

## Original Research Article

# Protective effect of ginkgolide B against isoproterenol-induced chronic heart failure in rats via modulation of Nrf2 and HO-1 signaling pathways

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### Abstract

**Purpose:** To determine the protective effect of ginkgolide B (GB) against isoproterenol (ISO)-induced chronic heart failure in a rat model.

**Methods:** A total of 32 male Wistar rats were randomly divided into 4 groups. Rats in control group received only saline, while rats in GB alone group were injected with GB at a dose of 20 mg/kg body weight (bwt) intraperitoneally (i.p.). Another group of rats was injected with ISO subcutaneously (s.c.) at a dose of 85 mg/kg for 2 days (ISO group). Rats in the GB+ISO group were administered GB at a dose of 20 mg/kg, i.p., for 7 days prior to exposure to ISO s.c. at a dose of 85 mg/kg.

**Results:** Rats pre-treated with GB for 7 days prior to ISO exposure showed a significant decrease in cardiac infarct size, and marked decreases in the levels of cardiac biomarkers, inflammatory and apoptotic biomarkers, and lipid peroxidation ( $p < 0.05$ ), but significant improvement in the levels of endogenous antioxidants ( $p < 0.05$ ). In addition, GB administration resulted in marked increases in the protein expression levels of heme oxygenase-1 (HO-1) and Nrf2 in cardiac tissue ( $p < 0.05$ ).

**Conclusion:** These results indicate that pre-treatment of chronic heart failure rats with GB for 7 consecutive days considerably lowered inflammatory and apoptotic markers via upregulation of Nrf2/HO-1 signaling pathway. Thus, GB has cardioprotective potential in humans.

**Keywords:** Ginkgolide B, Nrf2/HO-1, Inflammatory markers, Apoptotic markers, Antioxidants

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## INTRODUCTION

Myocardial infarction (MI) or heart attack (HA) is one of the most predominant and deadly forms of ischemic heart disease (IHD), based on the fact that it claims more lives than any other cardiovascular disease (CVD). A report from the World health organization (WHO) indicated that approximately 23 million people worldwide are

affected directly or indirectly by MI [1,2]. Similarly, the National Center for Cardiovascular Diseases in China reported that the number of CVD patients particularly MI patients, is continuously on the increase. Moreover, the cost of treating MI reached 2.3 billion US dollars in 2015, an indication of the indirect and considerable economic burden of the disease on China [3,4].

Myocardial infarction is caused by lack of sufficient blood supply to the heart muscles, leading to necrosis or apoptosis of the myocardium (heart muscle). This is accompanied by numerous pathophysiological and biochemical changes including hypoxia, hyperlipidemia, oxidative stress, and inflammatory responses [5,6]. The current treatment for MI is limited by the fact that many anti-MI medications result in serious adverse effects. Hence, many researchers have focused on natural antioxidant and anti-inflammatory agents for treating or managing MI-related conditions [7,8].

*Ginkgo biloba* is one of the oldest trees known to man. Its bark and leaves are traditionally recommended for improving immune and cognitive function, and for treating the common cold, tinnitus and migraine [9]. Ginkgolide B (GB; C<sub>20</sub>H<sub>24</sub>O<sub>10</sub>) is one of the major diterpenoids extracted from the leaves of the popular *Ginkgo biloba* tree. Ginkgolide B exerts an extensive range of pharmacological effects such as anti-inflammatory, anti-atherosclerotic, anti-tumor, antioxidant as well as hepatoprotective and neuroprotective properties [10-12]. Moreover, GB inhibits NF-κB activation, and protects the brain and heart from ischemia/reperfusion injury in *in vitro* and *in vivo* studies [13-15]. Studies have also shown that ginkgolide B exhibits cardioprotective effect against doxorubicin-induced cardiotoxicity via regulation of oxidative status [16]. Based on previous reports, it is clear that GB shows potent cardioprotective effect in various cardiotoxicity models as well as in ischemia/reperfusion conditions. Nonetheless, the mechanism behind the cardioprotective property of GB against isoproterenol-induced heart failure or myocardial infarction is not fully understood. The current animal study was aimed at investigating the protective effect of GB against isoproterenol-induced chronic heart failure in the rat.

## EXPERIMENTAL

### Chemicals

Ginkgolide B (GB) and isoproterenol (ISO) hydrochloride were purchased from Sigma-Aldrich (MA, USA). Other chemicals and reagents used in this study were of either HPLC or analytical grade.

### Experimental rats and ethical approval

The thirty-two male Wistar albino rats of mean weight 220 ± 10 g were bought from Experimental Animal Lab Vendor; Beijing, China.

The rats were raised in a polycarbonated cage at optimal temperature of 21 ± 2 °C and relative humidity of 55 - 60 %. The animals were handled with utmost care in line with the guidelines set by NRC (Care and handling of laboratory animals) to minimize animal suffering [17]. The animal ethical committee of Wuhan University gave approval for this animal study (approval no. WU/2019-45/3). All animal experiments were conducted at Wuhan University Animal house from March to April, 2018.

### Animal grouping

Healthy male Wistar rats (n = 32) were randomly divided into four groups (8 rats/group). Rats in the control group received only saline, whereas rats in the GB group were injected GB at a dose of 20 mg/kg bwt i.p. for 7 days. Rats in ISO group (MI group) were injected s.c. with ISO at a dose of 85 mg/kg bwt [7] for 2 days (on the 8<sup>th</sup> and 9<sup>th</sup> days), while rats in GB + ISO group were administered GB at a dose of 20 mg/kg i.p. for 7 days prior to ISO exposure (85 mg/kg bwt).

### Sample collection and analysis

On day 10, the rats were euthanized using isoflurane, and blood samples were collected in non-heparinized tubes for preparation of serum samples. The heart tissues were excised, and small portions were sliced and kept at -80 °C for use in triphenyl tetrazolium chloride (TTC) staining. The remaining heart samples were homogenized in Tris-HCl buffered solution (pH 7). The homogenates were centrifuged at 12000 g at 4 °C for 15 min, and the resultant supernatants were used for biochemical assays.

### Determination of antioxidant status and lipid peroxidation

Heart tissue homogenate was used to assay the activities of various endogenous antioxidants and levels of lipid peroxidation products. The levels of malondialdehyde (MDA, a lipid peroxidation product) was measured using an enzymatic assay kit (Kangchen Biotechnology (Shanghai, China) based on the manufacturer's instruction. The activities of cardiac antioxidants [catalase (CAT) and superoxide dismutase (SOD)] were assayed using enzymatic assay kit purchased from Nanjing Bioengineering Institute (Nanjing, China), in line with the manufacturer's protocol.

### Assessment of cardiac biomarkers

Serum lactate dehydrogenase (LDH), creatine kinase isoform MB (CK-MB), and cardiac troponin T (cTn T) were assayed using

commercial ELISA kits (MYBioSource, CA, USA; for LDH and CK-MB; and Teco Diagnostics, CA, USA for cTn T).

### Evaluation of inflammatory markers

Nuclear and cytosolic fractions from heart tissue homogenate were separated with commercial Nuclear/Cytosolic fractionation kit (Cellbiolabs Inc, CA, USA) using ultracentrifugation technique. Inflammatory markers (IL-6, IL-1 $\beta$  and TNF- $\alpha$ ) were measured in the cytosolic fraction of heart tissue homogenate with commercial rat specific ELISA kit (Thermo Fisher Scientific, MA, USA).

The level of NF- $\kappa$ B p65 was measured in the nuclear fraction using commercial ELISA NF- $\kappa$ B (p65) Transcription Factor Assay kit from Cayman Chemical (MI, USA), based on the manufacturer protocol.

### Assay of apoptosis markers

The major apoptotic markers i.e. caspase-3 and caspase-9 were assayed in cardiac tissue homogenate using a commercial assay kit from Abcam (Cambridge, UK) based on manufacturer's instructions.

### Determination of protein expression

The cardiac homogenate was used to determine the protein expressions of HO-1 (in cytosolic fraction) and Nrf2 (in nuclear fraction). The assay technique used was western blotting. The cardiac tissue homogenate was lysed using RIPA buffered solution containing various proteases, followed by determination of protein level using bicinchoninic acid (BCA) protein assay kit (BioVision Inc., CA, USA). An equal amount of protein (50  $\mu$ g) from each group was resolved with 8 % SDS-polyacrylamide gel electrophoresis apparatus, and transferred onto PVDF membrane.

After blocking non-specific binding, the membrane was probed overnight at 4 °C with the following primary antibodies: anti-Nrf2, anti-HO-1 (1:1000 dilution), anti-histone H3 (1:1000 dilution) and anti- $\beta$  actin (1:1000 dilution) (Santa Cruz Biotechnology Inc., TX, USA). Thereafter, the membrane was incubated with rabbit polyclonal anti-horseradish peroxidase (HRP) secondary antibody (1:10000 dilution) at 37 °C for 1 h. The protein bands in the PVDF membrane were developed using enhanced chemiluminescence (ECL) kit, and the protein expression was measured using image analyzing software.

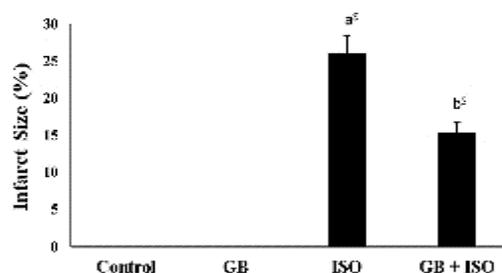
### Statistical analysis

Data are shown as mean  $\pm$  standard error of mean (SEM). Statistical analysis for determination of significant differences amongst groups was carried out with one-way ANOVA, followed by Tukey's multi-comparison test. All statistical analyses were done with GraphPad Prism (Ver 6; Graphpad Software Inc, CA, USA). Significant difference was set at  $p < 0.01$ .

## RESULTS

### Effect of GB on cardiac infarct size

Figure 1 depicts the effect of GB on cardiac infarct size in the control and experimental rats. The infarct size of ISO-treated rats was significantly higher than that of control rats ( $p < 0.01$ ). In contrast, rats injected i.p. with GB at a dose of 20 mg/kg of GB for 7 days prior to ISO injection (GB + ISO group) had marked decreases in infarct size, relative to ISO-treated MI model group ( $p < 0.01$ ).



**Figure 1:** Effect of GB on cardiac infarct size in control and experimental rats. Values are expressed as the mean  $\pm$  SEM; <sup>a\$</sup> $p < 0.01$ , control versus ISO group; <sup>b\$</sup> $p < 0.01$ , GB+ISO versus ISO group

### Effect of GB on cardiac antioxidants and lipid peroxidation

A pronounced increase ( $p < 0.01$ ) in MDA was observed in ISO-treated rats (Table 1). Moreover, the activities of CAT and SOD were significantly decreased in ISO-exposed rats ( $p < 0.01$ ). However, rats pre-treated with GB (GB + ISO) had significant reductions in MDA levels, as well as markedly elevation in the activities of CAT and SOD, when compared with ISO-induced group ( $p < 0.01$ ).

### Effect of GB on cardiac diagnostic markers

The effect of GB on cardiac diagnostic marker enzymes in control and experimental rats are shown in Table 2.

**Table 1:** Effect of GB on heart lipid peroxidation and antioxidant levels in control and experimental rats

Parameter	Control	GB	ISO	GB+ISO
MDA (nmols/mg protein)	0.44 ± 0.08	0.45 ± 0.06	0.78 ± 0.09 <sup>as</sup>	0.57 ± 0.06 <sup>bs</sup>
CAT (U/mg protein)	12.40 ± 2.45	12.72 ± 1.43	8.74 ± 0.10 <sup>as</sup>	10.81 ± 1.44 <sup>bs</sup>
SOD (U/mg protein)	4.89 ± 0.63	5.01 ± 0.70	3.23 ± 0.40 <sup>as</sup>	4.05 ± 0.75 <sup>bs</sup>

Values are expressed as mean ± SEM; <sup>as</sup> $p < 0.01$ , control versus ISO group; <sup>bs</sup> $p < 0.01$ , GB+ISO versus ISO group

**Table 2:** Effect of GB on heart diagnostic biomarkers in control and experimental rats

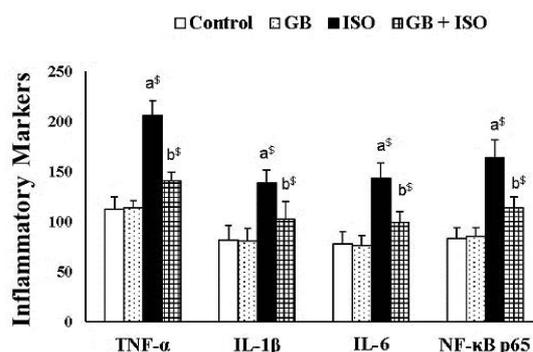
Parameter	Control	GB	ISO	GB+ISO
LDH (IU/L)	68.92 ± 8.10	70.12 ± 9.34	134.74 ± 18.03 <sup>as</sup>	85.56 ± 10.22 <sup>bs</sup>
CK-MB (IU/L)	59.29 ± 7.20	60.92 ± 7.45	130.86 ± 20.00 <sup>as</sup>	78.00 ± 10.05 <sup>bs</sup>
cTn T (ng/mL)	0.45 ± 0.07	0.47 ± 0.05	1.33 ± 0.35 <sup>as</sup>	0.64 ± 0.09 <sup>bs</sup>

Values are expressed as mean ± SEM. <sup>as</sup> $p < 0.01$ , control versus ISO group; <sup>bs</sup> $p < 0.01$ , GB+ISO versus ISO group

There were significant increases in the activities of cardiac diagnostic marker enzymes i.e. CK-MB, LDH, and cTn T in ISO-exposed MI model rats ( $p < 0.01$ ). However, these increases were significantly reversed to normal levels by treatment with GB i.e. in the GB + ISO group ( $p < 0.01$ ).

### Effect of GB on cardiac inflammatory biomarkers

As shown in Figure 2, the mean levels IL-6, IL-1 $\beta$ , TNF- $\alpha$  and NF- $\kappa$ B p65 subunit in cardiac tissue homogenate were significantly enhanced ( $p < 0.01$ ) in rats exposed to ISO (MI model), relative to control rats. However, administration of GB before ISO injection (GB + ISO) led to considerable decreases in the levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and NF- $\kappa$ B p65 subunit, when compared with ISO-exposed group ( $p < 0.01$ ).

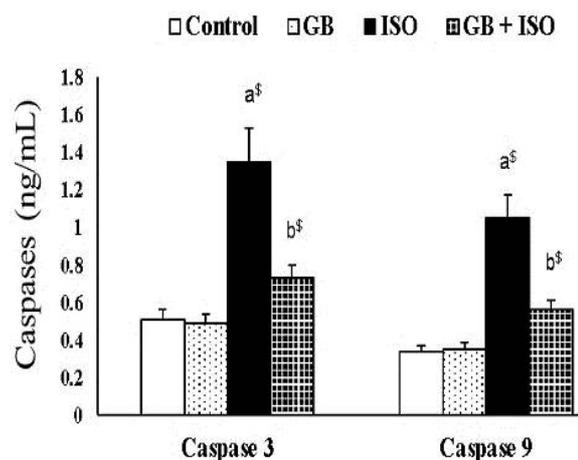


**Figure 2:** Effect of GB on cardiac inflammatory biomarkers (pro-inflammatory cytokines) in control and experimental rats. Values are expressed as mean ± SEM; <sup>as</sup> $p < 0.01$ , control versus ISO group; <sup>bs</sup> $p < 0.01$ , GB+ISO versus ISO group

### Effect of GB on cardiac apoptotic biomarkers

Figure 3 shows the effect of GB on cardiac apoptotic markers (caspase-3 and caspase 9) in control and experimental rats. The activities of

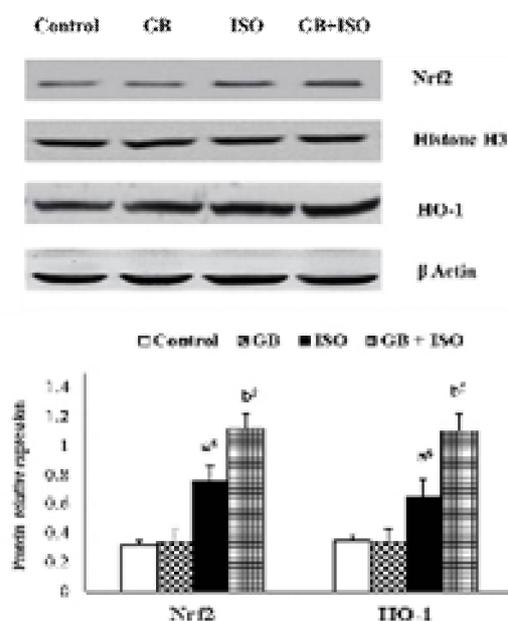
caspase-3 and caspase-9 were significantly upregulated in rats injected with ISO at a dose of 85 mg/kg (MI model), when compared with control rats ( $p < 0.01$ ). In contrast, rats injected with GB for 7 days before ISO-exposure (GB + ISO) showed a considerable decline in the activity of caspases-3 and caspase-9, relative to ISO-exposed rats ( $p < 0.01$ ).



**Figure 3:** Effect of GB on cardiac apoptotic markers (caspase-3 and caspase-9) in control and experimental rats. Values are expressed as the mean ± SEM. Values are expressed as mean ± SEM; <sup>as</sup> $p < 0.01$ , control versus ISO group; <sup>bs</sup> $p < 0.01$ , GB+ISO versus ISO group

### Effect of GB on protein expressions of HO-1 and Nrf2

The protein expression levels of HO-1 (in cytosolic fraction) and Nrf2 (in nuclear fraction) were markedly upregulated in rats exposed to ISO, when compared with control rats ( $p < 0.05$ ; Figure 4). Rats administered GB prior to ISO-treatment (GB + ISO rats) had higher levels of protein expressions of HO-1 and Nrf2 than ISO-exposed rats.



**Figure 4:** Effect of GB on cardiac protein expressions of Nrf2 and HO-1 in control and experimental rats; <sup>as</sup> $p < 0.01$ , control versus ISO group; <sup>bs</sup> $p < 0.01$ , GB+ISO versus ISO group

## DISCUSSION

The results obtained in this animal study have highlighted the cardioprotective potential of GB through significant decreases in cardiac infarct size and reduced levels of cardiac markers, inflammatory biomarkers and biomarkers of apoptosis. Moreover, GB decreased lipid peroxidation and brought about significant increases in the activities of endogenous antioxidants through upregulation of protein expressions of Nrf2 and HO-1. The hallmark of myocardial infarction is increased infarct size in the myocardium, which results in death of cardiomyocytes [6]. Thus, the effect of GB on cardiac infarct size was determined using TTC staining procedure. Rats treated with GB at a dose of 20 mg/kg had substantial decreases in the infarct size. Similarly, Zhang and his co-workers have reported that GB treatment significantly reduced infarct size in a myocardial ischemia/reperfusion injury (MI/RI) rat model [14].

Oxidative stress and inflammatory response constitute major pathophysiological processes of MI [5]. Thus, in this study, the effect of GB on cardiac antioxidants and lipid peroxidation was measured by assay of the antioxidant enzymes CAT and SOD. Injection of rats with GB i.p. for 7 days led to significant improvement in antioxidant capacity and lower MDA production owing to the potent free radical scavenging effect of GB. Recently, Li and his colleagues reported that administration of GB

markedly enhanced SOD and CAT activities, and significantly lowered the lipid peroxidation product (MDA) in a rat model of hypoxia-induced neuronal damage [18].

Myocardial infarction (MI) results in damage to cardiomyocyte membranes and the release of cardiac diagnostic marker enzymes (LDH, CK-MB and cTnT) into the blood. Thus, these enzymes are used as diagnostic parameters for monitoring the degree of MI [19]. In the present study, rats exposed to ISO had increased serum levels of the cardiac marker enzymes CK-MB, LDH and cTnT due to increased cardiomyocyte damage. However, GB treatment reversed these increases owing to its antioxidant and anti-lipid peroxidation properties. A study has shown that GB significantly reduced the levels of cardiac troponin T and LDH in a rat model of myocardial I/R [13].

Inflammation is one of the pivotal biochemical responses during MI. The levels of various inflammatory markers were markedly increased in ISO-treated rats owing to the activation of the NF- $\kappa$ B signaling cascade, thereby triggering excessive free radical generation. Pre-treatment of rats with GB significantly decreased the levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and NF- $\kappa$ B p65 subunit by inhibiting the activation of the NF- $\kappa$ B signaling pathway. It has been demonstrated that GB supplementation suppressed the activation of NF- $\kappa$ B p65 and triggered inflammatory response in various animal models [14,20].

Apoptosis (programmed cell death) is directly associated with various pathophysiological conditions, especially MI. In this study, rats exposed to ISO had increased levels of caspase 3 and caspase 9 due to enhanced oxidative stress and inflammatory response, leading to up-regulation of the caspase cascade and cardiomyocyte apoptosis. However, these changes were reversed by pre-treatment of the rats with GB for 7 consecutive days. These results are in agreement with the reports by Gu and coworkers, who found that GB markedly altered caspase cascade-related proteins in a mouse model of cerebral ischemia/reperfusion injury [15].

Nuclear transcription factor (Nrf2) plays a major role in the regulation of redox homeostasis by inducing various antioxidants and detoxifying enzymes including HO-1 and NADPH: quinone oxidoreductase 1 (NQO-1) [21]. The protein expressions of Nrf2 and HO-1 were significantly upregulated in ISO-exposed rats, resulting in protection of the myocardium from further damage (cytoprotective effect) triggered by

normal physiological redox response. The GB treatment also triggered on cytoprotective redox mechanism and resulted in further upregulation of Nrf2 and HO-1 protein expressions, and increased production of various antioxidant enzymes. Likewise, Jiang and co-workers have demonstrated that treatment with GB markedly upregulated protein expressions of Nrf2 and HO-1 in the renal tissue of diabetic rats [10].

### Limitations of the study

This study has some limitations: the left ventricular function was not subjected to electrocardiography and histopathological examination, and other signaling pathways (PI3K/Akt and GSK) were not investigated, due to financial constraints.

### CONCLUSION

Pre-treatment of rats with ISO-induced heart failure using GB for 7 consecutive days resulted in marked lowering of lipid peroxidation, inflammatory biomarkers and apoptotic indices, with enhanced antioxidant status via upregulation of Nrf2/HO-1 signaling pathway. Thus, GB exerts strong cardioprotective potential and might be beneficial in the clinical management of MI-related conditions. Nevertheless, clinical trials are needed to ascertain the cardioprotective and other effects of GB in humans.

### DECLARATIONS

#### Acknowledgement

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#### Conflict of interest

No conflict of interest is associated with this study

#### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lu Hui Zhi (LHZ), Dong Hui (DH) and Liu Li (LL) designed and conceived this animal experiment. LL, WB, and LHZ conducted most of the animal experiments. LL and LHZ involved in drafting and editing this research paper. Wang Bin ru (WB) and Tan Yun aided in statistical analysis.

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