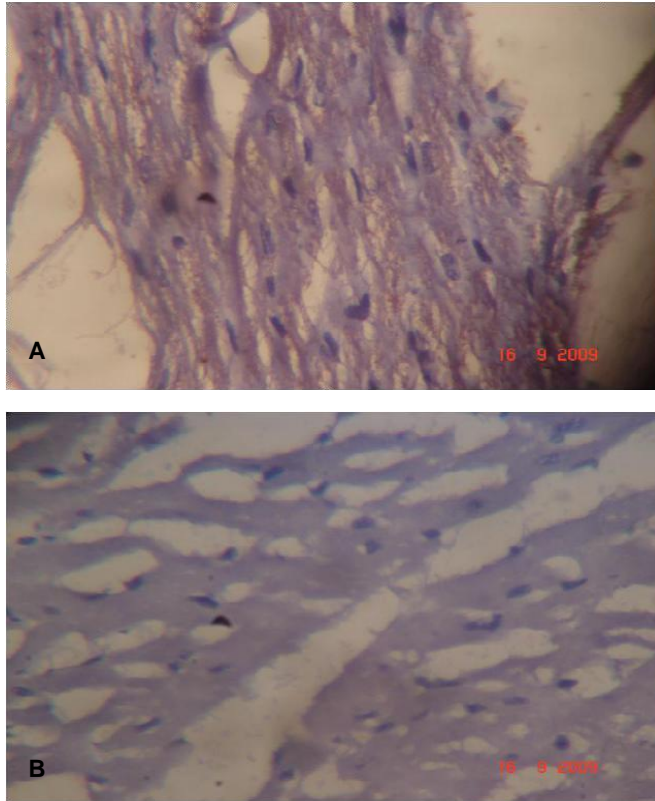
Figure 4. Immunohistochemical findings of Caspase 3 proteins: A. In the Control IR group significant immunostaining of subset of apoptotic cells within germinal center, as well as polymorphonuclear leukocytes within capillary was present; B. However, Caspase 3 expression was significantly attenuated in the Tt-IR treated group as compared to control IR. Figures are representative of 6 separate experiments.

Figure 5. Representative photomicrographs of ventricular tissue stained for nick-end labeling (TUNEL) for DNA breaks: A. TUNEL positive cells were significantly increased subsequent to I-R induced myocardial injury in the control IR group; B. Relative to the control IR group the number of TUNEL positive cells was significantly decreased by treatment with Tt (2.5 mg/kg). Figures are representative of 6 separate experiments.

myocardium was very faint in sham operated controls. In the control IR group post ischemic reperfusion led to increased intensity of immunostaining in the ischemic myocardium, and strong positive staining was seen in microvessels as well as myocytes (Figure 6A). In the Tt treated groups, few of the myocytes in the ischemic and nonischemic areas were weakly positive for HSP72 as compared to control IR group (Figure 6B).

Histopathology following IR induced myocardial injury

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 (Table 2). Contrastively, on histological evaluation, rat hearts, subjected to global I-R demonstrated marked edema, confluent areas of myonecrosis, myofiber loss and mild inflammation as compared to those in the sham group. In the Tt (2.5 mg/kg) treated rats occasional focal myofiber loss,
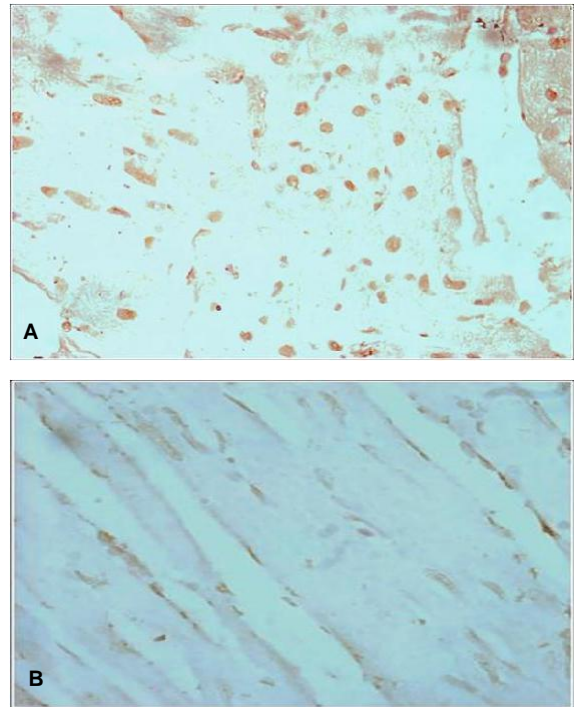
necrosis, edema and inflammation was observed but it was less as compared to control IR group (Table 2).

DISCUSSION

Over the last decade, increasing evidence has suggested that apoptosis is an important mechanism involved in the development and progression of cardiovascular disease (Mohanty et al., 2008). In the myocardium, apoptosis has been detected in a number of cardiac pathologies including, ischemia followed by reperfusion and myocardial infarction (Buttke and Sandstrom, 1994). The progressive loss of cardiomyocytes by apoptosis in a heart that is already compromised leads to further deterioration of cardiac function. Apoptosis is a genetically regulated process, hence, a better understanding of the cellular mechanisms that control apoptosis, could lead to defining novel and effective therapeutic strategies to limit the amount of tissue damage in patients with I-R (Narula, 1999).

TUNEL positivity and the immunohistochemical localization of Bax, an inducer of apoptosis and Bcl-2
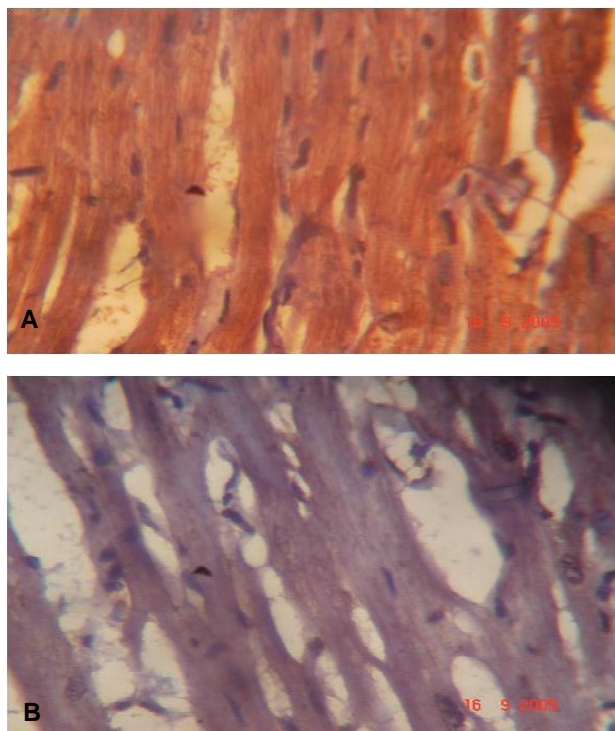
Figure 6. Immunohistochemical findings of HSP72 proteins: A. In the control IR group post ischemic reperfusion led to increased intensity of immunostaining in the ischemic myocardium; B. In the Tt treated groups, few of the myocytes in the ischemic and nonischemic areas were weakly positive for HSP72 as compared to control IR group. Figures are representative of 6 separate experiments.

Table 2. Light microscopic changes observed in the different groups.

	Necrosis	**Edema**	**Inflammation**
Sham	-	-	-
Control IR	++	+++	+
Tt-IR	+	+	+

The relative cardiovascular effects are ranked from **-** = No change; **+** = Focal change; **++** = Patchy change; **+++** = Confluent change and **++++** = Massive change.

proteins, inhibitor of apoptosis were incorporated to the study design to delineate the involvement of apoptosis in I-R induced injury. The TUNEL assay identifies single strand DNA breaks with free 3'-OH terminals. Several studies have raised doubts about the specificity of TUNEL staining (MacLellan and Schneider, 1997). Collective evidence suggests that the TUNEL assay is useful in identifying apoptosis but should be complemented by additional evidence of apoptosis, such as the up-regulation of pro- or anti-apoptotic gene products or structural criterion (Kumar and Jugdutt, 2003). Through the study of the molecular events in apoptosis systems, it is now clear that cleavage of protein

substrates by caspases is a pivotal cascade that is unique to apoptotic cells (Shobu, 1998).

With this point of view, the anti-apoptotic property of Tt was studied in the langendorff model of myocardial I-R injury, using a combination of techniques of TUNEL positivity and immunohistochemical localization of Caspase 3 enzyme, Bax and Bcl-2 proteins. On the basis of hemodynamic, biochemical and histopathological studies, the cardioptotective effects of Tt was evaluated. In addition, the effect of Tt (2.5 mg/kg) per se on basal endogenous antioxidants was also evaluated.

In the present study, oral administration of Tt (2.5 mg/kg) per sec to healthy experimental animals resulted in a significant increase in CAT, SOD activity and inhibition of basal lipid peroxidation. However, no significant effect of Tt on myocardial GSH content and GSHPx activity was observed. It has been reported that an augmented SOD activity, without a concomitant rise in the activity of CAT and/or GSHPx might be detrimental, since SOD activity, generates hydrogen peroxide as a metabolite, which is more cytotoxic than oxygen radicals and must be scavenged by CAT or GSHPx. Thus, simultaneous increase in myocardial SOD and CAT activities observed in the present study with administration of Tt underscores the distinct importance of enhanced beneficial effects of this herbal extract. This adaptogenic property may contribute to its cardioprotective effect and strengthen the antioxidant defense mechanisms of the heart. Although the precise mechanism of such an effect is not clear from the present protocol, several factors like induction of Bcl-2, an anti-apoptotic protein, and heat shock protein (HSP) as observed in the Tt control group might be playing contributing roles. In addition, following I-R induced myocardial injury, Tt pre-treatment demonstrated modest antioxidant activity.

Tt pretreatment did not significantly preserve MAP and CPP during the initial ischemic period; although during the latter half of the ischemia and early reperfusion period; Tt, significantly restored CPP. The fall in HR in the Tt treated group was not as pronounced as the control IR group during the latter half of reperfusion duration. However, the modest beneficial hemodynamic effects on HR and CPP exerted by Tt do not explain the marked cardioprotection observed during I-R injury. The vasodilatory properties of Tt may contribute to the observed effect. It has been reported that Tt saponin dilates coronary artery and improves coronary circulation (Wang et al., 2003).

In the ischemic myocardium loss of contractile cells during reperfusion poses an additional workload on the remaining viable myocytes that may be unbearable, resulting in pathologic stimuli and death signals. Most importantly, in the present study, Tt demonstrated significant anti-apoptotic property. Tt pre-treatment may have salvaged these myocytes and prevented cell loss induced by apoptosis. In association with a reduction in

the percentage TUNEL positive cells in the ischemic myocardium, Tt treatment upregulated the expression of anti-apoptotic protein, Bcl-2 and downregulated the expression of pro-apoptotic protein, Bax. Upregulation of Bcl-2 may result in formation of heterodimers with Bax, resulting in no/fewer free Bax protein available for homodimerization. It is well known that if Bax homodimers predominate cell death will occur, but when Bcl-2 and Bax heterodimererization prevails cells can survive (Buttke and Sandstrom, 1994). Our findings concur with the earlier reported anti-apoptotic property of Tt saponin in the neonatal rat ventricular myocytes isolated by collagenase digestion. Results suggested that the Tt saponin played a role in cardiomyocyte survival via PK Cepsilon and Bcl-2 (Rajak et al., 2004; Sun et al., 2008). In addition, in the present investigation, Tt treatment significantly prevented the intense caspase 3 immunoreactivity which is the central hallmark of almost all apoptotic systems.

Although the expression of HSP72 in the ischemic heart has been studied widely, the expression of HSP72 in the reperfused heart is still not well understood (Hong, 1999). In the present study, we showed that postischemic reperfusion markedly increased the immunoreactivity of HSP72 in myocytes, as well as microvascular endothelium, compared with non-ischemic myocardium. Strong expression of HSP72 appeared in microvascular endothelium after reperfusion for 30 min following a 20 min period of ischemia. This may be associated with the damage to the microvascular endothelium that occurred in reperfusion. In contrast to the ischemic reperfused myocardium, the immunoreactivity in the Tt treated group appeared much weaker and less frequent and was present mainly in the myocytes. These results indicate that expression of HSP72 in the Tt treated group is weak and temporary, and only occurs in myocytes, differing from the pattern observed in the ischemic reperfused myocardium.

Previous studies have reported the protective effect of Tt on the heart. Tt relieves left ventricular remodeling after myocardial infarction in a murine hyperlipemia model (Guo et al., 2003). The effect of Xinnao Shutong capsule, whose main ingredients gross saponins from Tt on cardiac muscle cell apoptosis and expressions of Bcl-2 compound rat model of and Bax in murine model of hyperlipemia after myocardial infarction has laso been studied. Tt reduced apoptosis through regulating protein expressions of Bcl-2 and Bax, which may be one of the mechanisms of its anti-ventricular-remodeling effects after myocardial infarction (Guo et al., 1990). In another study conducted in China, 406 cases of coronary heart disease was treated with saponin of Tt. Results shown that saponin of Tt has the action of dilating coronary artery and improving coronary circulation, and thus has better effects on improving ECG of myocardial ischemia (Wang et al., 1990).

The effect of Tt on myocardial I-R injury and apoptosis has not been studied so far in the langendorff model of myocardial injury. On the basis of the obtained hemodynamic, biochemical, immunohistochemical and histopathological data, it is concluded that Tt is an effective cardioprotective agent. The adaptogenic, antioxidant, anti-apoptotic properties and its beneficial effects on myocardial CPK, HSP 72, Caspase 3, Bax and Bcl-2 proteins may contribute to the beneficial effects of Tt. The study also provides scientific rationale for the use of Tt in Ayurveda, the ancient Indian System of Medicine.

Conclusions

Oral pretreatment with Tt (2.5 mg/kg) significantly ameliorated the I-R induced cardiomyocyte apoptosis, compromised antioxidant status and histopathologic alterations. Cardioprotection afforded by Tt treatment may be attributed to its significant antioxidant and anti-apoptotic properties. This finding also suggests that Tt may attenuate the HSP72 induced I-R mediated acute inflammation. Furthermore, Tt decreased the severity of pathological changes and significantly preserved the myocardial CPK confirming its myocardial salvaging effects.

REFERENCES

Aebi H (1974). Catalase: Methods of Enzymatic Analysis. In: Hans Elrich Bergmayer (eds) Academic Press, New York, pp. 213-215.

Becker LC, Ambrosio G (1987). Myocardial consequences of reperfusion. Prog. Cardiovasc. Dis., 30: 23-44.

Braunwald E, Kloner RA (1987). Myocardial reperfusion: A double-edged sword? J. Clin. Invest., 76(5): 1713-1719.

Buttke TM, Sandstrom PA (1994). Oxidative stress as a mediator of apoptosis. Immunol. Today, 15(1): 7-10.

David L, Aurelie de T, Sebastien M, Celine D, Carmen G (2007). Apoptosis Versus Cell Differentiation Role of Heat Shock Proteins HSP90, HSP70 and HSP27. Prion., 1(1): 53–60.

Ferrari R, Ceconi C, Curello S, Cargnoni A, De Giuli F, Visioli O (1992). Occurrence of oxidative stress during myocardial reperfusion. Mol. Cell. Biochem., 111(1-2): 61-69.

Guo Y, Shi DZ, Yin HJ, Chen KJ (2007). Effects of Tribuli saponins on ventricular remodeling after myocardial infarction in hyperlipidemic rats Am. J. Chin. Med., 35(2): 309-316.

Guo Y, Shi DZ, Yin HJ, Chen KJ (2007). Effects of Tribuli saponins on ventricular remodeling after myocardial infarction in hyperlipidemic rats. Am. J. Chin. Med., 35(2): 309-316.

Guo Y, Yin HJ, Shi DZ (1990). Effect of xinnao shutong capsule on cardiac muscle cell apoptosis and protein expressions of Bcl-2 and

Bax in hyperlipidemia rats after myocardial infarction. Zhong. Xi Yi. Jie He Za Zhi,10(2): 85-87.

Haunstetter A, Izumo S (1998). Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ. Res., 82(11): 1111-1129.

Hong Yu (1999). Time Course of Expression and Localization of Heat Shock Protein 72 in the Ischemic and Reperfused Rat Heart. Jpn. Circ. J., 63: 278–287.

Kavitha AV, Jagadeesan G (2006). Role of *Tribulus terrestris* (Linn.) (Zygophyllacea) against mercuric chloride induced nephrotoxicity in mice, *Mus musculus* (Linn.). J. Environ. Biol., 27(2): 397-400.

Kumar D, Jugdutt BI (2003). Apoptosis and oxidants in the heart. J. Lab. Clin. Med., 142(5): 288-297.

Lamprecht W (1994). Determination of creatine phosphate and adenosine triphosphate with creatine kinase. In Bergmeyer HU (eds), Methods of Enzymatic Analysis, New York, Academic Press, pp. 1776-1778.

Lowry OH, Rosebrough NJ, Farr AI (1951). Protein measurements with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

MacLellan WR, Schneider MD (1997). Death by design. Programmed cell death in cardiovascular biology and disease. Circ. Res., 81(2): 137-144.

Marczin N (2003). Antioxidants in myocardial ischemia-reperfusion injury: Therapeutic potential and basic mechanisms. Arch. Biochem. Biophys., 420(2): 222-236

Misao J (1996). Expression of bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. Circulation, 94(7): 1506-1512.

Misra HP, Fridovich I (1976). The oxidation of phenylhydrazine: superoxide and mechanisms. Biochemistry, 15: 681-687.

Mohanty IR, Arya DS, Gupta SK (2008). *Withania somnifera* provides cardioprotection and attenuates ischemia-reperfusion induced apoptosis. Clin. Nutr., 27(4): 635-642.

Mohanty IR, Maheshwari U, Daniel J, Deshmukh YA (2010). *Tribulus teresstris* augments cardiac endogenous antioxidants and prevents isoproterenol-induced myocardial necrosis in rats. Pharmaceut. Biol., (Under Revision).

Moron MS (1979). Level of glutathione, glutathione reductase and glutathione-s-transferase activity in rat lung and liver. Biochem. Biophys. Acta., 82: 67-78.

Narula J, Pandey P, Arbustini E, Haider N, Narula N (1999). Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc. Natl. Acad. Sci. USA, 96(14): 8144-8149.

Ohkawa H (1979). Assay of lipid peroxide in animal tissue by thiobarbituric acid reaction. Anal. Biochem., 95: 351-358.

Paglia D, Valentine WN (1967). Studies on the quantitative and qualitative characterization of erythrocyte peroxidase. J. Lab. Clin. Med., 2: 158-161.

Rajak S, Banerjee SK, Sood S, Dinda AK, Gupta YK, Gupta SK, Maulik SK (2004). *Emblica officinalis* causes myocardial adaptation and protects against oxidative stress in ischemic-reperfusion injury in rats. Phytother. Res., 18(1): 54-60.

Shobu N (1998). Activation and Cleavage of Caspase-3 in Apoptosis Induced by Experimental Cerebral Ischemia. J. Neurosci., 18(10): 3659-3668.

Singal PK (1993). Endogenous antioxidant changes in the myocardium in response to acute and chronic stress conditions. Mol. Cell. Biochem., 129(2): 179-186.

Sun W, Li H, Yang SJ (2008). A triterpene saponin from *Tribulus terrestris* attenuates apoptosis in cardiocyte via activating PKC signaling transduction pathway. J. Asian. Nat. Prod. Res., 10(1-2): 39-48.

Wang B, Ma L, Liu T (1990). 406 cases of angina pectoris in coronary heart disease treated with saponin of *Tribulus terrestris*. Zhong. Xi. Yi. Jie. He. Za. Zhi, 10(2): 85-87.