Ginkgo biloba extract prevents acute myocardial infarction and suppresses the inflammation- and apoptosis-regulating p38 mitogen-activated protein kinases, nuclear factor-κB and B-cell lymphoma 2 signaling pathways

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Abstract. *Ginkgo biloba* is a plant known from the Mesozoic and has been regarded as one of the first to be used in traditional Chinese medicine (TCM). The plant extract has attracted a great deal of attention in recent years. The *Ginkgo biloba* leaf contains flavones and diterpenes. In addition, *Ginkgo biloba* performs certain pharmacologic actions, including antioxidant and anti-aging activities. The aim of the present study was to examine whether *Ginkgo biloba* extract prevents acute myocardial infarction (AMI). The results demonstrated that *Ginkgo biloba* extract significantly inhibited infarct size, increased serum histamine levels and weakened creatine kinase (CK)-MB activity in AMI mice. *Ginkgo biloba* extract significantly inhibited serum interleukin (IL)-6 and IL-1β levels, and caspase-3/9 activity. In addition, it suppressed matrix metallopeptidase-9, transforming growth factor-β, p38 mitogen-activated protein kinases (MAPK) and nuclear factor (NF)-κB protein expression, and promoted B-cell lymphoma 2 (Bcl-2) protein expression in AMI mice. The results of *in vivo* assays demonstrated that *Ginkgo biloba* extract prevents AMI and suppresses inflammation- and apoptosis-regulating p38 MAPK, NF-κB and Bcl-2 signaling pathways.

Introduction

According to multi-level cooperative study results of cardiovascular health in random samples of urban and rural residents (aged 35-74 years) in China, the prevalence rate of congestive heart failure in females was identified to be 1.0% (1,2). The north had a higher prevalence rate compared with the south and as age increased, the prevalence rate markedly increased; however, there was no clear difference between urban and rural regions (2). With the increase of coronary heart disease and high blood pressure-associated morbidity, accelerating population aging and the increase of various dangerous factors, the number of patients presenting with congestive heart failure in China is also increasing (3). Ischemic heart disease caused by coronary artery disease has already become the most common pathogenesis resulting in congestive heart failure and seriously threatens the health of Chinese patients (4).

Acute myocardial infarction (AMI) causes oxidative stress reactions and inflammatory responses. These activate potential matrix metalloproteinases (MMPs) in cardiac muscle tissues (such as MMP-1, MMP-2, MMP-3 and MMP-9), degrade extracellular matrix and coronary vessel structures, promote inflammatory cell homing in the blood to the ischemic myocardium, as well as participating in enzymolysis and phagocytosis of infarct cardiac muscle tissues (5,6).

Subsequent to AMI, reactive oxygen species (ROS) and intracellular components generated by the damaged myocardium activate Toll-like receptor, NF-κB expression and complement activation, causing high expression of the vascular endothelial cell adherence factor in infarct cardiac muscle tissue and the increase in expression of damage-associated stress factors, and increasing the number of inflammatory cells in circulation, including neutrophil granulocytes and macrophages, which return to the infarct area, participating in enzymolysis and phagocytosis of the infract cardiac muscle tissue (7,8). Currently, the inflammatory response peaks one or two weeks after AMI, and 3 to 4 weeks after AMI, the inflammatory cells become apoptotic and diminish independently (9).

A previous study demonstrated that myocardial ischemia may be the initial factor of ventricular remodeling after AMI (10). Relevant damage-associated stress factors following AMI contribute to regulating myocardial cell death and progression of ventricular remodeling via other approaches (11). For example, AMI promotes Bcl-2 interacting protein 3 to express relevant genes, which results in apoptosis of myocardial cells via a caspase-dependent pathway (12).

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A previous study indicates that myocardial cells may be prevented from presenting ischemia and anaerobic conditions, so as to improve ventricular remodeling (11). Currently, researchers hope to regulate programmed cell death after AMI and improve the prognosis of ventricular remodeling.

Ginkgo biloba (also termed ginkgo) is a tall deciduous tree. The plant dates back to the Carboniferous period, 345 million years ago (13). Following Quaternary glaciation, it is the sole living representative of its genus and one of the oldest relic plants in the world. *Ginkgo biloba* was initially native to China and was subsequently introduced to Europe in 1710 (14). *Ginkgo biloba* extract may directly lead to anti-oxygenation, elimination of oxygen free radicals, regulation of superoxide dismutase activity and catalases, as well as eliminating nitric oxide (NO), thus contributing to protecting against ischemia damage and damage of vascular endothelial cells, potentially preventing atherosis (15). In the current study, whether *Ginkgo biloba* extract prevents AMI was investigated, in addition to the molecular mechanisms associated with its anti-inflammation effect.

Materials and methods

Animals. The current study was performed in strict accordance with the recommendations from the Guide for Animal Management Rules from Guangxi Medical University (Nanning, China). C57BL/6 mice (n=24, 8 mice per group) were purchased from the Department of Laboratory Animal Science (Guangxi Medical University) and housed together under specific-pathogen-free conditions (23-24˚C; humidity, 55-60%) in an animal room under a 12-h light/dark cycle with free access to water and food. All mice were randomly distributed into three groups as follows: Control, AMI and AMI + *Ginkgo biloba* extract (GBE, Guizhou Provincial Biochemical Engineering Center, Guiyang, China).

Induction of the AMI model. Anesthesia was performed by inhalation of 1.0-2.0% isoflurane gas and mechanically ventilated on a positive pressure ventilator. Left thoracotomy was performed and the pericardium was immediately stripped away to expose the heart. The coronary artery was identified and occluded with an 8-0 silk ligature; successful ligation was confirmed when the left ventricle turned pale. The chest cavity was closed and mice were placed in their cages on a heating pad. The control mice underwent the same surgical procedures without ligation. AMI mice were administered normal saline (200 μl) via daily gavage for 8 weeks. The AMI + GBE group mice received 100 mg/kg GBE via daily gavage for 8 weeks.

Infarct size assessment. Anesthesia was performed by inhalation of 1.0-2.0% isoflurane gas and the mice were sacrificed using decollation following the 8 weeks of the experiment. The hearts were immediately removed and cut into 1.0 mm vertical sections. These sections were stained with 1% 2,3,5-triphenyl-tetrazolium chloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBS for 10 min at 37˚C. Infarct size areas were determined using a microscope (model BX53M; Olympus Corporation, Tokyo, Japan). with Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

ELISA assay. Anesthesia was performed by inhalation of 1.0-2.0% isoflurane gas and then blood (100 μl) was collected from the eye sockets of the mice after treatment with *Ginkgo biloba* extract. Serum was collected by centrifugation at 2,000 x g for 10 min at 4˚C. ELISA kits were used to determine serum histamine, lactate dehydrogenase, creatine kinase (CK; A032) and CK-MB (H197), interleukin (IL)-6 (H007) and IL-1β (H002) levels, and caspase-3/9 activity (G015 and G018) were evaluated using ELISA kits (all from Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) according to the manufacturer's instructions.

Western blotting. Anesthesia was performed by inhalation of 1.0-2.0% isoflurane gas and mice were sacrificed using decollation following treatment with *Ginkgo biloba* extract. The hearts were immediately removed and homogenated using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) for 30-40 min at 4˚C. Lysates were centrifuged at 10,000 x g for 10 min to analyze the protein concentration via BCA assay (Beyotime Institute of Biotechnology) and then 50-80 μg protein was resolved on 8-10% SDS gel. Following electrophoresis, the proteins were electrotransferred (2.5A, 25 V for 30 min) onto a nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed with MMP-9 (cat. no. 13667; dilution, 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), TGF-β (cat. no. 5544; dilution, 1:2,000; Cell Signaling Technology, Inc.), p-p38 (cat. no. 4511; dilution, 1:2,000; Cell Signaling Technology, Inc.), NF-κB (cat. no. 8242; dilution, 1:2,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. AF0006; dilution, 1:3,000; Beyotime Institute of Biotechnology) antibodies overnight at 4˚C. The blot was washed with TBST three times for 5 min, exposed to horseradish peroxidase-conjugated secondary antibodies (cat. no. A0208; dilution, 1:5,000; Beyotime Institute of Biotechnology) for 1 h at 37˚C, and finally examined by chemiluminescence (ECL; GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. Data are presented as means ± standard error of the mean. Comparisons between the two groups were assessed by Student's t-test or two-way analysis of variance followed by Bonferroni's post-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GBE reduces the size of infarct areas. GBE was observed to reduce the size of infarct areas in the AMI model mice. A significant increase in the size of the infarct areas was observed in the AMI model group, when compared with the control group (Fig. 1). Subsequently, GBE treatment significantly inhibited the increase of infarct area size in the AMI mice, when compared with the AMI model group (Fig. 1).

GBE prevents AMI. The effects of GBE on AMI were subsequently evaluated. As compared with the control group, serum histamine was significantly decreased, and LDH, CK and CK-MB levels in the AMI mouse models were significantly increased (Fig. 2). However, GBE treatment significantly increased the serum histamine level, and decreased the LDH,

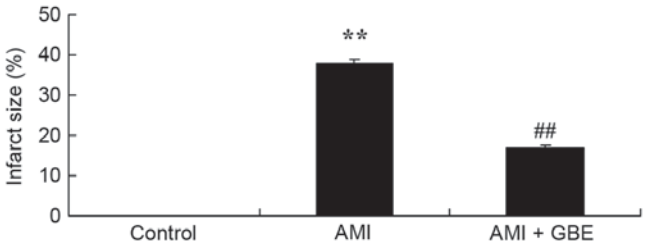

Figure 1. GBE treatment reduces infarct area size. **P<0.01 vs. control; ##P<0.01 vs. AMI. AMI, acute myocardial infarction; GBE, *Ginkgo biloba* extract.

CK and CK-MB levels in the AMI mice, when compared with AMI mouse model group (Fig. 2).

GBE reduces inflammatory reactions. To investigate the effect of GBE on inflammatory reactions, the expression levels of IL-6 and IL-1β in tissue samples were analyzed by ELISA. Significantly increased IL-6 and IL-1β activity levels were observed in the AMI mice, compared with the control group (Fig. 3). Treatment with GBE significantly inhibited the increased IL-6 and IL-1β activities in the AMI mice (Fig. 3).

GBE reduces caspase-3/9 activity. In order to investigate the effect of GBE on apoptosis, caspase-3/9 activities were analyzed by ELISA. Fig. 4 demonstrates the significantly increased caspase-3/9 activities in the AMI mice as compared with the control group. GBE treatment significantly reduced the caspase-3/9 activities in the AMI mice, when compared with AMI model group (Fig. 4).

GBE reduces MMP-9 protein expression levels. To evaluated the underlying mechanism of GBE against AMI, MMP-9 protein expression levels were analyzed using western blotting. The results indicated that MMP-9 protein expression was significantly induced in the AMI mouse model when compared with the control group. As compared with AMI model group, the group treated with GBE demonstrated significantly suppressed MMP-9 protein expression levels (Fig. 5).

GBE reduces TGF-β protein expression levels. TGF-β expression was examined to evaluate the underlying mechanism of GBE against AMI. The level of TGF-β protein expression observed in the AMI model group was significantly higher than that of control group. Treatment with GBE significantly suppressed TGF-β protein expression levels in the AMI mice, when compared with the AMI model mice (Fig. 6).

GBE reduces levels of p-p38 protein expression. Subsequently, the underlying mechanism of the effect of GBE against AMI was investigated by evaluating p-p38 protein expression levels using western blotting. The levels of p-p38 protein expression in the AMI model group were greater than that of the control group. In the AMI mice treated with GBE, p-p38 protein expression levels were significantly suppressed when compared with the AMI model mice (Fig. 7).

GBE reduces NF-κB protein expression levels. The underlying mechanism of GBE against AMI was evaluated by western blotting to detect NF-κB protein expression levels. A significant increase of NF-κB protein expression was observed in the AMI model mice when compared with the control group (Fig. 8). When the AMI mice were treated with GBE, NF-κB protein expression was significantly suppressed (Fig. 8).

GBE reduces Bcl-2 protein expression levels. The Bcl-2 protein expression levels were analyzed to investigate the underlying mechanism of GBE against AMI. Bcl-2 protein expression in the AMI model mice was significantly inhibited compared with the control group (Fig. 9). Following GBE treatment Bcl-2 protein expression in the AMI mice was significantly increased compared with the AMI model mice (Fig. 9).

Discussion

AMI leads to ischemic myocardium issues, particularly in the reperfusion area, where large quantities of ROS are generated, which cause direct damage to cytomembrane structures, such as inducing overloading in cells to increase mitochondrial membrane permeability and causing cell death (16). Furthermore, ROS promote the release of inflammatory factors, such as TNF-a, IL-1β and IL-6 in the ischemic region and surrounding area. Apoptosis is induced via the TNF-a/caspase signaling pathway to promote myocardial contraction (17). In addition, ROS and relevant inflammatory factors activate MMPs, degrade the extracellular matrix (ECM), resulting in sliding cardiac muscle fibers and finally causing expansion (18,19). The results of the current study demonstrated that GBE treatment significantly inhibited the increase of infarct area size, increased serum histamine levels, decreased LDH, CK and CK-MB levels, inhibited the increase of IL-6 and IL-1β activities and reduced caspase-3/9 activities in AMI mice. Li *et al* (20) reported that GBE inhibits experimental rat myocardial remodeling via TGF-β1, MMP-2 and MMP-9 (20).

Dynamic changes in the ECM occur following AMI and has an important role in ventricular remodeling. During the period of AMI, transforming growth factor in cardiac muscle tissue of TGF-β promoting fibrosis factor is activated (21). The fibrosis cell generates into type I and type III collagenous fibers, which gradually develop into scar tissue. Meanwhile, cardiac muscle tissue in non-infarct areas exhibits interstitial and peripheral fibrosis (22). Thus, these results demonstrate that GBE significantly suppresses TGF-β protein expression in AMI mice. Li *et al* (20) reported that GBE treatment inhibits myocardial remodeling via TGF-β1, MMP-2 and MMP-9 in experimental rats (20).

p38 MAPK is activated by phosphorylation, which increases the expression of inflammatory factors in rats. This causes thickening of the heart, interstitial fibrosis, serious cardiac insufficiency, myocardial apoptosis or mortality (23). p38 MAPK has previously been demonstrated to increase the expression levels of inflammatory factors following activation of myocardial ischemia reperfusion, by reactive activation of p38 MAPK (24). p38 MAPK is associated with myocardial remodeling and inflammatory factor expression in the myocardium following AMI (24). p38 MAPK influences the heart, by stimulating synthesis of inflammatory factors, promoting cell transformation into fibroblasts to integrate into the ECM,

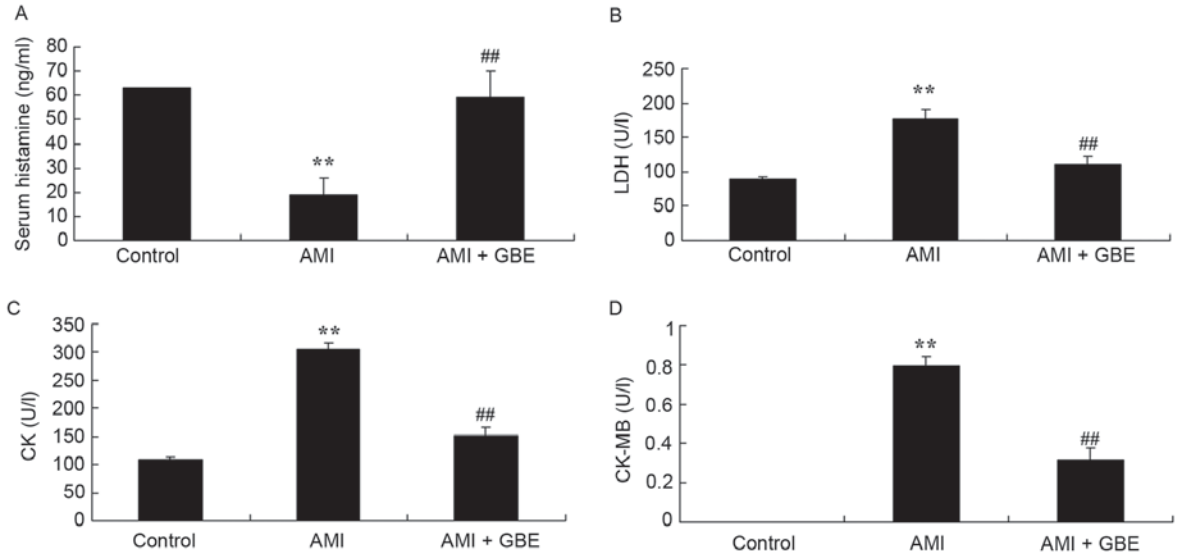

Figure 2. GBE prevents AMI. GBE treatment reduces serum levels of (A) histamine, (B) LDH, (C) CK and (D) CK-MB. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AMI. AMI, acute myocardial infarction; GBE, *Ginkgo biloba* extract; LDH, lactate dehydrogenase; CK, creatine kinase.

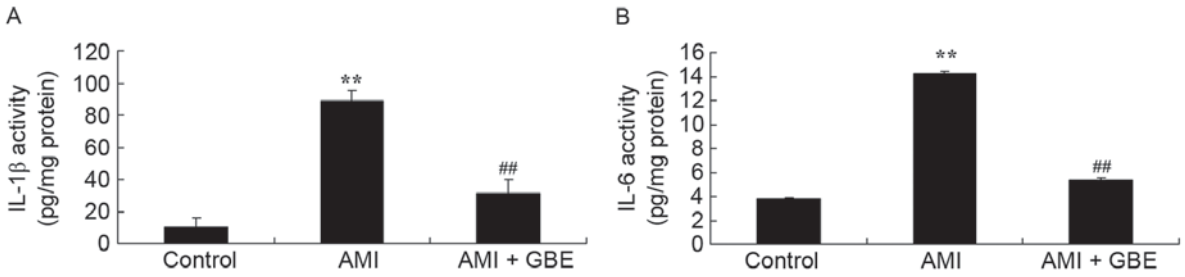

Figure 3. GBE reduces inflammatory reactions. GBE treatment reduces levels of (A) IL-6 and (B) IL-1β. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AMI. AMI, acute myocardial infarction; GBE, *Ginkgo biloba* extract; IL, interleukin.

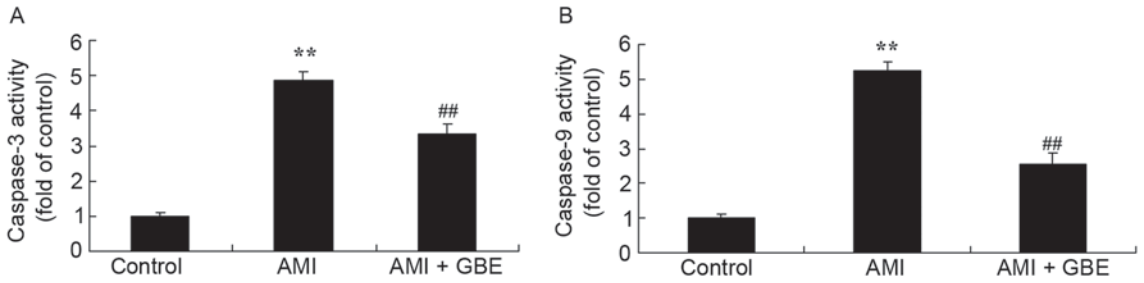

Figure 4. GBE reduces caspase-3/9 activities. Treatment with GBE reduces (A) caspase-3 and (B) caspase-9 activities. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AMI. AMI, acute myocardial infarction; GBE, *Ginkgo biloba* extract.

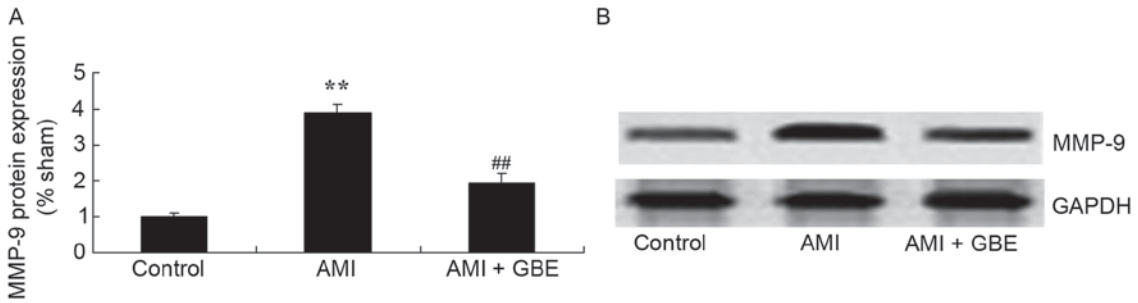

Figure 5. GBE treatment reduces MMP-9 protein expression levels. (A) Quantitative and (B) western blot analyses of MMP-9 protein expression levels. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AMI. GBE, *Ginkgo biloba* extract; MMP-9, matrix metalloproteinase-9; AMI, acute myocardial infarction.

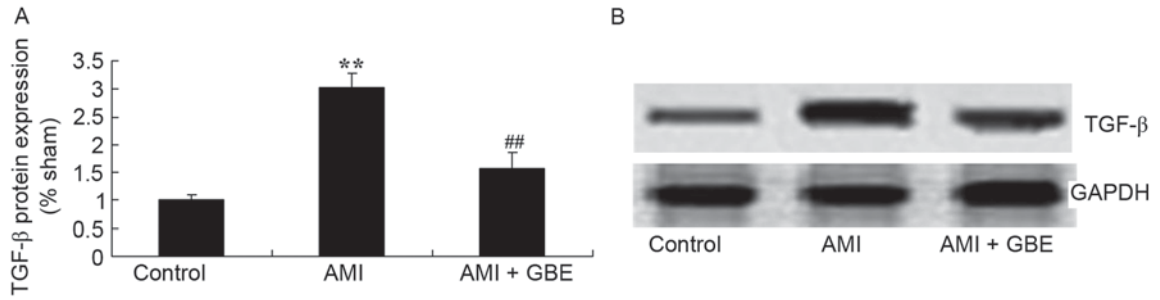

Figure 6. GBE treatment reduces TGF-β protein expression levels. (A) Quantitative and (B) western blot analyses of TGF-β protein expression levels. **P<0.01 vs. control; ##P<0.01 vs. AMI. GBE, *Ginkgo biloba* extract; TGF-β, transforming growth factor-β; AMI, acute myocardial infarction.

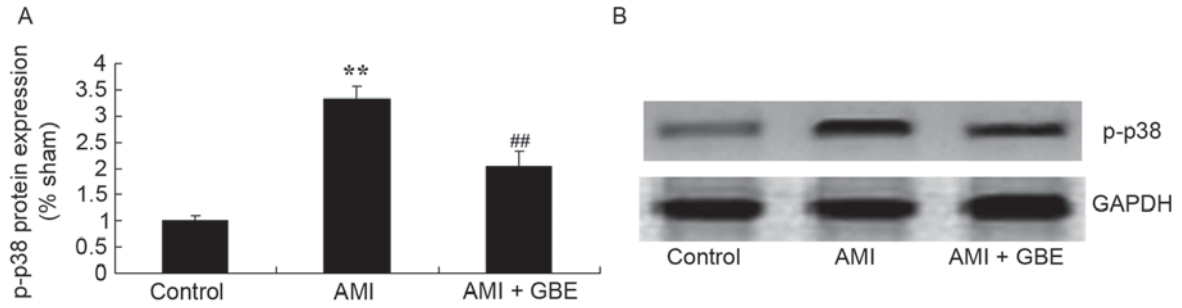

Figure 7. GBE treatment reduces p-p38 protein expression levels. (A) Quantitative and (B) western blot analyses of p-p38 protein expression levels. **P<0.01 vs. control; ##P<0.01 vs. AMI. GBE, *Ginkgo biloba* extract; AMI, acute myocardial infarction.

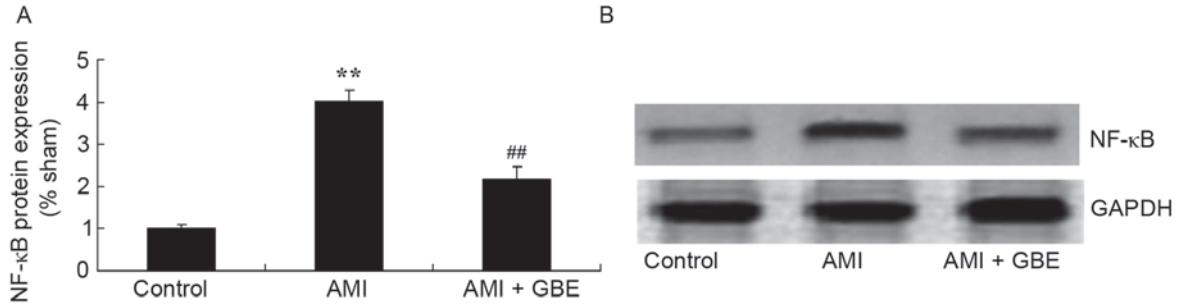

Figure 8. GBE treatment reduces NF-κB protein expression levels. (A) Quantitative and (B) western blot analyses of NF-κB protein expression levels. **P<0.01 vs. control; ##P<0.01 vs. AMI. GBE, *Ginkgo biloba* extract; NF-κB, nuclear factor-κB; AMI, acute myocardial infarction.

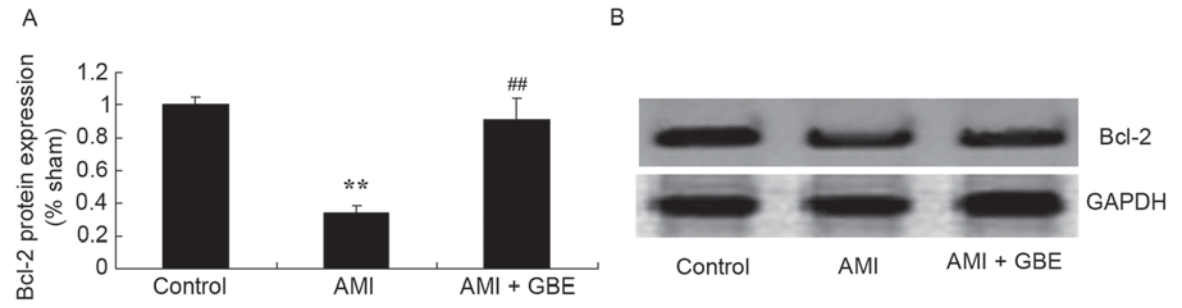

Figure 9. GBE treatment reduces Bcl-2 protein expression levels. (A) Quantitative and (B) western blot analyses of NF-κB protein expression levels. **P<0.01 vs. control; ##P<0.01 vs. AMI. GBE, *Ginkgo biloba* extract; Bcl-2, B-cell lymphoma 2; AMI, acute myocardial infarction.

and by inhibiting MMP degradation of the ECM. TGF-β1 also promotes hypertrophy of myocardial cells, differentiation and the increase of the number of lymphocytes (25). In addition, TGF-β1 is involved in stimulating tissue fibrosis, causing increases in fibrocytes, MMPs, collagen deposition and fiber binding proteins, and results in ventricular remodeling (23). TGF-β1 expression activity in cardiac muscle tissues of myocardial infarction rats was demonstrated to be markedly

enhanced (26). Corresponding MAP3K7, p38 MAPK, and p-p38 MAPK protein activity were also clearly enhanced (26). The possible underlying mechanism involves TGF-β1 activating MAP3K7, thus causing p38 MAPK to be phosphorylated into p-p38 MAPK and enhancing inflammatory factor expression levels in rats, finally resulting in cardiac hypertrophy, interstitial fibrosis, serious cardiac insufficiency, myocardial apoptosis or mortality (25). The present study demonstrates that GBE significantly suppressed MMP-9 and p-p38 protein expression in AMI mice. In addition, Tsai *et al* (15) demonstrated that GBE reduces high-glucose-induced endothelial ROS generation via Akt/endothelial NO synthase and p38 MAPK signaling pathways (15).

NF-κB, a nuclear transcription factor, was initially identified in mature B cells in 1986, specifically binding with the enhancer subsequence of the immune globulin κ light-chain gene (27,28). NF-κB regulates the relevant processes of AMI, including generation of NO, synthesis of prostaglandin, calcium and sodium treatment, growth factors, apoptosis, ECM, stress and reconstruction (29). In the smooth muscle cells of human atherosclerotic plaque, macrophages and endothelial cells, it has been demonstrated that activated NF-κB participates in lipid modification, chemotaxis and attachment (30). Inflammatory factors and tissue damage result in lesions and unstable plaque. NF-κB participates in and mediates the process by regulating NO (29). In addition, NF-κB participates in the immune response and cell apoptosis, and is the key transcription factor causing inflammatory reactions. An increasing number of studies demonstrates that NF-κB is important in AMI (28). The current study demonstrates that GBE significantly reduced NF-κB protein expression levels and induced Bcl-2 protein expression in AMI mice. Furthermore, Wang *et al* (13) indicated that GBE mitigates liver fibrosis via NF-κB, p38 MAPK and Bcl-2/Bcl-2-associated X protein signaling (13).

In conclusion, the present results demonstrates that treatment with GBE prevents AMI, by increasing serum histamine levels, decreasing LDH, CK and CK-MB levels, and suppressing inflammation- and apoptosis-regulating p38 MAPK, NF-κB and Bcl-2 signaling. Therefore, as GBE abrogates the activity of p38 MAPK and NF-κB signaling pathways in AMI, it may serve as an effective therapeutic strategy against various types of heart disease.

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