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Evaluation of Cardioprotective Activity of *Ginkgo biloba* and *Ocimum sanctum* in Rodents

Vandana S. Panda, PhD (Tech) and Suresh R. Naik, PhD, FRSC, CChem (Eng)

Abstract

This study investigates the cardioprotective activity of a combined treatment of *Ginkgo biloba* phytosomes (GBP) and *Ocimum sanctum* extract (Os) in isoproterenol (ISO)-induced myocardial necrosis in rats. Significant myocardial necrosis, depletion of the endogenous antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione (GSH), and increases in the serum marker enzymes aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) were observed in ISO-treated rats compared with normal rats. Co-administration of GBP (100 mg/kg) with Os at two doses (50 and 75 mg/kg) for 30 days to rats treated with ISO (85 mg/kg, sc) on the 29th and 30th days demonstrated a significant decrease in ISO-induced serum marker enzyme elevations and a significant attenuation of the ISO-elevated myocardial lipid peroxidation marker malondialdehyde (MDA). A significant restoration of ISO-depleted activities and levels of AST, LDH, CPK, GSH, SOD, CAT, GPx, and GR in the hearts of the treatment groups was observed. The combination of Os 75 mg/kg and GBP 100 mg/kg elicited greater protection than the combination of Os 50 mg/kg and GBP 100 mg/kg. It may be concluded that GBP-Os oral treatment to ISO-challenged rats demonstrates significant cardiac protection, decreases lipid peroxidation, and restores antioxidant activities. However, the combined treatment failed to enhance cardioprotective activity of either herb when used alone.

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Introduction

When a disease deprives a tissue of oxygen, re-establishing blood flow (reperfusion) and therefore re-introducing oxygen can result in tissue damage.[1] Myocardial ischemia-reperfusion (IR) can occur, resulting in damage to the myocardium following blood restoration after a critical period of blood occlusion. IR is associated with procedures such as thrombolysis, angioplasty, and coronary bypass surgery, procedures commonly used to re-establish blood and minimize damage to the heart due to severe myocardial ischemia. Reperfusion of the ischemic myocardium is accompanied by the generation of reactive oxygen species (ROS) that can cause vascular and microvascular injury, endothelial cell dysfunction, myocyte edema, increased myocyte apoptosis, increased myocyte necrosis, and cardiac contractile dysfunction.[2] Among the molecules attacked by oxygen free radicals are proteins, carbohydrates, neurotransmitters, nucleic acids, and cell membrane phospholipids.[3] Free radicals have been implicated in many conditions, including heart disease, diabetes, cancer, Alzheimer's disease, Parkinson's disease, cataracts, rheumatoid arthritis, and aging.[4]

Isoproterenol (ISO), a synthetic catecholamine and ß-adrenergic agonist, has been documented to produce myocardial infarction in large doses.[5] On autooxidation, ISO generates highly cytotoxic free radicals

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known to stimulate peroxidation of membrane phospholipids and cause severe damage to the myocardial membrane.

Herbal medicine is increasingly gaining acceptance from the public and medical professionals due to advances in the understanding of the mechanisms by which herbs positively influence health and quality of life. *Ginkgo biloba* (Ginkgoaceae family) is an important herb used in traditional Chinese medicine and is the only surviving species of Ginkgo, the oldest living tree species.[6] Extracts of *Ginkgo biloba* leaves have been found to possess cardioprotective, antiasthmatic, antidiabetic, hepatoprotective, and potent central nervous system activities.[7-10] Chemically, the active constituents of *G. biloba* leaf are glycosides of the flavonoids kaempferol, quercetin, and isorhamnetin; diterpene lactones namely ginkgolides A, B, C, M, J, and bilobalide; and the biflavones ginkgetin, isoginkgetin, and bilobetin.[11] The constituents of *G. biloba* are scavengers of free radicals.[12] By scavenging free radicals, *G. biloba* inhibits lipid peroxidation and augments levels of endogenous antioxidants.

Phytosomes are produced by binding individual components of herbal extracts to phosphatidylcholine, resulting in a dosage form that is better absorbed for greater effectiveness than conventional herbal extracts.[13]

Ocimum sanctum (Tulsi) is an Indian medicinal plant known to possess hypoglycemic, hypolipidemic, immunomodulatory, adaptogenic, hepatoprotective, and cardioprotective activities.[14,15] Initial investigations demonstrated that *Ocimum sanctum* (Os) possesses good cardioprotective activity due to its antioxidant activity.[16,17] Os constituents orientin and vicenin (flavonoids), phenolic compounds (eugenol, cirsilineol, apigenin), and anthocyanins are known to augment reduced glutathione (GSH) and antioxidant enzyme levels and scavenge lipid peroxides.[18]

Potent cardioprotective activity is expected by combining Os extracts with *Ginkgo biloba* phytosome (GBP). With this objective, the present study was designed to investigate the synergistic cardioprotective activity of GBP and Os combined treatment in ISO-induced cardiac necrosis (ischemia-reperfusion injury) using an *in vivo* rodent model. The authors studied the chronic effect of oral GBP-Os combination treatment on cardiac antioxidants, lysosomal enzymes, and histo-architectural changes in the rat myocardium to understand the underlying mechanism(s) of its cardioprotective effect.

Methods
Plant Material
Ginkgo biloba phytosome (Ginkgoselect®) was donated by Indena (Milan, Italy).

Ginkoselect phytosome is prepared by reacting a stoichiometric amount (3:1 w/w) of soy phospholipids with *Ginkgo biloba* extract (GBE). GBE contains ≥24-percent ginkgoflavonglucosides, ≥six-percent ginkgolides and bilobalide, and ≤five ppm ginkgoic acids.

Ocimum sanctum leaf extract was donated by Dabur Research Foundation (Ghaziabad, India). The hydro-alcoholic leaf extract of *Ocimum sanctum* was prepared by powdering authenticated dried leaves. The standard extraction procedure established at Dabur Research Centre is outlined below.

A hydro-alcoholic solution in the ratio 20:80 (water:alcohol) was prepared. A weighed quantity of the coarsely powdered leaves was mixed with the hydro-alcoholic solution eight times its volume and agitated for four hours with mild heating (40-45ºC). The solution was filtered and the filtrate kept aside. The residue was again extracted with the hydro-alcoholic mixture six times its volume for about four hours. This was filtered and both filtrates were mixed together. This extract was concentrated and spray dried to obtain the solid extract.

Drugs and Chemicals
Thiobarbituric acid (TBA), reduced glutathione, oxidized glutathione, and nicotine adenine dinucleotide phosphate (NADPH) were obtained from Himedia Laboratories (Mumbai, India). Isoproterenol, 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), and epinephrine were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from local sources and were of analytical grade.

Experimental Animals

Wistar albino rats (150-200 g) of either sex, procured from Haffkine Laboratories (Mumbai, India) were used. They were housed in clean polypropylene cages under standard conditions of humidity (50 ± 5%), temperature (25 ± 2°C) , and light (12 hour light/12 hour 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.

Preparation of GBP, Os, and ISO Solutions

Ginkgo biloba phytosomes were dissolved in distilled water. The dried powder of *Ocimum sanctum* leaf extract was dissolved in distilled water and used immediately. Isoproterenol was dissolved in distilled water and used immediately for subcutaneous injection.

Previous experiments for dose finding indicate ISO 85 mg/kg injected subcutaneously twice at an interval of 24 hours induces moderate necrosis in rat heart and a significant alteration in biochemical parameters;[10] therefore, 85 mg/kg was selected as the toxicant dose in the present study.

Experimental Procedure

Wistar albino rats after acclimatization (6-7 days) in the animal quarters were randomly divided into seven groups of six animals each and treated as follows:

- Group I – termed as normal control, received distilled water (1mL/kg, po) daily for 30 days and in addition received distilled water (0.5 mL/kg, sc) on the 29th and 30th days at an interval of 24 hours.
- Group II – termed as ISO control, received two injections of ISO (85 mg/kg, sc) at an interval of 24 hours.
- Group III – termed as IGBP100, received GBP (100 mg/kg, po) daily for 30 days and in addition received ISO (85 mg/kg, sc) on the 29th and 30th days at an interval of 24 hours.
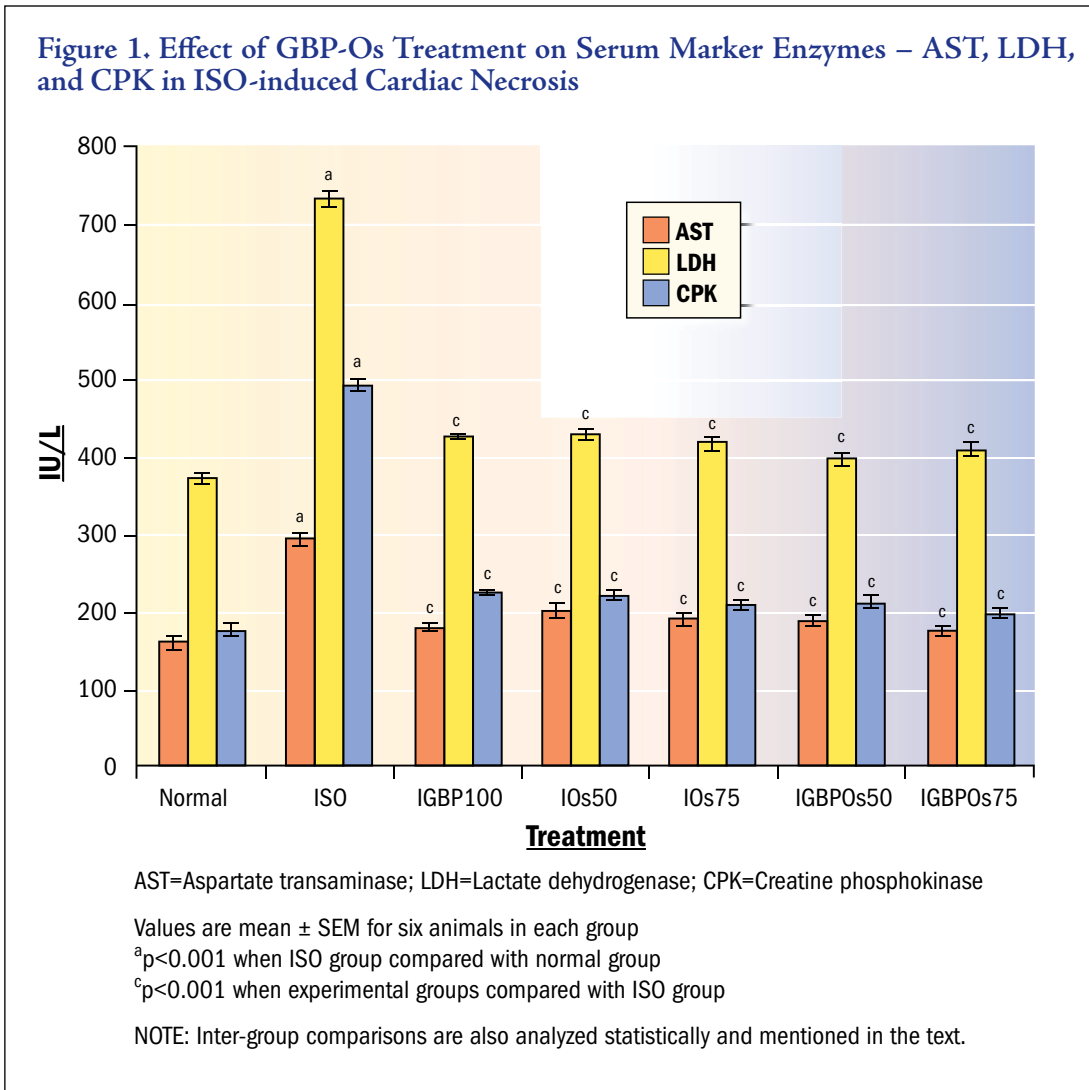
- Group IV – termed as IOs50, received Os (50 mg/kg, po) for 30 days and in addition received ISO (85 mg/kg, sc) on the 29th and 30th days at an interval of 24 hours.
- Group V – termed as IOs75, received Os (75 mg/kg, po) daily for 30 days and in addition received ISO (85 mg/kg, sc) on the 29th and 30th days at an interval of 24 hours.
- Group VI – termed as IGBPOs50, received GBP (100 mg/kg, po) and Os (50 mg/kg, po) daily for 30 days and in addition received ISO (85 mg/kg, sc) on the 29th and 30th days at an interval of 24 hours.
- Group VII – termed as IGBPOs75, received GBP (100 mg/kg, po) and Os (75 mg/kg, po) daily for 30 days and in addition received ISO (85 mg/kg, sc) on the 29th and 30th days at an interval of 24 hours.

Rats were weighed and put down 24 hours after the final subcutaneous injection of ISO. Blood collection was done by adhering to Good Laboratory Practices. Blood was collected by cardiac puncture under light ether anesthesia and allowed to clot for 30 minutes at room temperature. The serum was separated by centrifugation at 2500 rpm at 30°C for 15 minutes and used for the estimation of marker enzymes, including aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK). The hearts were dissected out immediately, chilled, and perfused with ice-cold saline. After washing with ice-cold saline, the hearts were patted dry, weighed, and divided into two halves. One half was used to prepare 10-percent (w/v) homogenate in phosphate buffer (50 mM, pH 7.4). An aliquot of the homogenate was used for the determination of lipid peroxidation (LPO). The homogenates were centrifuged at 7000 × g for 10 minutes at 4°C and the supernatants were used for the assays of AST, LDH, CPK, GSH, superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx), and glutathione reductase (GR). The remaining half was fixed in 10-percent (w/v) buffered formalin and used for histological studies.

Figure 1. Effect of GBP-Os Treatment on Serum Marker Enzymes – AST, LDH, and CPK in ISO-induced Cardiac Necrosis

AST=Aspartate transaminase; LDH=Lactate dehydrogenase; CPK=Creatine phosphokinase

Values are mean ± SEM for six animals in each group
[a]$p < 0.001$ when ISO group compared with normal group
[c]$p < 0.001$ when experimental groups compared with ISO group

NOTE: Inter-group comparisons are also analyzed statistically and mentioned in the text.

Marker Enzyme Assays

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

Protein Estimation

Total protein content was determined in heart homogenates of experimental animals by using the Lowry et al method, using bovine serum albumin as standard.[19]

Lipid Peroxidation

The quantitative estimation of lipid peroxidation was conducted by determining the concentration of thiobarbituric acid reactive substances in heart using the method of Ohkawa and Yagi.[20] 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 molar extinction coefficient of the chromophore (1.56×10^{-5}/M/cm) and 1,1,3,3-tetraethoxypropane as standard.

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Figure 2. Effect of GBP-Os Treatment on Myocardial Marker Enzymes – AST, LDH, CPK in ISO-induced Cardiac Necrosis
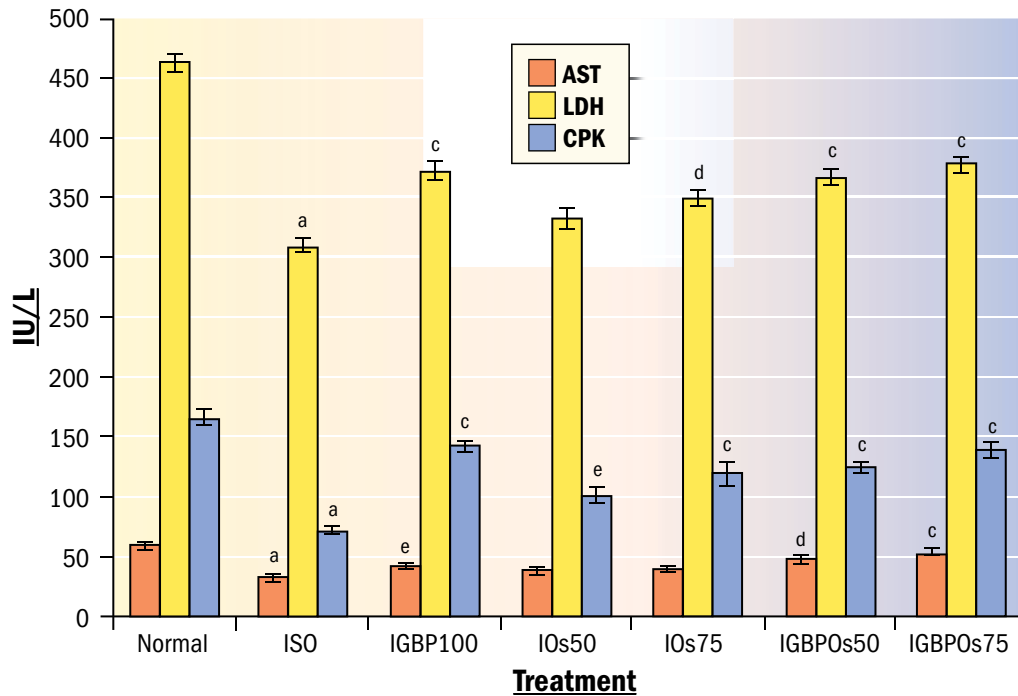
Values are mean ± SEM for six animals in each group
[a]p< 0.001 when ISO group compared with normal group
[d]p< 0.01 when experimental groups compared with ISO group
[e]p< 0.05 when experimental groups compared with ISO group
[c]p< 0.001 when experimental groups compared with ISO group

NOTE: Inter-group comparisons are also analyzed statistically and mentioned in the text.

Glutathione Estimation

GSH was estimated in the heart homogenate using DTNB by the method of Ellman.[21] 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

SOD was assayed by the method of Sun et al, in which the activity of SOD is inversely proportional to the concentration of its oxidation product adrenochrome, which is measured spectrophotometrically at 320 nm.[22] One unit of SOD activity is defined as the enzyme concentration required to inhibit the rate of auto-oxidation of epinephrine by 50 percent in one minute at pH 10.

CAT was estimated by the method of Clairborne et al, 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.[23]

GPx estimation was carried out using the method of Rotruck et al, which uses the following reaction:[24]

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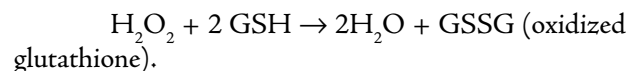
GPx in the tissue homogenate oxidizes gluta-thione and simultaneously H_2O_2 is reduced to water. This reaction is arrested at 10 minutes using trichloroa-cetic acid, and the remaining glutathione is reacted with DTNB solution to result in a colored compound which is measured spectrophotometrically at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

GR activity was determined by using the method of Mohandas et al, according to the following reaction:[25]

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

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

Histoarchitectural Studies

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

Statistical Analysis

The results of cardioprotective and antioxidant activities are expressed as mean ± SEM from six animals in each group. 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, CA), was used for statistical analysis.

Results

Biochemical Parameters

Serum Marker Enzymes

The effects of GBP and Os oral treatments on serum marker enzymes AST, LDH, and CPK for 30 days are outlined in Figure 1. Rats treated with ISO showed a significant increase ($p < 0.001$) in activities of serum marker enzymes compared with the normal rat group. Pretreatment of GBP 100 mg/kg (IGBP100 group), Os 50 mg/kg (IOs50 group), Os 75 mg/kg (IOs75 group), GBP 100 mg/kg plus Os 50 mg/kg (IGBPOs50 group), or GBP 100 mg/kg plus Os 75 mg/kg (IGBPOs75 group) to rats for 30 days, followed by ISO subcutaneous injection on the 29th and 30th days, elicited a significant ($p < 0.001$) reduction in the ISO-induced increased activities of AST, LDH, and CPK.

The combination treatments (IGBPOs50, IGBPOs75) were not significantly better than IGBP100, IOs50, or IOs75 treatment alone in lowering ISO-elevated serum enzyme activities.

Heart Marker Enzymes

The effect of GBP and Os oral treatments for 30 days on myocardial marker enzymes is presented in Figure 2. The ISO-treated rats showed a significant ($p < 0.001$) depletion of AST, LDH, and CPK activities compared with normal rats.

Oral administration of GBP-Os50 (IGBPOs50 group), GBP-Os75 (IGBPOs75 group), and GBP100 (IGBP100 group) to rats with subsequent ISO injection resulted in a significant restoration ($p < 0.01$ for IGBPOs50, $p < 0.001$ for IGBPOs75, and $p < 0.05$ for IGBP100) of ISO-induced depletion of AST activity. An insignificant increase in AST activity was observed in the IOs50 and IOs75 treated groups compared with the ISO-only group. The IGBP-Os treatment was significantly better than IOs50 ($p < 0.01$) and IOs75 ($p < 0.05$) treatments in restoring the ISO-induced depletion of AST activity.

ISO-depleted myocardial LDH activity was found to be restored significantly in the IGBP100 ($p < 0.001$), IOs75 ($p < 0.01$), IGBPOs50 ($p < 0.001$), and IGBPOs75 ($p < 0.001$) groups. The combination treatment groups IGBPOs50 and IGBPOs75 were significantly better ($p < 0.05$ and $p < 0.001$, respectively) than the IOs50-treatment group in restoring the ISO-induced depletion of LDH activity.

ISO-induced CPK depletion was restored significantly in the heart tissues of the IGBP100, IOs50, IOs75, IGBPOs50, and IGBPOs75 groups ($p < 0.05$ for IOs50; $p < 0.001$ for remaining groups). The IGBP-Os75 group elicited a significantly higher ($p < 0.01$) CPK activity than the IOs50 group.

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Table 1. Effect of GBP and Os on Heart MDA, GSH, SOD, CAT, GPx, and GR in ISO-induced Cardiac Necrosis

Treatment	MDA nmol/g wet tissue	GSH µmol/g wet tissue	SOD unit/mg protein	CAT unit/mg protein	GPX unit/mg protein	GR unit/mg protein
Normal	33.51 ± 2.61	2.02 ± 0.10	10.74 ± 1.01	21.24 ± 1.10	0.33 ±0.01	15.90 ± 1.55
ISO control	84.20 ± 4.93[a]	1.34 ± 0.08[a]	5.64 ± 0.48[a]	11.21 ± 0.64[a]	0.19 ± 0.01[a]	7.5 ± 0.72[a]
IGBP 100	44.20 ± 2.60[c]	1.83 ± 0.06[c]	8.42 ± 0.11[b]	19.76 ± 0.18[c]	0.32 ± 0.01[c]	13.23 ± 0.09[c]
IOs50	50.14 ± 3.54[c]	1.54 ± 0.04	8.51 ± 0.26[b]	18.66 ± 0.53[c]	0.28 ± 0.01[c]	14.11 ± 0.52[c]
IOs75	44.91 ± 2.90[c]	1.68 ± 0.04[b]	8.61 ± 0.28[b]	19.53 ± 0.46[c]	0.31 ± 0.01[c]	14.48 ± 0.28[c]
IGBPOs50	42.92 ± 2.73[c]	1.70 ± 0.03[b]	8.70 ± 0.30[c]	19.85 ± 0.42[c]	0.30 ± 0.01[c]	14.20 ± 0.26[c]
IGBPOs75	40.07 ± 2.89[c]	1.80 ± 0.03[c]	8.86 ± 0.17[c]	19.99 ± 0.36[c]	0.32 ± 0.01[c]	14.61 ± 0.27[c]

Values are mean ± SEM for six animals in each group
[a] $p < 0.001$ when ISO group compared with normal group
[b] $p < 0.01$ when experimental groups compared with ISO group
[c] $p < 0.001$ when experimental groups compared with ISO group

Note: Inter-group comparisons are also analyzed statistically and mentioned in the text.

MDA=malondialdehyde; GSH=glutathione; SOD=superoxide dismutase; CAT=catalase; GPx=glutathione peroxidase; GR=glutathione reductase

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

Heart Antioxidant Enzymes, GSH, and LPO

The effects of GBP and Os oral treatments for 30 days on antioxidant enzymes, GSH, and LPO are summarized in Table 1.

The ISO-treated group showed a significant ($p<0.001$) increase in MDA compared with normal untreated rats. A significant diminution ($p<0.001$) of ISO-induced MDA elevation was observed in all pretreated experimental groups (IGBP100, IOs50, IOs75, IGBPOs50, and IGBPOs75).

Myocardial GSH levels decreased significantly ($p<0.001$) in ISO-treated rats. These levels were restored significantly by pretreatment with GBP 100 mg/kg, Os 75 mg/kg, GBP 100 mg/kg + Os 50 mg/kg, and GBP 100 mg/kg + Os 75 mg/kg ($p<0.01$ for IOs75 and IGBPOs50; $p<0.001$ for IGBP100 and IGBPOs75 groups).

SOD, CAT, GPx, and GR activities were significantly lower ($p<0.001$) in ISO-treated rats compared with normal rats. Treatment of GBP 100 mg/kg, Os 50 mg/kg, Os 75 mg/kg, GBP 100 mg/kg plus Os 50 mg/kg, or GBP 100 mg/kg plus Os 75 mg/kg to different groups of rats, then challenged with ISO, restored the ISO depleted SOD activities significantly ($p<0.01$ for IGBP100, IOs50, and IOs75 groups; $p<0.001$ for IGBPOs50 and IGBPOs75 groups) in all groups of rats.

CAT, GPx, and GR activities were restored significantly ($p<0.001$) in all experimental groups compared with the ISO-only group.

IGBPOs50 and IGBPOs75 (combination treatments) were not significantly better in restoring the levels of GSH, SOD, CAT, GPX, and GR than IGBP100, IOs50, or IOs75 treatments alone. IGBPOs75 treatment was better than IGBPOs50, but not significantly.

Histoarchitectural Studies

Histopathological examination of the myocardium of normal rats showed clear integrity of myocardial cell membrane (Figure 3). Endocardium and pericardium were within normal limits. No inflammatory cell infiltration was observed.
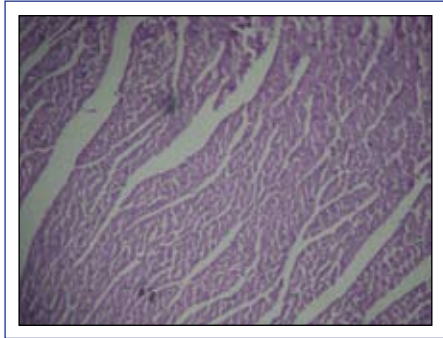
The group of ISO-treated rats showed moderate to marked myocytic necrosis with moderate infiltration of lymphocytes and macrophages (Figure 4). The changes were more prominent along the endocardium and in papillary muscles.
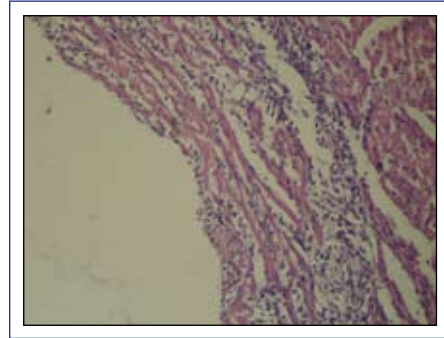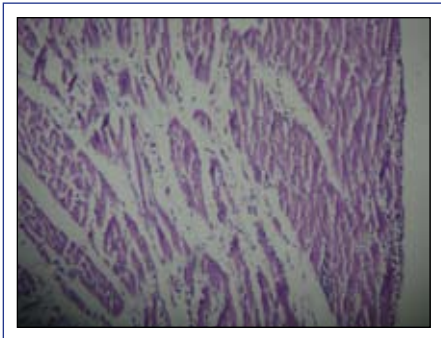
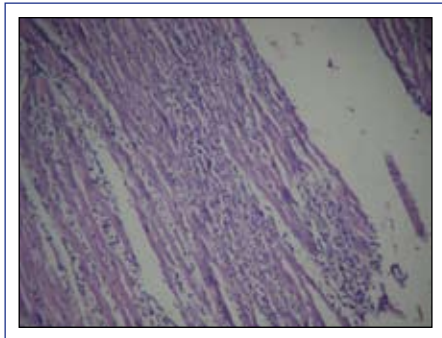**Figure 3. Hematoxylin and Eosin Staining of Heart of Normal Rats: 10 X 10x = 100x**

Figure 4. Hematoxylin and Eosin Staining of Heart of ISO-treated Rats: 10 X 10x = 100x
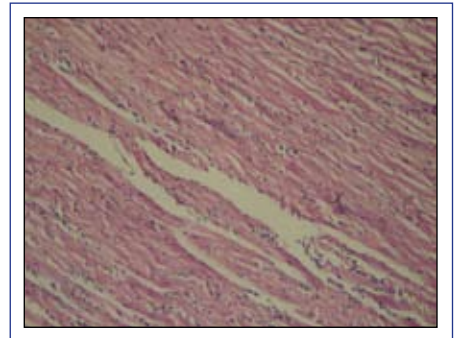

Figure 5. Hematoxylin and Eosin Staining of Heart of Rats Treated with Os 50 mg/kg and ISO: 10 X 10x = 100x
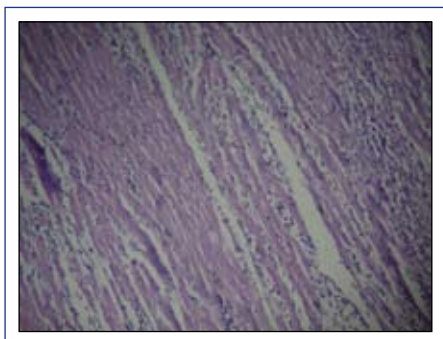

Figure 6. Hematoxylin and Eosin Staining of Heart of Rats Treated with Os 75 mg/kg and ISO: 10 X 10x = 100x
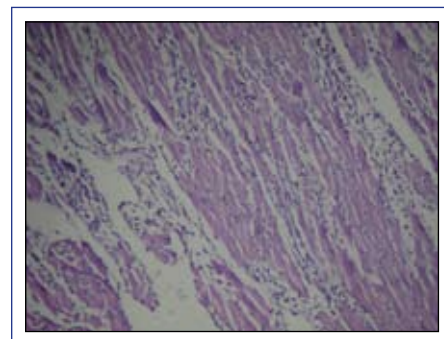

Figure 7. Hematoxylin and Eosin Staining of Heart of Rats Treated with GBP 100 mg/kg and ISO: 10 X 10x = 100x

Figure 8. Hematoxylin and Eosin Staining of Heart of Rats treated with GBP 100 mg/kg, Os 50 mg/kg, and ISO: 10 X 10x = 100x

Figure 9. Hematoxylin and Eosin Staining of Heart of Rats Treated with GBP 100 mg/kg, Os 75 mg/kg, and ISO: 10 X 10x = 100x

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The IOs50 treatment (Figure 5) showed mild multifocal myocytic necrosis with removal of sarcoplasm and mild diffuse lymphocytic infiltration along the endocardium. Minimal-to-mild multifocal myocytic necrosis with removal of sarcoplasm and mild diffuse inflammatory cell infiltration along the endocardium was observed in the IOs75 group (Figure 6).

Mild-to-moderate focal myonecrosis with moderate diffuse infiltration of lymphocytes was observed in heart sections of the IGBP100 group (Figure 7).

The IGBPOs50 treatment showed minimal-to-mild multifocal myocytic changes and minimal diffuse lymphocytic infiltration along the endocardium (Figure 8). Minimal-to-mild focal myocytic necrosis and minimal diffuse lymphocytic infiltration along the endocardium was seen in the heart sections of the IGBPOs75 group (Figure 9).

Discussion

In previous studies *Ginkgo biloba* phytosome has shown promising cardioprotective activity due to its antioxidant effects. The flavonoids of *Ginkgo biloba*, by scavenging free radicals, inhibit lipid peroxidation and augment activities of endogenous antioxidants.[10-12] GBP 100 mg/kg oral treatment for 30 days to rats injected with ISO (85 mg/kg, sc) on the last two days exhibited significant cardioprotective activity. Hence, this dose of GBP was selected for the present study.

Ocimum sanctum (Tulsi), an Indian medicinal plant, is reported to possess potential cardioprotective properties. The cardioprotective activity of *Ocimum sanctum* has been attributed largely to the antioxidant properties associated with its flavonoid and phenolic constituents, which are known to augment GSH and antioxidant enzyme levels and scavenge lipid peroxides.[16-18] From previous reports[17,18] on the cardioprotective activity of *Ocimum sanctum*, two effective doses (Os 50 mg/kg and Os 75 mg/kg) were selected for the present study to be combined with GBP 100 mg/kg. The combination of two herbs with potent cardioprotective and antioxidant activities was expected to have marked myocardial protective activity in ISO-induced cardiac necrosis.

Isoproterenol, a synthetic ß-adrenergic agonist by its positive inotropic and chronotropic actions, increases the myocardial oxygen demand that leads to ischemic necrosis of myocardium in rats. A number of pathophysiological mechanisms have been proposed to explain the ISO-induced myocardial damage, including altered permeability, increased turnover of norepinephrine, and generation of cytotoxic free radicals on auto-oxidation of catecholamine. Free radical-mediated lipid peroxidation and consequent changes in membrane permeability are the primary factors for cardiotoxicity induced by ISO.[26] Oxidative stress increases cAMP levels by exhausting ATP and decreases sarcolemmal Ca^{+2} transport, resulting in intracellular calcium overload, which leads to ventricular dysfunction and contractile failure in rat heart.[27,28]

The lesions produced by ISO in rat heart are similar to those found in myofibrillar degeneration in human ischemic heart disease (IHD).[29] Hence, the study of ISO-induced myocardial necrosis and its underlying mechanisms might provide better insight and new leads on the pathogenesis of IHD.

The diagnostic marker enzymes AST, LDH, and CPK serve as a sensitive index to assess the degree of myocardial necrosis. Rats treated with ISO exhibited increased activities of serum marker enzymes accompanied by concomitant reduction in the heart, indicative of the onset of myocardial necrosis.

ISO-generated free radicals are known to initiate peroxidation of membrane-bound polyunsaturated fatty acids, leading to damage of the structural and functional integrity of the myocardium. Metabolically impaired myocardium releases its marker enzymes into the bloodstream. Hence, the activities of AST, LDH, and CPK were found to be decreased in the heart tissue of ISO-treated animals compared with normal animals. This is indicative of cellular injury and can be attributed to excessive lipid peroxide formation.

Elevation was observed in the activities of serum AST, LDH, and CPK in ISO-control animals compared with normal animals due to the leaking of marker enzymes from a damaged myocardium into the bloodstream. Combination treatments of GBP (100 mg/kg) with Os at both doses (50 mg/kg and 75 mg/kg) to rats challenged with ISO significantly attenuated the elevated activities of the marker enzymes AST, LDH, and CPK in serum and significantly restored

their activities in the myocardium. These findings are suggestive of the cardioprotective activity of GBP-Os by its ability to maintain myocardial integrity, largely by preventing lipid peroxidation-induced myocardial damage.

Increased levels of MDA in animals treated with ISO reflect excessive formation of free radicals by auto-oxidation of ISO and greater formation of lipid peroxides, resulting in severe damage to the myocardium. The ISO-elevated MDA levels were significantly decreased by the combination treatments, probably 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 tissues.[30] Its functions are removal of reactive oxygen species, such as H_2O_2, superoxide anions, and alkoxy radicals; maintenance of membrane protein thiols; and provision of a substrate for GPx and glutathione S-transferase (GST).[31] Decreased GSH levels in ISO-treated rats may be due to its increased utilization to augment the activities of GPx and GST. The GSH levels depleted by ISO were significantly restored by GBP-Os oral administration. It may be that an increase in GSH levels are due to its enhanced synthesis or improved GR activity in the presence of GBP-Os.

Free-radical scavenging enzymes such as SOD, CAT, and GPx are known to be the first line cellular defense against oxidative damage, disposing of O_2 and H_2O_2 prior to the interaction to form the more harmful hydroxyl (OH·) radical.[32] In the present study SOD activity decreased significantly in the ISO-treated group, 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 and 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-Os to ISO-challenged rats effectively prevented the depletion of SOD, CAT, and GPx activities, which can be correlated to the scavenging of free radicals by GBP-Os.[33]

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-Os combination to ISO-myocardial infarcted rats restored the activity of GR, which accelerates the conversion of GSSG to GSH.

Summarizing the effect of GBP-Os combination treatments on marker enzymes and endogenous antioxidants, both combinations showed significant cardioprotective and antioxidant activities. GBP 100 mg/kg + Os 75 mg/kg elicited better cardiac protection than GBP 100 mg/kg + Os 50 mg/kg. The biochemical results are in agreement with the histological findings.

It is possible that GBP-Os exerts its cardioprotective effect by stabilizing the myocardial membrane. Membrane stabilization might be due to an augmentation of basal endogenous antioxidants, leading to increased myocardial antioxidant reserve and strengthened myocardial defense mechanisms.

Flavonoids of *Ginkgo biloba* and *Ocimum sanctum* stabilize ROS by reacting with them and being oxidized to more stable, less reactive radicals. Presumably, the high reactivity of the hydroxyl group of flavonoids is responsible for this free-radical scavenging activity.[34]

$$\text{Flavonoid (OH)} + R· \rightarrow \text{Flavonoid (O·)} + RH$$
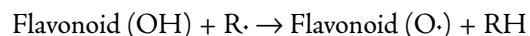
It may be concluded that GBP-Os oral treatment for 30 days to ISO-challenged rats results in significant cardiac protection, decreased lipid peroxidation, and restored antioxidant activities. However, neither combination treatment demonstrated better cardioprotective or antioxidant activities than GBP or Os treatment alone. Hence, the combination treatments failed to show significant synergistic activity in protection of experimental myocardial necrosis. This may be due to a ceiling effect of individual components of the combination. However, it would be interesting to determine whether a small dose of either GBP or Os can potentiate the other's activity.

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