4.1 Experimental animals

Laboratory bred 10-12 week old Wistar male albino rats weighing 150-200 g were used in the study. Animals were obtained from the central animal house facility of University College of Medical Sciences, Delhi. The rats were housed in polyacrylic cages (38x23x10 cm³) with not more than four animals per cage. They were housed under standard laboratory conditions with natural light and dark cycles (approximately 12 hr light/12hr dark) and maintained at humidity of 55±5% and an ambient temperature of 22±2°C. The animals were allowed free access to standard pellet diet (Durga Brothers Pvt. Ltd.) and tap water ad libitum. The commercial pellet diet fed to rat contained 22% protein, 5% fat, 5% fiber, 58% carbohydrate, 0.8% calcium, 0.2% phosphorus, 8% moisture, and 8% ash w/w. After acclimatization period of 1 week, the animals were assigned to experimental groups and all experiments were performed between 9.00-16.00 hr. The study protocol was reviewed and approved by the Institutional Animal Ethical Committee of University College of Medical Sciences, Delhi and conforms to the Indian National Science Academy guidelines for the use and care of experimental animals in research.

4.2 Chemicals

All chemicals used were of analytical grade, purchased from Sigma chemical co. St. Louis, MO, USA, and other drug companies viz BDH, Merck, Qualigens, SRL and SD Fine. CK-MB and SGOT assay kits were procured from Spinreact SA, Spain. Troponin-I ELISA kit from Calbiotech, USA, IL-6 and CRP ELISA kit from Biovendor USA. Immunohisto-staining kit based on horseradish peroxidase (HRP) polymer detection system was purchased from Thermo Fisher Scientific, USA and primary antibodies (Bax mouse monoclonal IgG2 and Bcl-2 mouse monoclonal IgGI) from Santa Cruz Biotechnology, USA. The TUNEL assay kit was purchased from Trivigen Inc Helgerman, Gaithersburg MD.
4.3 Preparation of herbal extracts

4.3.1 Hydroalcoholic extract of *T. arjuna* (HETA)

*Terminalia arjuna* (TA) was obtained from the campus of Institute of Human Behavior and Allied Sciences (IHBAS), Delhi and authenticated by Dr. Sayeed Ahmad, Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry Faculty of Pharmacy, Jamia Hamdard, New Delhi, India. The voucher specimen (BNPL/JH/078/2008) was kept for future reference. The bark was crushed in a mixer to coarse powder (sieve # 60) and then extracted with 50% v/v ethanol thrice by macerating the material in 1: 20 drug: solvent ratio for 24 hrs with occasional shaking at room temperature and sonicating the mixture for 30 min before filtration using 5-6 layers of muslin cloth. The filtrate was first centrifuged and then supernatant was lyophilized to store it for a longer duration. It was kept in a tightly closed bottle, protected from light in the refrigerator at 2 to 8ºC to be used throughout the experiment. The yield of the hydroalcoholic extract was 18.5 % w/w of the dried powder. The HPTLC fingerprint analysis and quantification of arjunolic acid was done for quality control / standardization of extract.

4.3.2 Hydroalcoholic extract of *E. Jambolana* (HEEJ)

*Eugenia jambolana* (EJ) was purchased from local market of Azadpur mandi at Delhi. The identification was done by a botanist and voucher specimen (P-96/6) was kept for future reference in Botanical Garden, Kolkata, India. Fresh fruits of *E. jambolana* were washed thoroughly and seeds were separated from fruit pulp. The pulp was grinded for 10 minutes in a mixer and mixed with 10 volumes of ethanol (50% v/v). It was kept at room temperature for 24 hrs with occasional shaking and sonicated for 30 min, before filtration using 5-6 layers of muslin cloth. The whole procedure was repeated twice again for complete extraction using residue. All three filtrates were pooled, centrifuge at 10,000 rpm and then supernatant was lyophilized.
It was kept in a tightly closed bottle, protected from light in the refrigerator at 2 to 8°C to be used throughout the experiment. The yield of hydroalcoholic extract was 4.7% w/w of fruit pulp.

4.3.3 Phytochemical screening *T. arjuna* and *E. jambolana*

Chemical tests were carried out on the hydroalcoholic extracts of *T. arjuna* and *E. jambolana* for qualitative determination of the presence of secondary metabolites using standard established methods (Trease and Evans, 1989; Harbone, 1973; Sofowora, 1993).

4.3.4 Quantitative analysis of the hydroalcoholic extract of *T. arjuna* by TLC and HPTLC

The sample was prepared by taking 750 mg of hydroalcoholic extract of *T. arjuna* in 25 ml of aqueous acidic solution (HCl 5.0 % v/v in water) and refluxed for one hr on water bath. The extract was filtered and taken into a separating funnel. It was then extracted with chloroform by taking same quantity and the process was repeated three times for complete extraction. The chloroform extracts were pooled and evaporated to dryness. The residue obtained was dissolved in 10 ml of HPLC grade methanol. The stock solution of standard arjunolic acid (purity 98%) was prepared in HPLC grade methanol to get 1.0 mg ml\(^{-1}\) solution. The estimation was carried out as per the method described by Singh *et al* (2010). The samples were spotted in the form of bands of width 4 mm using micro liter syringe on pre-coated silica aluminum sheet 60F\(_{254}\) (5.0×10 cm 0.2 μm thickness) using Camag Linomat V sample applicator (Switzerland). The plates were pre-washed with methanol and activated at 60°C for 20 min prior to chromatography. A constant application rate of 120 nl /sec was employed and space between two bands was 15 mm. The slit dimension was kept at 4×0.30 mm and 20 mm sec\(^{-1}\) scanning speed were employed.
Materials & Methods

The mobile phase consisted of chloroform: toluene: ethanol (4:4:1, v/v/v) and 10 ml of mobile phase was used for per chromatography. Linear ascending development was carried out in 10×10 cm twin trough glass chamber, which was previously saturated with mobile phase for 15 min. The length of the chromatogram run was 80 mm. After the development, TLC plates were dried in a current of air with the help of an air dryer and sprayed with anisaldehyde sulphuric acid reagent and again air dried then kept in oven for 10 min at 110°C. The densitometric scanning was performed on Camag TLC scanner III operated by win Cats software using wavelength 600 nm.

4.3.5 Quantitative analysis of the hydroalcoholic extract of fruit pulp of *E. jambolana* by TLC and HPTLC

The sample was prepared by taking 1g of hydroalcoholic extract of fruit pulp of *E. jambolana* in 10 ml methanol by sonication and centrifuged at 1500 rpm. The supernatant was filtered with 0.22 μm membrane filter and used for HPTLC analysis. The stock solution of standard gallic acid (purity 98%) was prepared in HPLC grade methanol to get 1.0 mg ml⁻¹ solution. The estimation was carried out as per the method described by Singh *et al* (2010). The samples were spotted in the form of bands of width 4 mm using micro liter syringe on pre-coated silica aluminum sheet 60F₂₅₄ (5.0×10 cm 0.2 μm thickness) using Camag Linomat V sample applicator (Switzerland). A constant application rate of 120 nl/sec was employed and space between two bands was 15 mm. The slit dimension was kept at 4×0.30 mm and 20 mm sec⁻¹ scanning speed were employed. The mobile phase consisted of Toluene: Acetone: Glacial acetic acid: Formic acid (7:10:2:1 v/v/v/v) and 10 ml of mobile phase was used for per chromatography. Linear ascending development was carried out in 10×10 cm twin trough glass chamber, which was previously saturated with mobile phase for 15 min. The length of the chromatogram run was 80 mm. After the
development, TLC plates were dried in a current of air with the help of an air dryer.

The densitometric scanning was performed on Camag TLC scanner III operated by win Cats software using wavelength 254 nm.

5. **Induction of myocardial ischemia**

Isoproterenol (ISP) was freshly prepared in normal saline and injected subcutaneously (s. c.) at a dose of 85 mg/kg b. w. to the rats for two consecutive days (28\textsuperscript{th} and 29\textsuperscript{th} day) of the study at an interval of 24 hrs (Rona \textit{et al.}, 1959; Mohanty \textit{et al.}, 2004; Goyal \textit{et al.}, 2010).

6. **Experimental Groups and treatment protocol**

Animals were divided into the following experimental groups and subgroups for the evaluation of various biochemical parameters, cardiac markers, pro-inflammatory cytokines and histopathological changes. Number of animals in each groups were considered eight (n=8/group).

**Group 1: Healthy control**

Rats were administered normal saline for 30 days and then sacrificed on 30\textsuperscript{th} day.

**Group 2: Ischemic control (ISP control)**

Rats were administered isoproterenol (ISP) at dose 85 mg/kg b. w. subcutaneously for two consecutive days (28\textsuperscript{th} and 29\textsuperscript{th} day) at an interval of 24 hr and then sacrificed on 30\textsuperscript{th} day.

**Group 3: Hydroalcoholic extract of \textit{Terminalia arjuna} (HETA) + ISP**

(a)- 100 mg/kg b. w.

(b)- 200 mg/kg b. w.

(c)- 400 mg/kg b. w.
The above treatments were given once a day for 30 days and ISP was injected subcutaneously on 28th and 29th day and then sacrificed on 30th day.

**Group 4: Hydroalcoholic extract of *Eugenia jambolana* (HEEJ) + ISP**

(a) - 100mg/kg b. w.  
(b) - 200mg/kg b. w.  
(c) - 400mg/kg b. w.  

The above treatments were given once a day for 30 days and ISP was injected subcutaneously on 28th and 29th day and then sacrificed on 30th day.

**Group 5: Combination of *T. arjuna* and *E. jambolana* (HETA+HEEJ) + ISP**

(a) - 100mg/kg b. w. (HETA 50+HEEJ 50 mg/kg)  
(b) - 200mg/kg b. w. (HETA 100+HEEJ 100 mg/kg)  
(c) - 400mg/kg b. w. (HETA 200+HEEJ 200 mg/kg)  

The above treatments were given once a day for 30 days and ISP was injected subcutaneously on 28th and 29th day and then sacrificed on 30th day.

**Group 6: α-tocopherol (Vit E) 100 mg/kg b. w. + ISP**

The above treatment was given once a day for 30 days and ISP was injected subcutaneously on 28th and 29th day and then sacrificed on 30th day.

Healthy control and ISP control rats were given normal saline once a day for 30 days. Hydroalcoholic extract of *T. arjuna* (HETA) were administered to group 3 rats in three different doses (100, 200 and 400 mg/kg b. w.) once a day for 30 days. Group 4 rats were administered hydroalcoholic extract of *E. jambolana* (HEEJ) at a dose of 100, 200 and 400 mg/kg b. w. once a day for 30 days. Group 5 rats were administered the combination of HETA and HEEJ at different doses viz.100 (HETA 50 + HEEJ 50 mg/kg), 200 (HETA 100+HEEJ 100 mg/kg) and 400 (HETA 200+HEEJ 200 mg/kg) once a day for 30 days. Group 6 rats were administered Vit E at a
dose of 100 mg/kg b. w. once a day for 30 days. The hydroalcoholic extracts were dissolved in normal saline and supplemented to rats by standard orogastric intubation along with a good excipients gum acacia (5%). Vit E is also supplemented with vehicle gum acacia.

Isoproterenol was freshly prepared in normal saline before injection and injected subcutaneously (s. c) to rats for two consecutive days on 28th and 29th days at an interval of 24hr.

Blood was drawn by retro-orbital venepuncture technique using microcapillary (Sorg and Buckner, 1964). Blood samples were collected in plain vial for estimation of different parameters (MDA, SGOT, CK-MB, Trop I, IL-6 and CRP). Whole blood collected in EDTA vials were used for estimation of GSH and SOD.

7. Estimation of oxidative stress parameters

7.1 Reduced Glutathione (GSH)

Reduced glutathione in erythrocytes was estimated by the method of Beutler et al., (1963).

Principle

The method is based on the development of yellow color when 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) is added to sulphydryl compounds.

Reagents

1. Precipitating reagent: Metaphosphoric acid (1.67 g), Na₂EDTA (0.2 g) and sodium chloride (30 g) were dissolved in distilled water and make up the volume up to 100 ml.
2. Na₂HPO₄ (0.3 M)
3. Na₂EDTA (1 g/l)
4. Sodium citrate (10 g/l)
5. DTNB reagent: 40 mg of DTNB was dissolved in 100 ml of 10 g/l sodium citrate.

6. Glutathione standard (GSH)

**Procedure**

1. 0.2 ml of blood collected in EDTA vial was lysed by addition of 1.8 ml of EDTA.
2. 3 ml of precipitating reagent was added to the hemolysate and mixed. The mixture was allowed to stand for 5 min and then filtered.
3. 4 ml of Na$_2$HPO$_4$ and 1 ml of DTNB reagent were added to 2 ml of filtrate. DTNB was added just before taking the absorbance.
4. A blank was prepared containing 1.2 ml of precipitating reagent, 0.8 ml of EDTA solution, 4 ml of Na$_2$HPO$_4$ and 1 ml of DTNB reagent.

   The absorbance was measured at a wavelength of 412 nm. Results were expressed as mg/dl.

**7.2 Serum malondialdehyde (MDA)**

Serum MDA levels were measured as an index of lipid peroxidation using the colorimetric method as described by Satoh (1978).

**Principle**

Lipid peroxides are precipitated from serum with trichloroaccetic acid (TCA) and heated with thiobarbituric acid (TBA). The reaction results in formation of a pink colored chromogen, which is extracted with n-butyl alcohol. Absorbance of organic phase is determined at 530 nm.

**Reagent**

1. TCA (20%)
2. Sulphuric acid (0.05 M)
3. Sodium sulphate solution (2 M)
4. TBA reagent (0.22% w/v in 2 M sodium sulphate)
5. n-Butyl alcohol
**Procedure**

1. 2.5 ml of TCA was added to 0.5 ml of serum in a test tube and left for 10 min at room temperature.

2. The tube was centrifuged at 3500 rpm for 10 min. After centrifugation, the supernatant was discarded and precipitate was washed twice with sulphuric acid.

3. 2.0 ml of sulphuric acid and 3.0 ml of TBA were added to the precipitate and the mixture was kept in boiling water bath for 30 min to allow coupling of lipid peroxides with TBA.

4. Thereafter, the tube was kept in cold water. After cooling, the resultant chromogen was extracted with 4.0 ml of n-butyl alcohol by vigorous shaking.

5. Organic phase was separated by centrifugation at 3000 rpm for 10 min and its absorbance was determined at wavelength of 530 nm. The values were expressed in terms of MDA concentration in nmol/ml.

**7.3 Erythrocyte superoxide dismutase activity**

Activity of superoxide dismutase (SOD) in erythrocytes was assayed by the method described by Marklund and Marklund (1974) as modified by Nandi and Chatterjee (1988).

**Principle**

The method is based on the ability of the enzyme SOD to inhibit the auto oxidation of pyrogallol.

**Reagent**

1. Na$_2$EDTA (30 mM) Ethylene diamine tetraacetic acid

2. Tris HCl buffer (50 mM, pH 8.5)

3. Pyrogallol solution (2.6 mM), prepared fresh in 10 mM HCl
Procedure

RBCs were washed twice with normal saline and hemolysed with three volumes of cold distilled water. Hemoglobin concentration was measured in the hemolysate by cyanometh hemoglobin method using Drabkin’s reagent. For Hb estimation, 20 µl of hemolysate was mixed with 5 ml of Drabkin’s reagent and incubated for 10 min at room temperature. The absorbance was taken at a wavelength of 530 nm against blank (Drabkin’s reagent). For the assay of SOD, an extract called Tsuchihasi extract was prepared by adding 3.5 ml of cold distilled water, 1 ml of ethanol and 0.6 ml of chloroform to 0.5 ml of hemolysate and vortexed the mixture for 5 min. The tube was centrifuged for 10 min at 3000 rpm. The clear supernatant was used for the enzyme assay.

To adjust the concentration of pyrogallol required to get the rate of change of absorbance per min approximately 0.020-0.023, the reaction was initiated by addition of 100 µl (variable) of freshly prepared 2.6 mM pyrogallol solution in assay mixture containing 100 µl of EDTA and 2.8 ml of Tris buffer (variable to get final volume of 3 ml). As a blank, a mixture of 2.9 ml of Tris buffer and 100 µl of EDTA was used. The rate of increase in absorbance at 420 nm was recorded for 2 min from 1 min to 3 min. The lag of 1 min was allowed for steady state auto oxidation of pyrogallol to be attained.

The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the addition of SOD. To get the volume of supernatant (containing enzyme) required for 50% inhibition, the reaction was carried out by taking different amounts of supernatant (50-300 µl). The reaction was initiated by addition of pyrogallol solution as amount set by prior reaction in a assay mixture containing supernatant (variable, 50-300 µl), 100 µl of EDTA and 2.7 ml of Tris buffer (variable to get final volume of 3 ml). One unit of SOD is described as the amount of enzyme
required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture.

50% inhibition was considered when change in OD/min from 1 min to 3 min at 420 nm was 0.010 to 0.012. Finally the results were expressed as unit per g of hemolysate Hb (U/g Hb).

8. Estimation of Cardiac markers

8.1 Serum Glutamate Oxaloacetate Transaminase (SGOT)

Also known as Aspartate transaminase (AST), present in cardiac muscles, skeletal muscles, kidney and brain. SGOT is a marker of choice for detection for myocardial infarction and it may be elevated due to damage. SGOT was estimated by U.V kinetic method, without pyridoxal phosphate activation, modified by IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) using kits from Centronic Gmbh Germany (Bergmeyer et al., 1986).

Principle

SGOT catalyzes the transfer of amino group between L-Aspartate and α-Ketoglutarate to form Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with NADH in the presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance at 340 nm, which is proportional to the SGOT activity in the sample.

\[
\text{SGOT} \\
\text{L-Aspartate} + \alpha-\text{Ketoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-Glutamate}
\]

\[
\text{MDH} \\
\text{Oxaloacetate} + \text{NADH} + H^+ \rightarrow \text{Malate} + \text{NAD}^+
\]

Reagents

Buffer (R1)

1. Tris-buffer pH 7.8
2. L-aspartate
3. LDH
4. MDH

**Starter reagent (R2)**

1. NADH
2. α-Ketoglutarate

**Procedure**

1. Five volume of buffer (R1) containing (Tris-buffer pH 7.8, L-aspartate, LDH and MDH) was mixed in one volume of starter regent R2 (NADH and α-Ketoglutarate) in 5:1 ratio.

2. After mixing, 100 µl of reaction mixture were taken.

3. 100 µl of serum sample were taken.

4. Both were mixed and incubated for 30 sec.

5. After incubation absorbance (O.D) was taken at 340 nm using kinetic method for 3 min.

6. Difference in absorbance/min (δA/min) was determined.

7. The absorbance was taken at 340 nm and results were expressed in IU/L.

**8.2 Creatine Phosphokinase-MB (CPK-MB)**

Creatine phosphokinase (CPK) is an enzyme that catalyses the transfer of phosphate from creatine phosphate to ADP to form ATP. There are 3 major CPK isoenzymes identified (MM, MB, and BB) that exhibit some degree of tissue specificity. CPK-MM is the principal form in skeletal muscle, both CPK-MM and CPK-MB are present in myocardium and CPK-BB is the predominant form in brain and kidney. By virtue of this differential tissue distribution, there is diagnostic utility in measuring serum levels of CPK isoenzymes. CPK-MB is a well known diagnostic marker of myocardial infarction. Elevation of serum CPK-MB is considered a
reasonably specific marker of acute myocardial infarction (AMI). CPK-MB was measured by immunoenzymatic method (Okinaka et al., 1961).

**Principle**

CPK-MB catalyses the reaction between creatine phosphate (CP) and adenosine di-phosphate (ADP) resulting in the formation of creatine and adenosine triphosphate (ATP). The formed ATP is utilized to phosphorylate glucose producing glucose-6-phosphate (G-6-P) in the presence of hexokinase (HK). Subsequently, G-6-P is converted to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalyzed by glucose-6-phosphate dehydrogenase (G-6-PD). During this oxidation, an equivalent amount of NAD is reduced to NADH. The rate of change of NAD is directly proportional to CPK-MB activity. The change in NAD can be measured spectrophotometrically at 340nm.

\[
\text{CK} \quad \text{CP} + \text{ADP} \rightleftharpoons \text{Creatine} + \text{ATP}
\]

\[
\text{HK} \quad \text{ATP} + \text{Glucose} \rightleftharpoons \text{Glucose-6-phosphate} + \text{ADP}
\]

\[
\text{G-6-PDH} \quad \text{Glucose-6-phosphate} + \text{NADP}^{+} \rightleftharpoons \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+}
\]

**Reagents**

**Buffer**

1. Imidazole pH 6.7
2. Magnesium acetate
3. Glucose
Lyophilisate

1. N-acetylcysteine
2. Phospho creatine
3. EDTA
4. ADP
5. NADP
6. AMP
7. Adenosine5’pentaphospho5’-adenosine
8. Glucose-6-phosphate dehydrogenase hexokinase
9. CK-MM inhibiting antibodies

Procedure

1. The lyophilisate material containing (N-acetylcysteine, Phospho creatine, EDTA, ADP, NADP, AMP, Adenosine 5’pentaphospho5’-adenosine, Glucose-6 phosphate dehydrogenase hexokinase and CPK-MM inhibiting antibodies) was added to 10 ml of buffer (Imidazole pH 6.7, Magnesium acetate, Glucose).
2. Mixed both and kept it for 30 min.
3. Pipetted 1000 µl of reaction mixture (mixture of lyophilisate and buffer) and 40 µl of serum
4. Mixed both properly and leave it for 2 min at 37°C.
5. The increase in absorbance was measured after 2 min following kinetic for every minute for 3 min (δA/min).
6. The absorbance was taken at 340nm and results were expressed in IU/L.

8.3 Cardiac Troponin-I (cTnI)

cTnI and cTnT are specific, sensitive and robust biomarkers of myocardial damage that are released into the serum soon, following tissue pathogenesis and reflect the extent of irreversible myocardial cell injury caused by both natural and
drug-induced diseases in humans and common laboratory species. Concentrations in serum are increased as a result of direct myocardial injury, myocardial ischemia, ventricular strain caused by disease (Fromm, 2007) or drug-induced toxicity (Babuin and Jaffe, 2005; Adamcova et al., 2007). Troponin levels may remain elevated for 7-10 days after an episode of myocardial infarction (Gupta and deLemos, 2007).

**Principle**

The cTnI ELISA test is based on the principle of a solid phase ELISA (O’Brien et al., 2006). The assay system utilizes four unique monoclonal antibodies directed against distinct antigenic determinants on the molecule. Three mouse monoclonal anti-troponin-I antibodies are used for solid phase immobilization. The fourth antibody is in the antibody-enzyme conjugate solution. The test sample is allowed to react simultaneously with the four antibodies, resulting in the troponin-I molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 90 min incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. TMB solution is added and incubated for 20 min, resulting in the development of a blue color. The color development is stopped with the addition of stop solution changing the color to yellow. The concentration of Troponin-I is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

**Kit components**

1. Microwell strips
2. Troponin standards
3. Troponin enzyme conjugate
4. TMB chromogenic substrate
5. Stop solution
Materials & Methods

Assay Procedure

1. 100 μl of standards, specimens and controls into appropriate wells was dispensed
2. 100 μl of enzyme conjugate reagent into each well was dispensed and mixed for 30 sec
3. Incubated at room temperature (18-25°C) for 90 min
4. Incubated mixture was removed by flicking plate contents into a waste container
5. Liquid was removed from all wells and wells were washed three times with 300 μl of 1X wash buffer.
6. 100 μl of TMB reagent was dispensed into each well and gently mixed for 5 sec
7. Incubated at room temperature for 20 min
8. Stopped the reaction by adding 100 μl of stop solution to each well
9. Gently mixed for 30 sec
10. Absorbance was taken at 450nm

Calculations

1. Mean absorbance was taken at 450 nm for each set of reference standards, controls and samples.
2. Standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on graph paper with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, corresponding concentration of troponin-I (ng/ml) from the standard curve was determined.
9. Assessment of pro-inflammatory cytokines

9.1 Interleukin-6 (IL-6)

IL-6 is a predominantly pro-inflammatory cytokine. IL-6 is a non antibody protein and intercellular mediator. Elevated serum IL-6 levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune diseases, inflammations, malignancies and CVD. At the protein sequence level, there is approximately 39% identity between rat and human, and 87% identity between mouse and rat IL-6.

Principle

This assay employs the quantitative sandwich enzyme immunoassay technique as described by Mikaelian et al., 2008. A monoclonal antibody specific for rat IL-6 has been pre-coated onto a micro plate. Standards control and samples are pipetted into the wells. Rat IL-6 bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-6 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of rat IL-6 bound in the initial step. The sample values are then read off the standard curve.

Materials

1. Rat IL-6 micro plate
2. Rat IL-6 conjugate
3. Rat IL-6 standard
4. Rat IL-6 control
5. Assay diluents
6. Calibrator diluents
7. Wash buffer concentrate
8. Color reagent A
9. Color reagent B
10. Stop solution

**Assay procedure**

All reagents and samples were brought to room temperature before use.

1. All reagents, standard dilutions, control and samples were prepared.
2. Added 50 µl of diluent to each well.
3. Added 50 µl of standard, control or sample per well.
4. Mixed by gently tapping and incubated for 2 hrs at room temperature.
5. Aspirated each well and washed.
6. Added 100 µl of Rat IL-6 conjugate to each well.
7. Incubated for 2 hrs at room temperature.
8. Repeated the aspiration/wash as in step 5.
9. Added 100 µl of substrate solution to each well and Incubated for 30 min at room temperature.
10. Added 100 µl of stop solution to each well.
11. The optical density was determined within 30 min, using a micro plate reader at 450 nm.
12. The final IL-6 result in pg/ml

**9.2 C-reactive protein (CRP)**

CRP is the acute-phase protein produced by the liver. The CRP level is elevated in tissue injury, infection or inflammation. CRP is prothrombotic and promotes tissue factor production, macrophage uptake of low-density lipoprotein,
vascular cell adhesion molecule expression and induces monocyte chemo attractant protein-1. Elevated levels of CRP are associated with an increased risk of recurrent events in all of the acute coronary syndromes. Circulating CRP values correlate closely with other markers of inflammation, some of which show similar, albeit generally less significant, predictive associations with coronary events. The attention focused on CRP reflects in part the fact that it is an exceptionally stable analyte in serum or plasma and those immunoassays for it are robust, well standardized, reproducible and readily available. Tissue necrosis is a potent acute-phase stimulus during myocardial infarction. In MI, there is a major CRP response, the magnitude of which reflects the extent of myocardial necrosis. The C-reactive protein assay is intended for the detection and quantification of rat CRP in serum.

**Principle**

Rat serum was diluted (1:4000) and allowed to react with antibodies coated on specially treated micro-wells. After appropriate incubation, the wells were washed to remove non reacted serum proteins, and an enzyme-labeled rabbit anti-rat CRP (conjugate) is then added to react with and tag the antigen-antibody complexes. Following another incubation period, the wells were again washed to remove non reacted conjugate. A urea peroxide substrate with TMB as chromogen was added to start color development. Development of a blue color indicates a positive reaction while negative reactions appear colorless or with a trace of blue. The reaction was interrupted with a stop solution that turns the blue positive reactions to yellow. Negative reactions remain colorless or with a hint of yellow. The absorbance was taken at 450nm.
Kit components

1. 96-well plate containing an affinity purified rabbit anti-rat CRP-IgG
2. Conjugate, HRP-labeled rabbit anti-rat CRP-IgG
3. CRP standard
4. TMB substrate
5. Stop solution

Assay procedure

1. Wash buffer was prepared by adding 1 packet of powder to 1L of distilled water.
2. Standards were prepared as follows.
   - Standard # 1 = 133 ng/ml (diluted provided standard 1:10).
   - Standard # 2 = 44.5 ng/ml (diluted Standard #1 three-fold).
   - Standards # 3 (14.8 ng/ml), standard #4 (4.9 ng/ml) and standard # 5 (1.6 ng/ml) were prepared by serial three-fold dilutions following standard #2.
3. Sample preparation at 1:4000: a) - Serum was diluted 1:1000 as follows.
4. 2 ml of wash buffer was added. To this, added 2 µl of serum. b) Then, diluted 1:4 by adding 1 part of a 1:1000 sample to 3 parts wash buffer.
5. 100 µl was added to each well and incubate at ambient temperature for 30 min.
6. Washed plates 4-5 times with a gentle stream of wash buffer.
7. Diluted stock conjugate (100x) to the desired working dilution (1x) with the tris buffer, to 5 ml buffer, added 50 µl stock conjugate was added.
8. To each microwell, 100 µl of conjugate was added.
9. Plate was covered and incubates for 30 min.
10. To each micro well, 100 μl TMB/substrate solution was added and allowed reaction to proceed at ambient temperature for 5-10 min.

11. Stop reaction by adding 100 μl of Stop solution to each well.

12. Absorbance was taken at 450 nm.

13. Results were expressed in μg /ml

10. **Histopathological studies**

After forty eight hours of first injection of ISP, biochemical parameters, cardiac markers and pro-inflammatory cytokines were estimated. After blood sampling, rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and sacrificed for histopathological studies. Excised hearts were cut in to 3-4 transverse section and processed for histopathological evaluation. Myocardial tissues were fixed in 10% buffered neutral formalin. The tissues were carefully processed for paraffin embedding in automated tissue processor. Blocks were made using plastic moulds and kept under freezing plates to allow the paraffin to solidify. Sections (5 μm thick) of the fixed myocardial tissue were cut using a microtome. These sections were stained with haematoxylin and eosin (H&E) and mounted in DPX and visualized under light microscope to study the histoarchitectural changes of the myocardium. The pathologist performing the histological evaluations was masked to the treatment allocations to reduce bias.

**Procedure outline**

H&E staining method is well established method for paraffin embedded tissues to demonstrate the cell components in their native localization.

- The tissue sections were de-paraffinized as follows: 3 changes of xylene for 5 min each, 2 changes of 100% ethanol for 10 min each, 2 changes of 95% ethanol for 10 min each and 1change of 70% ethanol for 10 min.

- Passed through two changes of distilled water.
Materials & Methods

- Stained with 5% Harris hematoxylin for 10 min.
- Washed under running tap water for 5 min.
- Passed through a change of acid alcohol (25% HCl in 70% alcohol).
- Washed in running tap water for 3-5 min and stained with eosin for 30 sec.
- Dehydrated by dipping in ascending grades of ethanol 70%, 90%, 100%.
- Cleared in 2 changes of xylene for 5 min each and mounted with DPX [189-(2-chloro-N-(4-methoxy-1, 3, 5-triazin-2-yl amino carbonyl) benzene sulphonamide)].
- The slides were examined under light microscope (Nikon, Tokyo, Japan) and photomicrographs were taken.

**Histopathological scoring**

There was a scoring for the degree of severity of histopathological changes. The degree of myocardial necrosis and interstitial edema was graded and scored as follows:

- Absence of any inflammation, edema and necrosis : score (-)
- Single focal area of inflammation, edema and necrosis : score (+)
- Multiple focal areas of inflammation, edema and necrosis : score (++)
- Confluent area of inflammation, edema and necrosis : score (+++)

The histopathological changes were judged as significant if seen in three or more high-power fields.

11. **Determination of myocardial apoptosis and necrosis**

11.1 **Immunostaining for localization of Bax and Bcl-2 proteins**

To determine whether the reduction in cardiomyocyte apoptosis was accompanied by a change in the Bcl-2 and Bax protein, the expression of Bax and Bcl-2 protein in the myocardium was visualized by immunohistochemical analysis. The Bcl-2 and Bax proteins were expressed as percentage of total normal nuclei. The
cardiac tissue specimens preserved in 10% formalin were routinely processed for paraffin embedding. Tissue sections (4-5 µm thick) were cut from paraffin-embedded blocks on a microtome and mounted from warm water (40°C) on to poly-L-lysine coated slides and dried completely. Mouse monoclonal anti-bcl-2 and Bax proteins were used as the primary antibodies for Bcl-2/Bax immunohistochemical staining in the dilution of 1:100. The Ultravision ONE HRP polymer detection system was used to locate primary antibodies by a universal secondary antibody polymer formulation. The amino acid polymer is conjugated to horseradish peroxidase (HRP) and the Fab fragments of secondary antibody. The polymer complex is visualized with an appropriate chromogen/substrate. Briefly, formalin-fixed paraffin-embedded myocardial sections were subjected to the immunohistochemical procedure for the localization of Bax and Bcl-2 proteins using specific mouse monoclonal primary antibodies. Sections are first blocked for endogenous peroxidase and then incubated in primary antibody followed by Ultra Vision One HRP polymer. The target protein (Bax/Bcl-2) was visualized by incubation in peroxidase substrate (H₂O₂) using 3, 3′ diaminobenzidine (DAB) as the chromogen (Miselli et al., 2008).

Test principle

Immuno enzymatic staining methods utilize enzyme substrate reaction to convert color less chromogens in to coloured end products. The hematin of peroxidase forms a complex with H₂O₂ and causes it to decompose in to H₂O and atomic O₂. However, peroxidase activity in the presence of electron donor (DAB) first results in the formation of an enzyme-substrate complex and then in the oxidation of the electron donor. DAB upon oxidation produces a brown product, which is highly insoluble in alcohol.
Tissue section preparation

- The glass slide were cleaned with hydrochloric acid (1%), ethanol (95%), treated with subbing solution, coated with poly-L-lysine (10%) and then air-dried.

- 4-5µ thick tissue sections were cut using microtome and applied to slides. The section were deparaffinized as follows: 3 changes of xylene for 5 min each, 2 changes of 100% ethanol for 10 min each, 2 changes of 95% ethanol for 10 min each and 1 change of 70% ethanol for 10 min. These sections were then washed in de-ionized water. The excess liquid was aspirated from slides.

- To unmask antigens by heat treatment, the slides were placed in coupling jars containing 10 mM sodium citrate, pH 6.0, covered and heated at 95 °C for 5 min. The slides were allowed to cool in the buffer for 20 min and then washed in de-ionized water three times for 2 min each on a stir plate. The excess liquid was aspirated from slides.

Immunostaining for Bax and Bcl-2 proteins

- All subsequent steps were carried out room temperature in a humidified chamber. Prior to the use of immunostaining system, regents were allowed to stand at room temperature. Tissue sections were not allowed to dry out at any time during the procedure.

- To quench endogenous peroxidase activity, the specimens were incubated in 1-3 drops of peroxidase block (3% H₂O₂ prepared in methanol) for 10 min. The slides rinsed in PBS for 2 min and the excess wash buffer was aspirated from slides.

- The specimens were then incubated in 1-3 drops of serum block for 30 min to prevent non-specific binding to collagen and connective tissue. The excess buffer was wiped from the slides.
• The blocking serum was drained out. Antibody against Bax/Bcl-2 was pre-diluted (1:100) in the serum block (5% normal goat serum in PBS). The specimens were incubated with one of the primary antibody: Bax or Bcl-2. Precaution was taken to see that the diluted primary antibody added was of sufficient volume to cover the tissues. The specimens were incubated overnight in refrigerator. After the completion of incubation duration the slides were washed in PBS thoroughly.

• The sections were thereafter incubated in 1-3 drops of HRP-polymer detection fluid for 30 min. Subsequently, the sections were rinsed in PBS for 2 min on a stir plate. The excess liquid was aspirated from slides.

• 1-3 drop of HRP substrate chromogen reaction mixture was added to each slide for 10 min for color product formation at the site of reaction. The sections were rinsed in de-ionized water after viewing for color development under light microscope.

• As described earlier, the slides were counterstained with haematoxylin for 1 min and immediately washed with several changes of deionized water. The specimens were de stained with acid alcohol and subsequently washed with tap water.

• The sections were then dehydrated by passing through ascending grades of ethanol: 1 change of 70% ethanol and 1 change of 100% ethanol each and 2 changes of xylene for 5 min each. The excess xylene was washed from edges of slides.

• Subsequently, 1-2 drops of permanent mounting medium (DPX) was added and covered with glass cover slip. The slides were then observed under light microscope.
Immunohistochemical control

The immunohistochemical negative tissue control included eliminating the primary antibody and replacing species specific antiserum with normal serum of the appropriate species (normal goat serum) i.e. do not contain the relevant tissue marker. However positive tissue controls: tonsil for Bcl-2 and carcinoma breast for Bax was also run. These controls were processed identically to the specimen and contained the target protein. These procedure controls (both positive and negative controls) serve to ascertain primarily whether the staining protocols were followed correctly, whether day to day, worker to worker variation have been occurred and whether reagents continue to be in good working condition and there is no background due to non-specific staining. All descriptions and the pictures are based on the specific staining adjusted against these controls.

11.2 Western blot analysis for quantification of Bax, Bcl-2 and TNF-α

Principle

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electro-transferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate. A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to ‘block’ the nonspecific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated
with a second antibody, which is specific for the first antibody. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (eg. crude cell extract) of other proteins by western blotting.

**Material**

Primary antibodies (mouse monoclonal), anti Bcl-2, anti Bax, anti TNF-α were utilized, which was obtained from Cell Signaling Technology Inc (Danvers, MA, USA) and Goat anti rabbit/mouse-HRP conjugate secondary antibody was procured from Santa Cruz Biotechnology.

**Procedure**

Western blotting was performed as described by Maheshwari et al., (2009 and 2011). Cardiac tissue lysates were prepared in 200 μl lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM β-glycerophosphate, 1% Triton X-100, 150 mM NaCl, 10% Glycerol and Protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were clarified by centrifugation at 12,000 x g at 4°C for 20 min and the protein concentration of the supernatant was determined with the Bradford assay. For SDS-PAGE, protein lysates were mixed with Laemmli sample buffer (Bio-Rad Laboratories, PA, USA) and boiled for 10 min. Total protein (50-100 μg) was separated on a 12/15% gel and transferred to nitrocellulose membrane (Millipore, Billerica, USA) using wet-blotting apparatus (Bio-Rad Laboratories, Philadelphia, PA, USA). After blocking in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.6) with 5% skimmed milk, membranes were incubated with the primary
antibodies (1:500) diluted in TBST for 2 hours at room temperature. Next, membranes were washed three times with TBST and incubated for an additional 2 hrs with HRP linked secondary antibody (1:2000) diluted in TBST. Again membranes were washed three times with TBS and labelled protein bands were visualized with the DAB system (Bangalore Genei, Bangalore, India). β-actin was used to monitor equal loading of protein. Densitometric analysis was performed with the help of Image analysis software (Lab Works Image analysis software 4.0, UVP, Upland, CA, USA.

11.3 Terminal Deoxynucleotidyl Transferase-mediated dUTP nick end labeling (TUNEL assay)

Myocardial apoptosis can be quantitatively analyzed by detecting DNA fragmentation using TUNEL technique (Gavrieli et al., 1992; Gorczyca et al., 1993). This method takes advantage of DNA fragmentation, characteristic of apoptosis. The DNA breaking points (nick) expose the 3'OH ends of DNA, which can be labeled thus, allowing the identification of apoptotic cells. Residues of digoxigenin nucleotides can be incorporated in to 3' OH ends of DNA with the aid of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) to identify apoptotic cells. A secondary reaction with antibodies is used to detect the nicks.

Procedure

- Paraffin embedded tissue sections
- Dewaxed/rehydrated
- Permeabilization of samples
- Addition of TUNEL reaction mixture
- Incubate with converter-POD
Materials & Methods

- Addition of substrate solution
- Analysis of substrate solution

The sections were de-paraffinized and rehydrated according to the standard protocols. The tissue sections were heated at 60\(^\circ\)C and dewaxed by washing in 3 changes of xylene for 5 min each. Deparaffinized tissue sections were washed by immersing the slides in 100% ethanol followed by rehydration of the samples by subsequently immersing the slides through graded alcohol washes 2 changes of 100% ethanol for 10 min each, 2 changes of 95% ethanol for 10 min each, followed by 1 change of 70% ethanol wash for 10 min. The sections were subsequently washed in deionized water. The excess liquid was aspirated from slides.

- All slides were washed in PBS for 10 min and thereafter slides were processed for TUNEL assay using kit from Roche Diagnostics, USA.
- The slides were incubated in 0.1% Triton X prepared in 0.1% sodium citrate for 8 min to permiabilize the cell membrane. The slides were rinsed with PBS twice.
- Blocking solution (3% H\(_2\)O\(_2\) in methanol) was added to the tissue specimens for 10 min at 15-25\(^\circ\)C. The slides were rinsed thoroughly with PBS twice.
- TUNEL reaction mixture was prepared by adding 50 µl of enzyme solution (terminal deoxynucleotidyl transferase enzyme isolated from calf serum) to 450 µl of label solution (biotinylated nucleotide mixture in reaction buffer).
- The free ends of cellular DNA were labeled by incubating the slides in 50 µl of TUNEL reaction mixture for 60 min at 37\(^\circ\)C in a humidified chamber. To ensure a homogenous spread of TUNEL reaction mixture across the tissue specimens and to avoid evaporative loss, samples were covered with cover slip and sealed the
tissue with a hydrophobic barrier using a barrier pen during incubation. The slides were rinsed thoroughly with PBS thrice for 5 min each.

• The area around the sample was dried. The samples were incubated in 50-100µl of POD substrate containing streptavidin conjugated to horseradish peroxidase (HRP) for 10 min at 15-25°C. The slides were rinsed thoroughly with PBS thrice for 5 min each.

• 1-3 drops of HRP substrate(1.6 ml de-ionized water, 5 drops of substrate buffer and 1 drop of 50x DAB (3,3’ diaminobenzidine) chromogen and 1 drop of peroxidase substrate was added to each slides for 10 min until a light brown color developed. The reaction was stopped by incubating the sections in de-ionized water for 10 min.

• The slides were counterstained in haematoxylin for 1-2 min, immediately washed with several changes of de-ionized water. The specimens were subsequently de-stained with acid alcohol and washed with tap water.

• The sections were then dehydrated by passing through ascending grades of ethanol: 1 change of 70% ethanol, 1 change of 90% ethanol each and 1 change of 100% ethanol, 2 changes of xylene for 5 min each. The excess xylene was washed from edges of the slides.

• Subsequently, 1-2 drops of permanent mounting medium (DPX) was added and covered with glass cover slip. The slides were then observed under light microscope.

• Total cell counts and TUNEL positive cells in the specimens were determined by means of light microscope. 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.

**TUNEL assay control**

For positive control, subsequent cells were treated with DNAase for 10 min at 37°C and the same procedure of TUNEL assay as discussed above was performed. All descriptions and pictures are based on specific staining as adjusted against controls.

**12. Statistical analysis**

The results were presented as mean ± SEM for 8 animals in each group. The data was analyzed by analysis of covariance (ANCOVA) taking baseline as a covariant. Multiple comparisons among the groups were done by Bonferroni adjustment method and statistical differences between mean values were determined using SPSS software version 17.0. A value of p<0.05 was considered statistically significant.

In this study out of three time points (0th, 21st, 30th) only two time point (0th and 30th) were used, 21st days data were excluded from the study because ischemia was induced on 28th and 29th days and 21st days rats were normal. There was no significant difference between 0th day and 21st day values, therefore they were excluded from the study.
Figure 20- Experimental and operative setup