Cardioprotective effect of a chronic treatment of *Ginkgo biloba* Phytosomes in isoproterenol-induced cardiac necrosis in rats: Involvement of antioxidant system

Vandana S. Panda*, Suresh R. Naik

Abstract

The present study investigates the cardioprotective effect and antioxidant activity of a chronic treatment of Ginkgo biloba Phytosomes (GBP) in isoproterenol (ISO) induced cardiac necrosis in rats. Pretreatment of GBP 100 mg/kg daily for 30 & 45 days to rats treated with ISO (85 mg/kg, s.c) on the last 2 days, resulted in a significant cardioprotective activity reflected by attenuation of the ISO-elevated levels of serum marker enzymes (aspartate aminotransferase, lactate dehydrogenase & creatine phosphokinase) and malondialdehyde, and restoration of the activities and levels of the ISO-depleted marker enzymes, reduced glutathione and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase & glutathione reductase) in the heart. However, oral administration of GBP 200 mg/kg to ISO treated rats exhibited lesser cardioprotective effect than GBP 100 mg/kg. It may be concluded that GBP 100 mg/kg oral treatment to ISO challenged rats augments endogenous antioxidants of rat heart, enhances scavenging of free radicals and inhibits lipid peroxidation of membrane, thereby salvaging the myocardium from the deleterious effects of ISO.

Keywords: *Ginkgo biloba*, Phytosomes, Chronic treatment, Cardioprotective, Isoproterenol, Antioxidant activity.

Introduction

Myocardial Infarction (MI) is the acute condition of necrosis of the myocardium that occurs as a result of prolonged myocardial ischemia, where there is oxygen deprivation to the heart muscle due to interruption of blood supply to an area of the heart. Restoration of blood flow to the ischemic myocardium (reperfusion) is accompanied by the generation of ROS which cause vascular and microvascular injury, dysfunction of endothelial cells, myocyte edema, increased myocyte apoptosis, increased myocyte necrosis and cardiac contractile dysfunction. Among the molecules attacked by oxygen free radicals are proteins, carbohydrates, nucleic acids & phospholipids of plasma membranes. Free radicals have been implicated in diseases such as heart disease, diabetes, cancer, Alzheimer’s, Parkinson’s, cataracts, rheumatoid arthritis and in the process of aging.

Isoproterenol (ISO), a synthetic catecholamine and β adrenergic agonist is documented to produce myocardial infarction in large dose. On auto-oxidation, it generates highly cytotoxic free radicals, which are known to stimulate peroxidation of membrane phospholipids and cause severe damage to the myocardial membrane.
**Ginkgo biloba** (Family: Ginkgoaceae) is an important plant of the Traditional Chinese Medicine (TCM) and is the only surviving member of Ginkgo, which is the oldest living tree species on earth. The extracts of the leaves of Ginkgo biloba have been found to possess cardioprotective, antiasthmatic, antidiabetic, hepatoprotective and potent CNS activities. Chemically, the active constituents of *G. biloba* leaf is flavone glycosides (kaempferol, quercitin and isorhamnetin), diterpene lactones namely Ginkgolides A, B, C, M, J & bilobalide and the biflavones ginkgetin, isoginkgetin, bilobetin. The constituents of *G. biloba* are potent scavengers of free radicals. By scavenging free radicals and ROS, *G. biloba* inhibits lipid peroxidation and augments levels of endogenous antioxidants.

Literature reports extensive work on the cardioprotective activity of *Ginkgo biloba* extracts (EGb). Most studies have shown EGb to improve the recovery of post ischemic cardiac function (coronary flow, aortic flow, LVDp and its first derivative) in the ischemic reperfused myocardium. It has been demonstrated that EGb protects the heart by its antioxidant properties and its ability to adjust fibrinolytic activity. In one study, EGb diminished the increase of myocardial ascorbate content after 40 min of ischemia and 20 min of reperfusion and also suppressed the increase of dehydroascorbate. In another study, vitamin E depletion observed in case of ischemia/reperfusion in rat heart was spared by EGb. Microvascular endothelium protecting effects of EGb on the myocardium of rats subjected to acute isobaric hypoxia as well as oxidized LDL in separate studies have been reported. Research work of Varga et al indicates that EGb may act as a potent inhibitor of NO production under the conditions of ischemia/reperfusion, thereby improving the recovery of post ischemic cardiac function.

Phytosomes are advanced herbal liposomes produced by binding individual components of herbal extracts to Phosphatidyl choline, resulting in a dosage form that is better absorbed and thus, produces a better therapeutic effect than the conventional herbal extracts. EGb has been reported to significantly increase the total antioxidant plasma capacity (by 24.5% TRAP; 27.9% FRAP) only when complexed with phospholipids.

With this background, the present study was designed to investigate the cardioprotective effect of chronic treatment of *Ginkgo biloba* Phytosomes (GBP) in ISO induced cardiac necrosis using an in vivo rat model. It is proved that in clinical conditions of myocardial ischemia, chronic drug treatment results in maximum recovery of cardiac function. This study evaluates the effect of GBP treatment on the battery of antioxidant enzymes, marker enzymes and histoarchitectural changes in the rat myocardium.

**Materials and Methods**

**Plant material**

*Ginkgo biloba* Phytosomes (Ginkgoselect) was a gift sample from Indena, Italy.

Ginkgoselect Phytosomes are prepared by reacting a stoichiometric amount (3:1 w/w) of soy phospholipids with Ginkgo biloba extract (EGb). EGb contains ≥ 24% of ginkgoflavonglucosides, ≥ 6% of ginkgolides & bilobalide and ≤ 5 ppm of ginkgoic acids.

**Drugs and chemicals**

Thiobarbituric acid (TBA), reduced glutathione, oxidized glutathione and NADPH were obtained from Himedia Laboratories, Mumbai, India. 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB), isoproterenol and epinephrine were purchased from Sigma Chemical Co., St. Louis, USA. All other chemicals were obtained from local sources and were of analytical grade.

**Experimental animals**

Wistar albino rats (150-200 g) and Haffkine albino mice (20-25 g) of either sex were used. They were housed in clean polypropylene cages under standard conditions of humidity (50 ± 5 %), temperature (25 ± 2°C), light (12h light/12h dark cycle) and fed with standard diet (Amrut Laboratory Animal Feed, Nava Maharashtra Chakan Oil Mills, Pune, India) and water ad libitum. All animals were handled with humane care.

Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No.25/1999/CPCSEA) and conform to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research.

**ISO dose fixation**

Our earlier results of dose finding studies indicated that ISO 85 mg/kg given subcutaneously produced moderate necrosis in rat heart and a significant alteration in

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biochemical parameters, hence this dose was chosen as the toxicant dose in the present study.\textsuperscript{23}

**Preparation of GBP and ISO solutions**

*G. biloba* Phytosomes were dissolved in distilled water and used.

Isoproterenol was dissolved in distilled water and used immediately for subcutaneous administration.

**Experimental procedure**

Wistar rats after acclimatization (6-7 days) in the animal quarters were randomly divided into 10 groups of 6 animals each. The treatment regimen was as follows:

- **Group I** – termed as N30 served as Normal Control and received distilled water (1 ml/kg, p.o.) daily for 30 days and in addition received distilled water (0.5 ml/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group II** – termed as N45 served as Normal Control and received distilled water (1 ml/kg, p.o.) daily for 45 days and in addition received distilled water (0.5 ml/kg, s.c.) on the 44th and 45th day at an interval of 24h.

- **Group III** – termed as I30 served as ISO Control and received distilled water (1 ml/kg, p.o.) daily for 30 days and in addition received ISO (85 mg/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group IV** – termed as I45 served as ISO Control and received distilled water (1 ml/kg, p.o.) daily for 45 days and in addition received ISO (85 mg/kg, s.c.) on the 44th and 45th day at an interval of 24h.

- **Group V** – termed as GBP\textsubscript{100}30, received GBP (100 mg/kg, p.o.) daily for 30 days and in addition received distilled water (0.5 ml/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group VI** – termed as GBP\textsubscript{200}30, received GBP (200 mg/kg, p.o.) daily for 30 days and in addition received distilled water (0.5 ml/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group VII** – termed as GBP\textsubscript{100}45, received GBP (100 mg/kg, p.o.) daily for 45 days and in addition received ISO (85 mg/kg, s.c.) on the 44th and 45th day at an interval of 24h.

- **Group VIII** – termed as GBP\textsubscript{200}45, received GBP (200 mg/kg, p.o.) daily for 45 days and in addition received ISO (85 mg/kg, s.c.) on the 44th and 45th day at an interval of 24h.

- **Group IX** – labeled as IGBP\textsubscript{100}30, received GBP (100 mg/kg, p.o.) daily for 30 days and in addition received ISO (85 mg/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group X** – labeled as IGBP\textsubscript{200}30, received GBP (200 mg/kg, p.o.) daily for 30 days and in addition received ISO (85 mg/kg, s.c.) on the 29th and 30th day at an interval of 24h.

- **Group XI** – labeled as IGBP\textsubscript{100}45, received GBP (100 mg/kg, p.o.) daily for 45 days and in addition received ISO (85 mg/kg, s.c.) on the 44th and 45th day at an interval of 24h.

- **Group XII** – labeled as IGBP\textsubscript{200}45, received GBP (200 mg/kg, p.o.) daily for 45 days and in addition received ISO (85 mg/kg, s.c.) on the 44th and 45th day at an interval of 24h.

Rats were sacrificed 24 h after the last subcutaneous injection by cervical dislocation. Blood was collected by cardiac puncture and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and used for the estimation of marker enzymes viz., AST, LDH & CPK.

The hearts were dissected immediately, washed with ice-cold saline and divided into 2 equal parts. One part was used to prepare 10% homogenate in phosphate buffer (50mM, pH 7.4). An aliquot was used for the determination of lipid peroxidation (LPO). The homogenates were centrifuged at 7000 × g for 10 min at 4°C and the supernatants were used for the assays of AST, LDH, CPK, GSH, SOD, CAT, GPX and GR. The remaining part of heart was fixed at 10% buffered formalin and used for histological studies.

**Marker Enzyme Assays**

The marker enzymes AST, LDH & CPK were assayed in serum and heart using standard kits supplied from Accurex Biochemicals, Mumbai, India and Erba Mannheim, Germany. The results were expressed as IU/L for AST, LDH & CPK.

**Protein Estimation**

The levels of total proteins were determined in heart homogenates of experimental animals by the method of Lowry et al using bovine serum albumin as standard.\textsuperscript{24}

**Lipid Peroxidation**

The quantitative estimation of LPO was done by determining the concentration of Thiobarbituric Acid
Reactive Substances (TBARS) in the heart using the method of Ohkawa, Ohishi, and Yagi. The amount of malondialdehyde (MDA) formed was quantified by reaction with TBA and used as an index of lipid peroxidation. The results were expressed as nmol of MDA/g of wet tissue using a molar extinction coefficient of the chromophore (1.56 × 10-5/M/cm) and 1,1,3,3-tetraethoxypropane as standard.

**Glutathione Estimation**

GSH was estimated in the heart homogenate using DTNB by the method of Ellman. The absorbance was read at 412 nm and the results were expressed as µmol of GSH/g of wet tissue.

**Antioxidant Enzyme Assays in heart homogenate**

Superoxide dismutase (SOD) was assayed by the method of Sun & Zigman in which the activity of SOD was inversely proportional to the concentration of its oxidation product adrenochrome, which was measured spectrophotometrically at 320 nm. 1 unit of SOD activity is defined as the enzyme concentration required to inhibit the rate of auto-oxidation of epinephrine by 50% in 1 min at pH 10.

Catalase (CAT) was estimated by the method of Clairborne, which is a quantitative spectroscopic method developed for following the breakdown of H2O2 at 240 nm in unit time for routine studies of catalase kinetics.

Glutathione peroxidase (GPx) estimation was carried out using the method of Rotruck et al, which makes use of the following reaction.

\[
\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG} \text{ (oxidized glutathione)}.
\]

GPx in the tissue homogenate oxidizes glutathione and simultaneously, H2O2 is reduced to water. This reaction is arrested at 10 min using trichloroacetic acid and the remaining glutathione is reacted with DTNB solution to result in a colored compound, which is measured spectrophotometrically at 420 nm.

Glutathione reductase (GR) activity was determined by using the method of Mohandas et al in which the following reaction is implicated.

\[
\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2 \text{GSH}
\]

In the presence of GR, oxidized glutathione undergoes reduction and simultaneously, NADPH are oxidized to NADP+. Enzyme activity is quantified at room temperature by measuring spectrophotometrically at 340 nm, the disappearance of NADPH/min.

**Histological Studies**

The parts of the hearts which were stored at 10% (w/v) buffered formalin were embedded in paraffin, sections cut at 5 µm and stained with hematoxylin and eosin. These sections were then examined under a light microscope for histo-architectural changes.

**Statistical Analysis**

The results of cardioprotective and antioxidant activities are expressed as mean ± SEM from 6 animals in each group. The results were statistically analyzed using one-way ANOVA followed by Tukey-Kramer post test for individual comparisons. P<0.05 was considered significant. GraphPad InStat version 3.00 of GraphPad Software Inc., San Diego, USA was the software used for statistical analysis.

**Results**

**Biochemical parameters**

The effect of GBP of serum marker enzymes AST, LDH & CPK for 30 and 45 days is shown in Figures 1 & 2 respectively. Rats treated with ISO showed a significant increase (p<0.001) in the serum activities of the marker enzymes when compared with the normal group of rats. Pretreatments with GBP 100 mg/kg (IGBP 100 30 group) & 200 mg/kg (IGBP 200 30 group) for 30 days followed by ISO treatment in rats decreased significantly (p<0.001) the elevated activities of these enzymes, but not to normal. GBP 100 mg/kg (IGBP 100 45 group) & 200 mg/kg (IGBP 200 30 group) administered orally to rats for 45 days with subcutaneous ISO treatment on the 44th and 45th day showed a significant decrease (p<0.001) in the serum activities of AST, LDH & CPK when compared to the ISO control group. It was observed that GBP 100 mg/kg treatment to rats for 45 days (IGBP 100 45 group) was more effective than the corresponding GBP 200 mg/kg treatment (IGBP 200 45 group) in lowering the ISO elevated serum marker enzyme activities.
Marker enzyme activities were also assayed in hearts. The effect of GBP treatment for 30 and 45 days on marker enzymes in heart is shown in Figures 3 & 4 respectively. The ISO control group exhibited a significant decrease (p<0.001) in AST, LDH & CPK activities in the heart when compared with the normal group of rats. Treatment of GBP 100 mg/kg (IGBP100 30 group) as well as 200 mg/kg (IGBP200 30 group) for 30 days with ISO treatment on the 29th & the 30th day to rats, increased significantly the activities of AST (p<0.05), LDH (p<0.001) & CPK (p<0.001) when compared with only ISO treated rats. In the GBP 45 day treatment, GBP 100mg/kg (IGBP1045 group) increased significantly the activities of AST (p<0.01), LDH (p<0.001) & CPK (p<0.001) in the heart homogenates of ISO myocardial infarcted animals. GBP 200 mg/kg dose (IGBP20045 group), however, could significantly increase the activities of only AST (p<0.05) & CPK (p<0.001).
The effects of GBP on antioxidant enzymes, GSH and LPO are summarized in Tables 1& 2 for 30 days & 45 day treatments respectively.

Table 1: Effect of GBP on heart MDA, GSH, SOD, CAT, GPx and GR in ISO intoxicated rats in a 30-day treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA nmol /g Wet tissue</th>
<th>GSH μmol /g Wet tissue</th>
<th>SOD u/mg protein</th>
<th>CAT u/mg protein</th>
<th>GPX u/mg protein</th>
<th>GR u/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N45)</td>
<td>33.51 ± 2.61</td>
<td>2.02 ± 0.10</td>
<td>10.74 ± 1.01</td>
<td>21.24 ± 1.09</td>
<td>0.33 ±0.01</td>
<td>15.9 ± 1.55</td>
</tr>
<tr>
<td>ISO control (I45)</td>
<td>84.2± 4.93a</td>
<td>1.34 ± 0.07a</td>
<td>5.64± 0.48a</td>
<td>11.21±0.64a</td>
<td>0.19 ± 0.01a</td>
<td>7.5 ± 0.72</td>
</tr>
<tr>
<td>GBP10045</td>
<td>31.8 ± 1.0</td>
<td>2.17 ± 0.08</td>
<td>10.30 ± 0.02</td>
<td>22.45 ± 0.46</td>
<td>0.35 ± 0.01</td>
<td>15.6 ± 0.33</td>
</tr>
<tr>
<td>GBP20045</td>
<td>36.9 ± 1.82</td>
<td>1.95 ± 0.11</td>
<td>10.12 ± 0.076</td>
<td>20.68 ± 0.49</td>
<td>0.32 ± 0.02</td>
<td>15.02 ± 0.34</td>
</tr>
<tr>
<td>IGBP10045</td>
<td>40.44 ± 1.13b</td>
<td>1.88 ± 0.06b</td>
<td>8.68 ± 0.05b</td>
<td>20.61 ± 0.53b</td>
<td>0.31±0.01b</td>
<td>13.07 ± 0.28</td>
</tr>
<tr>
<td>IGBP20045</td>
<td>72.53 ± 2.26</td>
<td>1.55 ± 0.05</td>
<td>6.42 ± 0.20</td>
<td>14.63 ± 0.47</td>
<td>0.22 ± 0.01</td>
<td>7.82 ± 0.36</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.; N = 6 in each group

P values: a < 0.001 when ISO group compared with Normal group; b < 0.05 when IGBP groups compared with ISO control; c < 0.01 when IGBP groups compared with ISO control; d <0.001 when IGBP groups compared with ISO control

1 unit of CAT = μmol H₂O₂ consumed / min / mg protein
1 unit of GPX = μg GSH utilized / min / mg protein
1 unit of GR = nmol NADPH oxidized / min / mg protein

Table 2: Effect of GBP on heart MDA, GSH, SOD, CAT, GPx and GR in ISO intoxicated rats in a 45-day treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA nmol /g Wet tissue</th>
<th>GSH μmol /g Wet tissue</th>
<th>SOD u/mg protein</th>
<th>CAT u/mg protein</th>
<th>GPX u/mg protein</th>
<th>GR u/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N30)</td>
<td>33.51 ± 2.61</td>
<td>2.02 ± 0.10</td>
<td>10.74 ± 1.00</td>
<td>21.24 ± 1.09</td>
<td>0.33 ±0.01</td>
<td>15.9 ± 1.54</td>
</tr>
<tr>
<td>ISO control (I30)</td>
<td>84.20± 4.93a</td>
<td>1.34 ± 0.07a</td>
<td>5.64± 0.48a</td>
<td>11.20±0.64a</td>
<td>0.19 ± 0.01a</td>
<td>7.5 ± 0.72a</td>
</tr>
<tr>
<td>GBP10030</td>
<td>37.73 ± 1.74</td>
<td>2.22 ± 0.06</td>
<td>10.28 ± 0.04</td>
<td>20.86 ± 0.3</td>
<td>0.31 ± 0.01</td>
<td>15.75 ± 0.03</td>
</tr>
<tr>
<td>GBP20030</td>
<td>35.68 ± 2.11</td>
<td>1.91 ± 0.13</td>
<td>9.90 ± 0.16</td>
<td>20.18 ± 0.25</td>
<td>0.32 ± 0.01</td>
<td>15.9 ± 1.55</td>
</tr>
<tr>
<td>IGBP10030</td>
<td>44.20±2.60d</td>
<td>1.83 ± 0.05d</td>
<td>8.42 ± 0.11 b</td>
<td>19.76 ± 0.18d</td>
<td>0.32 ± 0.01d</td>
<td>13.23 ± 0.09d</td>
</tr>
<tr>
<td>IGBP20030</td>
<td>65.16±1.70c</td>
<td>1.65 ± 0.05</td>
<td>7.83 ± 0.24</td>
<td>17.00 ± 1.45c</td>
<td>0.3 ± 0.01d</td>
<td>10.26 ± 0.48</td>
</tr>
</tbody>
</table>
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Values are mean ± S.E.M.; N = 6 in each group

**P values:** # < 0.001 when ISO group compared with Normal group; * < 0.05 when IGBP groups compared with ISO group; a < 0.01 when IGBP groups compared with ISO group; b < 0.001 when IGBP groups compared with ISO control

1 unit of CAT = µmol H₂O₂ consumed / min / mg protein
1 unit of GPX = µg GSH utilized / min / mg protein
1 unit of GR = nmol NADPH oxidized / min / mg protein

MDA, the myocardial lipid peroxidation marker was significantly elevated (p<0.001) in the ISO control group of rats in comparison with the normal group. Pretreatment of GBP 100 mg/kg (IGBP₁₀₀₃₀ group) as well as 200 mg/kg (IGBP₂₀₀₃₀ group) for 30 days to ISO intoxicated rats, depleted significantly (p<0.001) the increased levels of MDA due to ISO. For the 45 days treatment, GBP 100 mg/kg (IGBP₁₀₀₄₅ group) significantly lowered (p<0.001) the ISO elevated levels of MDA, whereas pretreatment with GBP 200 mg/kg (IGBP₂₀₀₄₅ group) for 45 days did not elicit any significant depletion of the ISO elevated MDA levels.

Significant decline in myocardial GSH (p<0.001) was observed in the ISO treated group when compared with the normal rats. Pretreatment with GBP 100 mg/kg orally for 30 as well as 45 days followed by ISO treatment of rats, significantly increased the myocardial levels of GSH (p<0.001); whereas pretreatment with GBP 200 mg/kg orally for 30 & 45 days failed to restore significantly the ISO depleted GSH levels.

ISO treatment induced a significant depletion of antioxidant enzymes SOD (p<0.001), CAT (p<0.001), GPx (p<0.001) & GR (p<0.001) when compared to normal values. Pretreatment with GBP 100 mg/kg for 30 days (IGBP₁₀₀₃₀ group) followed by ISO injection on the 29th & the 30th day to rats, significantly restored the activities of SOD (p<0.05), CAT (p<0.001), GPx (p<0.001) & GR (p<0.001) depleted by ISO. Similarly, pretreatment of rats with GBP 100 mg/kg for 45 days (IGBP₁₀₀₄₅ group) and ISO injection on the 44th & 45th day significantly restored the depleted activities of SOD (p<0.01), CAT (p<0.001), GPx (p<0.001) & GR (p<0.01) when compared to only ISO treatment. GBP 200 mg/kg treatment for 30 & 45 days to ISO challenged rats could only significantly increase the activities of CAT (p<0.01) and GPx (p<0.001).

Oral treatment of GBP 100 mg/kg (GBP₁₀₀₃₀ & GBP₁₀₀₄₅ groups) as well as 200 mg/kg (GBP₂₀₀₃₀ & GBP₂₀₀₄₅ groups) per se to rats did not adversely affect the basal levels of MDA, GSH, SOD, CAT, GPx & GR, nor were they significantly elevated in comparison with the normal rats.

**Histopathology**

Histopathological examination of the myocardium of normal rats showed the clear integrity of myocardial cell membrane (Figure 5). Endocardium and pericardium were seen within normal limits. No inflammatory cell infiltration was seen. 85 mg/kg ISO treated rats showed moderate to marked myocytic necrosis with moderate infiltration of lymphocytes and macrophages (Figure 6). The changes were more prominent along the endocardium and in papillary muscles. Hearts of rats treated with GBP 100 mg/kg for 45 days and challenged with ISO (IGBP₁₀₀₄₅ group) showed minimal myonecrotic patches with minimal inflammatory cell infiltration (Figure 7). There was mild to moderate focal myonecrosis with moderate diffused infiltration of lymphocytes in heart sections of GBP 100 mg/kg treatment for 30 days to ISO infarcted rats (IGBP₁₀₀₃₀ group), (Figure 8). Figure 8 showed almost same cardioprotection as Figure 7.

In rats treated with GBP 200 mg/kg for 30 days followed by ISO injection (IGBP₂₀₀₃₀ group), gradation of myocardial damage was more than in IGBP₁₀₀₃₀ & IGBP₁₀₀₄₅ groups of rats (Figure 9). Moderate multifocal and hemorrhagic areas of myocardial necrosis with mild infiltration of inflammatory cells was observed in hearts of IGBP₂₀₀₄₅ group of rats (Figure 10). Amelioration of the myonecrosis of hearts attained histopathologically was more pronounced in Figure 7 & 8 when compared with Figures 9 & 10. Figure 7 showed most effective cardioprotection while least effective cardioprotection was seen in Figure 10.

*Per se* treatments of GBP 100 mg/kg (GBP₁₀₀₃₀ & GBP₁₀₀₄₅ groups) as well as GBP 200 mg/kg (GBP₂₀₀₃₀ & GBP₂₀₀₄₅ groups) to rats showed the heart sections to have histoarchitecture similar to that of normal rats.
**Figure 5:** Haematoxylin and eosin staining of heart of normal rat 10 X 10x = 100x

**Figure 6:** Haematoxylin and eosin staining of heart of ISO (85 mg/kg, sc) treated rat 10 X 10x = 100x

**Figure 7:** Haematoxylin and eosin staining of heart of rat treated with GBP 100 mg/kg for 45 days and ISO (85 mg/kg, sc) 10 X 10x = 100x

**Figure 8:** Haematoxylin and eosin staining of heart of rat treated with GBP 100 mg/kg for 30 days and ISO (85 mg/kg, sc) 10 X 10x = 100x

**Figure 9:** Haematoxylin and eosin staining of heart of rat treated with GBP 200 mg/kg for 30 days and ISO (85 mg/kg, sc) 10 X 10x = 100x

**Figure 10:** Haematoxylin and eosin staining of heart of rat treated with GBP 200 mg/kg for 45 days and ISO (85 mg/kg, sc) 10 X 10x = 100x
Discussion

Isoproterenol, a synthetic β adrenergic agonist with its positive inotropic and chronotrophic actions increases the myocardial oxygen demand that leads to ischemic necrosis of myocardium in rats. A number of patho-physiologic mechanisms have been postulated to explain the ISO-induced myocardial damage, viz., altered permeability, increased turnover of norepinephrine and the generation of cytotoxic free radicals on auto-oxidation of catecholamine. Free radical mediated lipid peroxidation and consequent changes in membrane permeability are the major causative factors for cardiotoxicity induced by ISO.31 Oxidative stress increases cAMP levels by exhausting ATP, depresses sarcolemmal Ca\(^{2+}\) transport resulting in intracellular calcium overload, leading to ventricular dysfunction and contractile failure in rat heart.32, 33

The lesions produced by ISO in rat heart are similar to those found in myofibrillar degeneration in Ischemic Heart Disease (IHD) in man.34 Hence, the study of ISO induced myocardial necrosis and its underlying mechanisms might provide scientific leads for a better understanding of the pathogenesis of IHD.

The diagnostic marker enzymes AST, LDH & CPK serve as sensitive probes to assess the degree of myocardial necrosis. Rats treated with ISO exhibited a decline in activities of the marker enzymes in heart homogenate followed by their concomitant elevation in serum, indicating the onset of myocardial necrosis.

Free radicals generated by ISO are known to initiate lipid peroxidation of membrane bound PUFA, leading to structural and functional damage of the myocardium. The myocardium once metabolically impaired, releases its marker enzymes into the blood. Hence, the activities of AST, LDH & CPK were found to be decreased in the heart homogenates of ISO treated animals when compared with normal animals, which is indicative of cellular injury possibly due to lipid peroxide formation.

Elevation in the activities of serum AST, LDH & CPK in ISO control animals in comparison with normal animals was observed. This was due to the leaking out of marker enzymes from a damaged myocardium into the blood stream. Attenuation of the ISO elevated activities of AST, LDH & CPK in serum, and their partial restoration in the heart by GBP treatment is suggestive of a cardioprotective effect of GBP.

The increased levels of MDA reflect the excessive formation of free radicals by auto-oxidation of ISO and greater formation of lipid peroxides, resulting in severe damage to the myocardium of animals treated with ISO. GBP treatment significantly reduced the MDA levels by preventing formation of lipid peroxides from fatty acids of the myocardium.

Reduced glutathione is one of the most abundant non-enzymatic antioxidant bio-molecules present in the tissues.35 Its functions are removal of free oxygen species such as H\(_2\)O\(_2\), superoxide anions & alkoxy radicals, maintenance of membrane protein thiols and to act as a substrate for GPx and glutathione S- transferredase.36 Decreased GSH levels in ISO intoxicated rats may be due to its increased utilization for augmenting the activities of GPx & GST. GSH levels depleted by ISO were significantly restored by GBP, either due to increased synthesis of GSH or by stimulation of GR activity.

Free radical scavenging enzymes such as SOD, CAT & GPx are known to be the first line cellular defense against oxidative damage, disposing O\(_2\) & H\(_2\)O\(_2\) before their interaction to form the more harmful hydroxyl (OH\(^-\)).37 In the present study SOD activity decreased significantly in the ISO treated group of animals, which might be due to an excessive formation of superoxide anions. These excessive superoxide anions might inactivate SOD and decrease its activity. In the absence of adequate SOD activity, superoxide anions are not dismuted into H\(_2\)O\(_2\) which is the substrate for the H\(_2\)O\(_2\) scavenging enzymes CAT & GPx. As a result, there is an inactivation of the H\(_2\)O\(_2\) scavenging enzymes CAT and GPx, leading to a decrease in their activities. Administration of GBP to ISO challenged rats effectively prevented the depletion of SOD, CAT & GPx activities, which can be correlated to the scavenging of free radicals by GBP, resulting in the protection of these enzymes.38

GR is an antioxidant enzyme involved in the reduction of GSSG (an end product of GPx reaction) to GSH. In ISO treated rats, there was a marked reduction in GPx activity, leading to reduced availability of substrate for GR, thereby decreasing the activity of GR. Oral treatment of GBP to ISO myocardial infarcted rats restored the activity of GR, which accelerates the conversion of GSSG to GSH.
Conclusion

In conclusion, our present biochemical and histological studies have demonstrated that GBP 100 mg/kg administered for 45 days most effectively alleviated ISO induced myocardial injury. GBP 100 mg/kg treatment for 30 days was found to be as effective as GBP 100 mg/kg given for 45 days in salvaging the myocardium from the deleterious effects of ISO.

Based on our present findings it is suggested that GBP exerts its cardioprotective effect by stabilizing the myocardial membrane. The membrane stabilizing activity of GBP may be due to an augmentation of basal endogenous antioxidants, which in turn increase the myocardial antioxidant reserve and strengthen the defense mechanism(s) operating in the myocardium.

Flavonoids of GBP stabilize the ROS by reacting with them and getting oxidized in turn, to more stable less reactive radicals. Presumably, the high reactivity of the OH group of flavonoids is responsible for this free radical scavenging activity. Flavonoid (OH) + R. → Flavonoid (O.) + RH

However, a down regulation of β receptors by GBP during its chronic treatment, leading to decreased pharmacological and toxic effects of ISO cannot be ruled out.

Surprisingly, GBP 200 mg/kg chronic treatment for ISO intoxicated rats for 30 days and 45 days failed to elicit cardioprotective activity greater than that of GBP 100 mg/kg. GBP 200 mg/kg treatment for 30 days was marginally better than GBP 200 mg /kg treatment for 45 days in alleviating myocardial damage. One wonders whether GBP 200 mg/kg administered in longer treatment exhibits pro-oxidant activity, either due to the tachyphylaxis phenomenon or a negative feedback mechanism. This warrants further study at the molecular level to understand the underlying mechanism(s) associated with such biphasic effects.

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References


