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

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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.[1] 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.[2] Among the molecules attacked by oxygen free radicals are proteins, carbohydrates, nucleic acids & phospholipids of plasma membranes.[3] 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.[4]

Isoproterenol (ISO), a synthetic catecholamine and ß adrenergic agonist is documented to produce myocardial infarction in large dose.[5] 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. [6] The extracts of the leaves of Ginkgo biloba have been found to possess cardioprotective, antiasthmatic, antidiabetic, hepatoprotective and potent CNS activities. [7-9] 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. [10] The constituents of *G. biloba* are potent scavengers of free radicals.[11, 12] 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.[13, 14] It has been demonstrated that EGb protects the heart by its antioxidant properties and its ability to adjust fibrinolytic activity.[15] In one study, EGb diminished the decrease of myocardial ascorbate content after 40 min of ischemia and 20 min of reperfusion and also suppressed the increase of dehydroascorbate.[16] In another study, vitamin E depletion observed in case of ischemia/reperfusion in rat heart was spared by EGb.[17] 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.[18, 19] 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.[20]

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. [21] EGb has been reported to significantly increase the total antioxidant plasma capacity (by 24.5% TRAP; 27.9% FRAP) only when complexed with phospholipids. [22]

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 $\geq 24\%$ of ginkgoflavonglucosides, $\geq 6\%$ 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

biochemical parameters, hence this dose was chosen as the toxicant dose in the present study. [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 $GBP_{100}30$, 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 $GBP_{200}30$, 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 $GBP_{100}45$, received GBP (100 mg/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 VIII – termed as $GBP_{200}45$, received GBP (200 mg/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 IX – labeled as $IGBP_{100}30$, 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 $IGBP_{200}30$, 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 $IGBP_{100}45$, 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 $IGBP_{200}45$, 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 $7000 \times g$ 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.[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.[25] 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 ($1.56 \times 10\text{-}5$/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.[26] 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. [27] 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[28], which is a quantitative spectroscopic method developed for following the breakdown of H_2O_2 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.[29]

$$H_2O_2 \; + \; 2\,GSH \; \rightarrow \; 2H_2O \; + \; GSSG \text{ (oxidized glutathione)}.$$
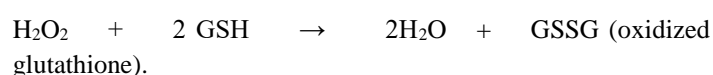
GPx in the tissue homogenate oxidizes glutathione and simultaneously, H_2O_2 is reduced to water. This reaction is arrested at 10 min using trichloroacetic acid and the remaining glutathione is reacted with DTNB solution to result in a colored compound, which is measured spectrophotometrically at 420 nm.

Glutathione reductase (GR) activity was determined by using the method of Mohandas et al in which the following reaction is implicated.[30]

$$NADPH \; + \; H^+ \; + \; GSSG \; \rightarrow \; NADP^+ \; + \; 2\,GSH$$

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 ($IGBP_{200}45$ 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.
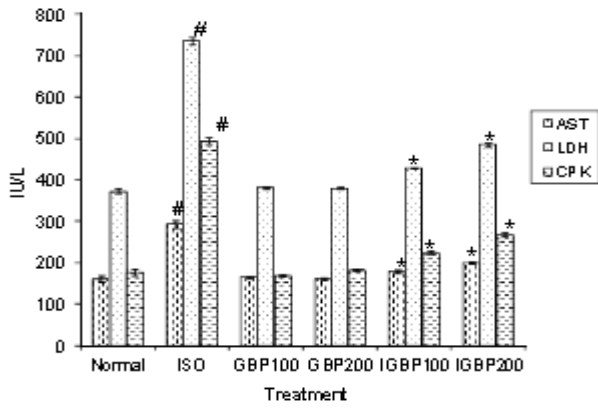
Figure 1: Effect of GBP of serum marker enzymes in ISO intoxicated rats in a 30-day treatment
Values are mean ± SEM for 6 animals in each group
#p< 0.001 when an ISO group compared with Normal group
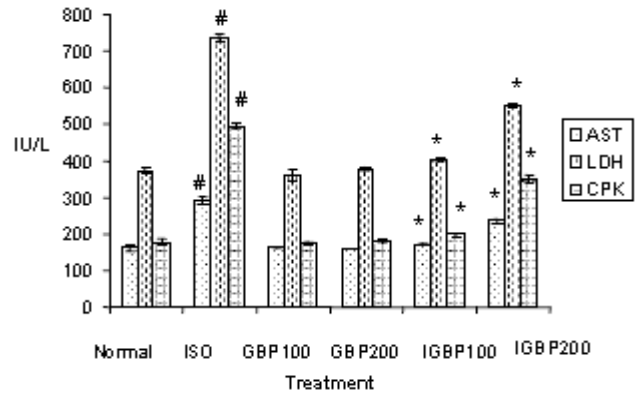*p< 0.001 when IGBP groups compared with ISO group

Figure 2: Effect of GBP of serum marker enzymes in ISO intoxicated rats in a 45-day treatment
Values are mean ± SEM for 6 animals in each group
#p< 0.001 when an ISO group compared with Normal group
*p< 0.001 when IGBP groups compared with ISO group

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 (IGBP$_{100}$30 group) as well as 200 mg/kg (IGBP$_{200}$30 group) for 30 days with ISO treatment on the 29th & the 30th day to rats, increased significantly the activities of AST (p<0.05), LDH (p<0.001) & CPK (p<0.001) when compared with only ISO treated rats. In the GBP 45 day treatment, GBP 100mg/kg (IGBP10045 group) increased significantly the activities of AST (p<0.01), LDH (p<0.001) & CPK (p<0.001) in the heart homogenates of ISO myocardial infarcted animals. GBP 200 mg/kg dose (IGBP$_{200}$45group), however, could significantly increase the activities of only AST (p<0.05) & CPK (p<0.001).
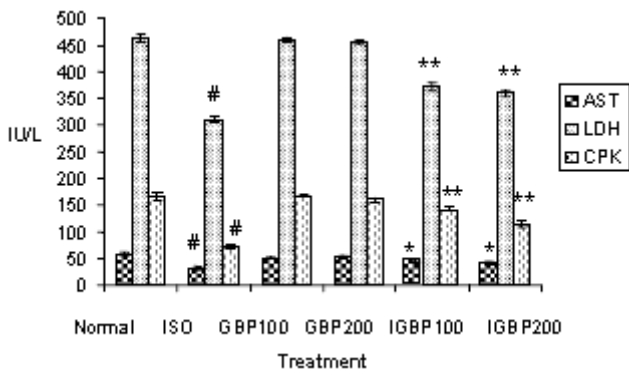

Figure 3: Effect of GBP of heart marker enzymes in ISO intoxicated rats in a 30-day treatment
Values are mean ± SEM for 6 animals in each group
#p< 0.001 when an ISO group compared with Normal group
*p< 0.05 when IGBP groups compared with ISO group
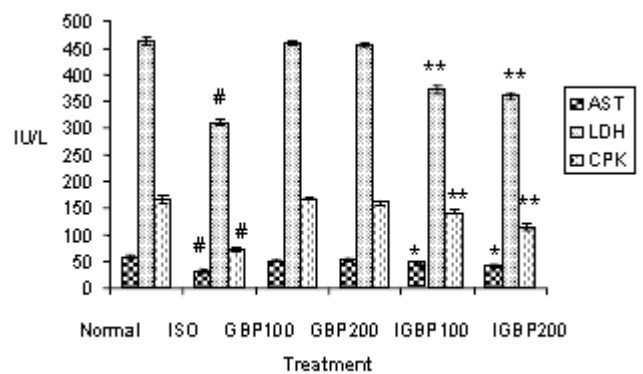**p< 0.001 when IGBP groups compared with ISO groups

Figure 4: Effect of GBP of heart marker enzymes in ISO intoxicated rats in a 45-day treatment
Values are mean ± SEM for 6 animals in each group
#p< 0.001 when an ISO group compared with Normal group
*p< 0.05 when IGBP groups compared with ISO group
**p< 0.01 when IGBP groups compared with ISO group
***p< 0.001 when IGBP groups compared with ISO group

The effects of GBP on antioxidant enzymes, GSH and   treatments respectively.
LPO are summarized in Tables 1& 2 for 30 days & 45 day

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

Treatment	MDA nmol /g Wet tissue	GSH μmol /g Wet tissue	SOD u/mg protein	CAT u/mg protein	GPX u/mg protein	GR u/mg protein
Normal (N45)	33.51 ± 2.61	2.02 ± 0.10	10.74 ± 1.01	21.24 ± 1.09	0.33 ± 0.01	15.91 ± 1.55
ISO control (I45)	$84.2 \pm 4.93^{\#}$	$1.34 \pm 0.07^{\#}$	$5.64 \pm 0.48^{\#}$	$11.21 \pm 0.64^{\#}$	$0.19 \pm 0.01^{\#}$	$7.50 \pm 0.72^{\#}$
GBP$_{100}$45	31.8 ± 1.0	2.17 ± 0.08	10.30 ± 0.02	22.45 ± 0.46	0.35 ± 0.01	15.6 ± 0.33
GBP$_{200}$45	36.9 ± 1.82	1.95 ± 0.11	10.12 ± 0.076	20.68 ± 0.49	0.32 ± 0.02	15.02 ± 0.34
IGBP$_{100}$45	40.44 ± 1.13^{b}	1.88 ± 0.06^{b}	8.68 ± 0.05^{a}	20.61 ± 0.53^{b}	0.31 ± 0.01^{b}	13.07 ± 0.28^{a}
IGBP$_{200}$45	72.53 ± 2.26	1.55 ± 0.05	6.42 ± 0.20	$14.63 \pm 0.47^{*}$	0.22 ± 0.01	7.82 ± 0.36

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 H_2O_2 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

Treatment	MDA nmol /g Wet tissue	GSH μmol /g Wet tissue	SOD u/mg protein	CAT u/mg protein	GPX u/mg protein	GR u/mg protein
Normal (N30)	33.51 ± 2.61	2.02 ± 0.10	10.74 ± 1.00	21.24 ± 1.09	0.33 ± 0.01	15.90 ± 1.54
ISO control (I30)	84.20 ± 4.93^{a}	1.34 ± 0.07^{a}	5.64 ± 0.48^{a}	11.20 ± 0.64^{a}	0.19 ± 0.01^{a}	7.5 ± 0.72^{a}
GBP$_{100}$30	37.73 ± 1.74	2.22 ± 0.06	10.28 ± 0.04	20.86 ± 0.3	0.31 ± 0.01	15.75 ± 0.03
GBP$_{200}$30	35.68 ± 2.11	1.91 ± 0.13	9.90 ± 0.16	20.18 ± 0.25	0.32 ± 0.01	15.9 ± 1.55
IGBP$_{100}$30	44.20 ± 2.60^{d}	1.83 ± 0.05^{d}	8.42 ± 0.11^{b}	19.76 ± 0.18^{d}	0.32 ± 0.01^{d}	13.23 ± 0.09^{d}
IGBP$_{200}$30	65.16 ± 1.70^{c}	1.65 ± 0.05	7.83 ± 0.24	17.00 ± 1.45^{c}	0.3 ± 0.01^{d}	10.26 ± 0.48

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 H_2O_2 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 (IGBP$_{100}$30 group) as well as 200 mg/kg (IGBP$_{200}$30 group) for 30 days to ISO intoxicated rats, depleted significantly (p<0.001) the increased levels of MDA due to ISO. For the 45 days treatment, GBP 100 mg/kg (IGBP$_{100}$45 group) significantly lowered (p<0.001) the ISO elevated levels of MDA, whereas pretreatment with GBP 200 mg/kg (IGBP$_{200}$45 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 (IGBP$_{100}$30 group) followed by ISO injection on the 29th & the 30th day to rats, significantly restored the activities of SOD (p<0.05), CAT (p<0.001), GPx (p<0.001) & GR (p<0.001) depleted by ISO. Similarly, pretreatment of rats with GBP 100 mg/kg for 45 days (IGBP$_{100}$45 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<0.001) 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 (GBP$_{100}$30 & GBP$_{100}$45 groups) as well as 200 mg/kg (GBP$_{200}$30 & GBP$_{200}$45 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 (IGBP$_{100}$45 group) showed minimal myonecrotic patches with minimal inflammatory cell infiltration (Figure 7). There was mild to moderate focal myonecrosis with moderate diffused infiltration of lymphocytes in heart sections of GBP 100 mg/kg treatment for 30 days to ISO infarcted rats (IGBP$_{100}$30 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 (IGBP$_{200}$30 group), gradation of myocardial damage was more than in IGBP$_{100}$30 & IGBP$_{100}$45 groups of rats (Figure 9). Moderate multifocal and hemorrhagic areas of myocardial necrosis with mild infiltration of inflammatory cells was observed in hearts of IGBP$_{200}$45 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 (GBP$_{100}$30 & GBP$_{100}$45 groups) as well as GBP 200 mg/kg (GBP$_{200}$30 & GBP$_{200}$45 groups) to rats showed the heart sections to have histoarchitecture similar to that of normal rats.
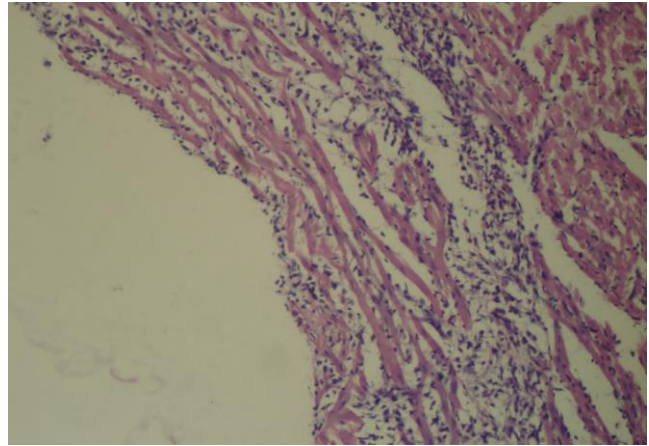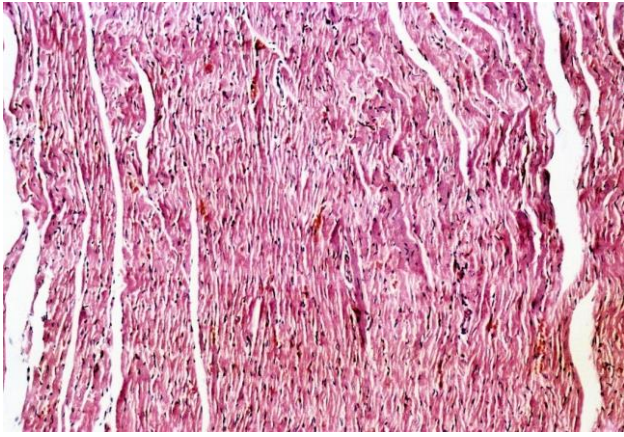
Figure 5: Haematoxylin and eosin staining of heart of normal rat 10 X 10x = 100x

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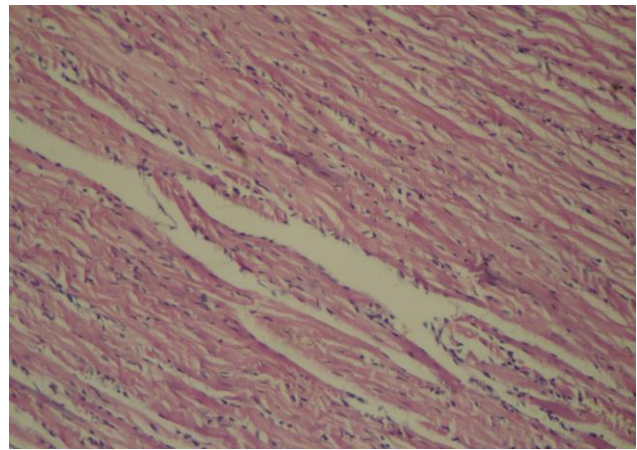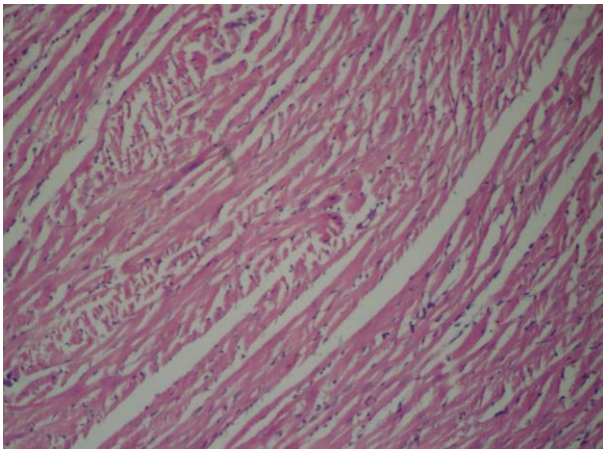

Figure 7: Haematoxylin and eosin staining of heart of rat treated with GBP 100 mg/kg for 45 days and ISO (85 mg/kg, sc) 10 X 10x = 100x

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
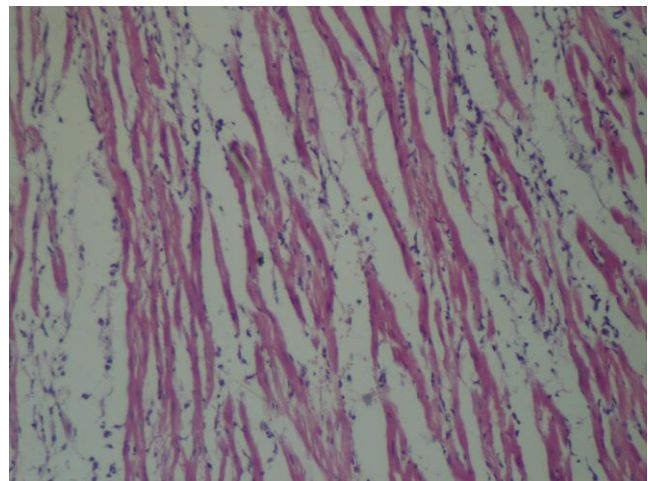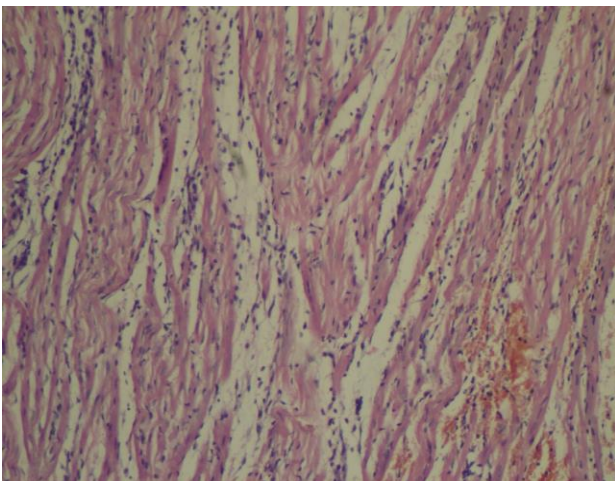

Figure 9: Haematoxylin and eosin staining of heart of rat treated with GBP 200 mg/kg for 30 days and ISO (85 mg/kg, sc) 10 X 10x = 100x

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 ß adrenergic agonist with its positive inotropic and chronotropic 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_2O_2, superoxide anions & alkoxy radicals, maintenance of membrane protein thiols and to act as a substrate for GPx and glutathione S- transferase.[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_2O_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_2O_2, which is the substrate for the H_2O_2 scavenging enzymes CAT & GPx. As a result, there is an inactivation of the H_2O_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. [39]

Flavonoid (OH)   +   R.   →   Flavonoid (O.) + RH

However, a down regulation of ß 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.

Acknowledgement

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