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Original Article

Cardioprotective effect of embelin ameliorates acute myocardial infarction through the inhibitions of inflammatory reactions and oxidative stress in rats

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Abstract: Embelin possesses a variety of pharmacological activities, including anti-oxidative anti-inflammation, anti-bacteria, anti-fertility, protecting brain injury, anticonvulsant, promoting wound healing, anti-depressant effects. However, the cardio-protective effect of embelin on acute myocardial infarction (AMI) is largely unknown. This study investigated the cardio-protective effect and possible mechanism of embelin on AMI rats. Rats were pretreated for three days with embelin (50 mg/kg, p.o) after rat inducing AMI model. Serum specific cardiac injury biomarkers (CK-MB, LDH and AST) and infarct size were analyzed. Inflammatory reactions, oxidative stress and caspase-3/9 expressions were evaluated using commercial kits. Cytochrome c, Bcl-2, Bax and PARP protein expressions were appraised using western blot analysis. Results showed that administering embelin significantly reduced the elevated serum levels of CK-MB, LDH, AST and infarct size of AMI rats, when compared to AMI rat group. Meanwhile, pretreatment with embelin significantly decreased inflammatory reactions and oxidative stress of AMI rats. Exploration of the underlying mechanisms of embelin revealed interrupted mitochondria dependent apoptotic damage through downregulating the expression of caspase-3/9, cytochrome c, Bax and PARP, and increasing the myocardial expression of Bcl-2. Result suggested that the cardioprotective effect of embelin ameliorates acute myocardial infarction through the inhibitions inflammatory reactions and oxidative stress in rats.

Keywords: Embelin, acute myocardial infarction, inflammatory, oxidative stress

Introduction

Acute myocardial infarction (AMI) is an important cause of death for cardiovascular disease, which is also a common clinical symptom of sudden death [1]. Mostly, based on long and sharp coronary lesions, coronary blood flow is drastically reduced or interrupted, which leads to serious and lasting acute ischemia of the corresponding myocardial, resulting in ischemic myocardial necrosis. AMI is one of the problems to be solved in medicine field in the 21st century [2].

AMI is widely recognized as an inflammatory injury. Inflammation exists throughout the occurrence and development of AMI and inflammatory cells as well as NF-κB, Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 play an important role in the early stage of immuno-inflammatory response and late ventricular reconstruction of AMI [3, 4]. Meng et al. demonstrated that oxysophoridine attenuates the injury caused by AMI in rats through anti-oxidative and anti-inflammatory pathways [5]. Doi et al. showed that early eicosapentaenoic acid treatment reduces acute inflammatory responses in patients with AMI after percutaneous coronary intervention [6].

Ventricular remodeling refers to the progressive expansion and shape changes of left ventricle after AMI, including the changes in ventricular volume, shape, wall thickness, cardiac structure, the ultrastructure and other aspects. Recent studies have shown that oxidative stress plays an important role in pathological and physiological changes after myocardial infarction, associated with the severity of the disease [7]. Animal studies have shown that oxi-

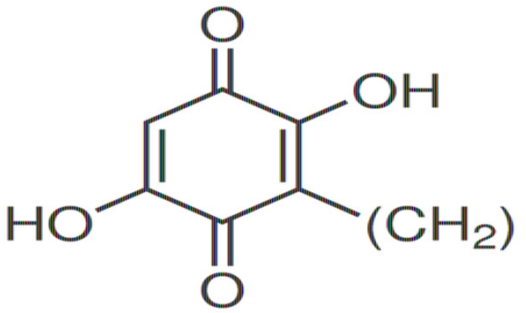

Figure 1. The chemical structure of embelin.

dative stress is involved in the pathophysiologic process of ventricular remodeling, and interference with oxidative stress can be taken as a means of prevention and treatment for ventricular remodeling. Freitas et al. indicated that polycyclic aromatic hydrocarbons contribute to oxidative stress (Malondialdehyde (MDA), superoxide dlismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX) and exogenous antioxidants) and are associated to acute myocardial infarction [8]. Meanwhile, Tan's study also showed baicalein protects against AMI-induced injury by inhibiting oxidative stress [9].

Embelin (2,5-dihydroxy, 3-undecyl-p-benzoquinone) is the active ingredient of acid vine fruit, with a variety of pharmacological activities, including anti-cancer, anti-inflammation, analgesia, inhibiting hepatitis C virus, anti-bacteria, anti-fertility, cytotoxic activity, protecting brain injury, anticonvulsant, promoting wound healing, anti-depressant effects [10-12]. However, the effect of embelin on AMI has not been examined. In the current study, we aimed to evaluate the cardio-protective effect of embelin on acute myocardial infarction and to elucidate the mechanism of protection by AMI in rats.

Materials and methods

Chemicals

Creatine kinase (CK-MB), lactate dehydrogenase (LDH) and AST (aspartate transaminase) commercial kits were purchased from Sangong Biotech (Shanghai, China). NF-κB, TNF-α, IL-1β, IL-6, SOD, CAT, GR, GSH-PX, caspase-3 and caspase-9 commercial kits was purchased from Sigma-Aldrich Co (MO, USA). Bicinchoninic acid (BCA) commercial kits were purchased from Pierce Biotechnology (Rockford, USA).

Experimental animals

Male Sprague-Dawley rats (150-180 g) were housed under optimal conditions of temperature, relative humidity 40-60% and light-cycle (12 L:12 D) and allowed food and water. All experimental protocols were approved by the University Committee on Research Practice at ZhongNan Hospital of Wuhan University.

Induction of AMI

The left anterior descending coronary artery was ligated as AMI model [13]. All Sprague-Dawley rats were intraperitoneally (i.p.) anesthetized with intraperitoneally anesthetized and then given an operation. Successful establishment of AMI models was ascertained by ST-segment elevation and regional cyanosis of myocardial surface.

Experimental design

The chemical structure of embelin (purity > 98%, Sigma, USA) was indicated in **Figure 1**. The Sprague-Dawley rats were randomly divided into four groups consisting of 8 rats in each group. (1) control group (Con), which was normal mice that received sodium pentobarbital (i.p., 0.1 mL/100 g); (2) control + embelin group (Embelin), which was normal mice that received embelin (i.p., 50 mg/kg) for 3 consecutive days; (3) AMI model group (AMI), which was AMI rat that received sodium pentobarbital (i.p., 0.1 mL/100 g); (4) AMI model + embelin group (AMI + embelin) (n = 10), which was AMI rat that received which was AMI rat that received embelin (i.p., 50 mg/kg) for 3 consecutive days.

Estimation of serum myocardial injury markers

Serum samples were extracted from vena cava after treatment with embelin. Then, serum samples were centrifuged at 2000 g for 15 min and saved for at -20°C. Serum levels of CK-MB, LDH and AST were estimated using respective commercial kits (commercial kits were purchased from Sangong Biotech, Shanghai, China) and employing auto blood analyzer (Siemens, Dimension Xpandplus, USA), according to the manufacture's protocols.

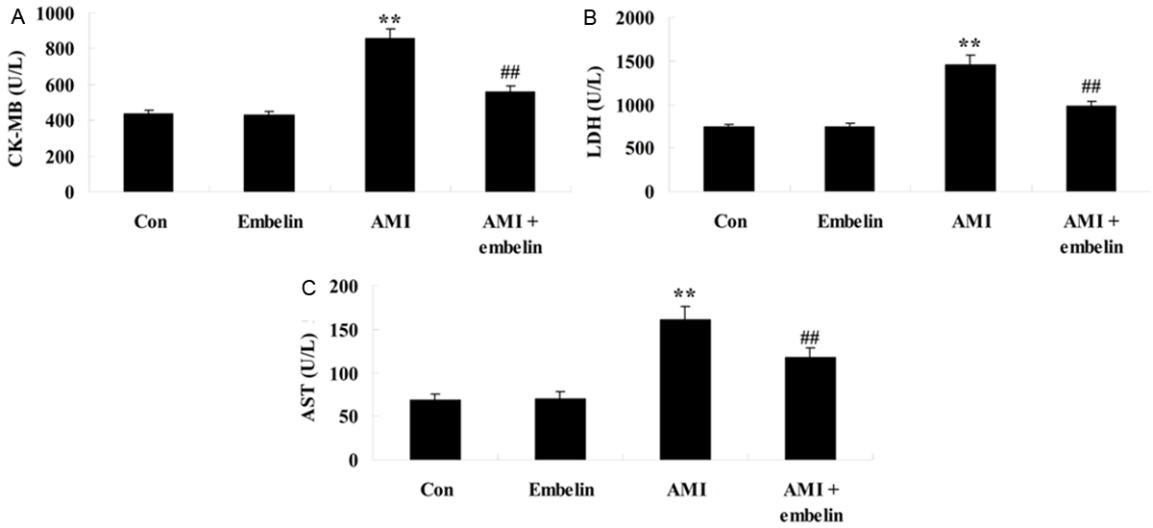

Figure 2. Embelin suppresses serum levels of CK-MB, LDH and AST in a rat model of AMI. Embelin suppresses serum levels of CK-MB (A), LDH (B) and AST (C) in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

Estimation of infarct size

Heart tissue was extracted from vena cava after treatment with embelin. Immediately, hearts were measured through the aorta and washed with physiological saline. Left ventricle was placed at -80°C for 10 minutes. Refrigerated left ventricle was sliced into 2 mm thick sections for infarct size measurement. Infarct size was dyed with 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Co, MO, USA) for 30 min in the dark [14, 15]. The volume and weight of infarct size were measured as a percentage of the left ventricle.

Estimation of NF-κB, TNF-α, IL-1β and IL-6 levels

Heart tissue was extracted from vena cava after treatment with embelin. Immediately, heart tissue was incubated in a solution buffer at 37°C for 30 min. NF-κB, TNF-α, IL-1β and IL-6 levels were estimated with commercial kits (Sigma-Aldrich Co, MO, USA), according to the manufacture's protocols.

Estimation of SOD, CAT, GR and GSH-PX

Heart tissue was extracted from vena cava after treatment with embelin. Immediately, heart tissue was incubated in a solution buffer at 37°C for 30 min. SOD, CAT, GR and GSH-PX activities were estimated with commercial kits (Sigma-Aldrich Co, MO, USA), according to the manufacture's protocols.

Estimation of caspase-3 and caspase-9 activities assay

Heart tissue was extracted from vena cava after treatment with embelin. Immediately, heart tissue was incubated in a solution buffer at 37°C for 30 min. Briefly, caspase-3 and caspase-9 activities were estimated with commercial kits (Sigma-Aldrich Co, MO, USA), according to the manufacture's protocols. 50 heart tissue were added 120 μL reaction buffer with 15 μL substrate (Ac-DEVD-pNA for caspase-9, Ac-LEHD-pNA for caspase-3) and incubated for 6 h. Caspase-3 and caspase-9 activities was measured with a Microplate Reader (Bio-Rad, Hercules, CA) at an absorbance of 405 nm.

Western blot analysis of cytochrome c, Bcl-2/Bax and PARP expression

Heart tissue was extracted from vena cava after treatment with embelin. Immediately, heart tissue was incubated in a solution buffer for 30 min on ice. The protein concentration was measured with Bicinchoninic acid (BCA) commercial kits (Pierce Biotechnology, Rockford, USA). Equal amount of heart tissue were loaded by SDS-PAGE and then transferred to

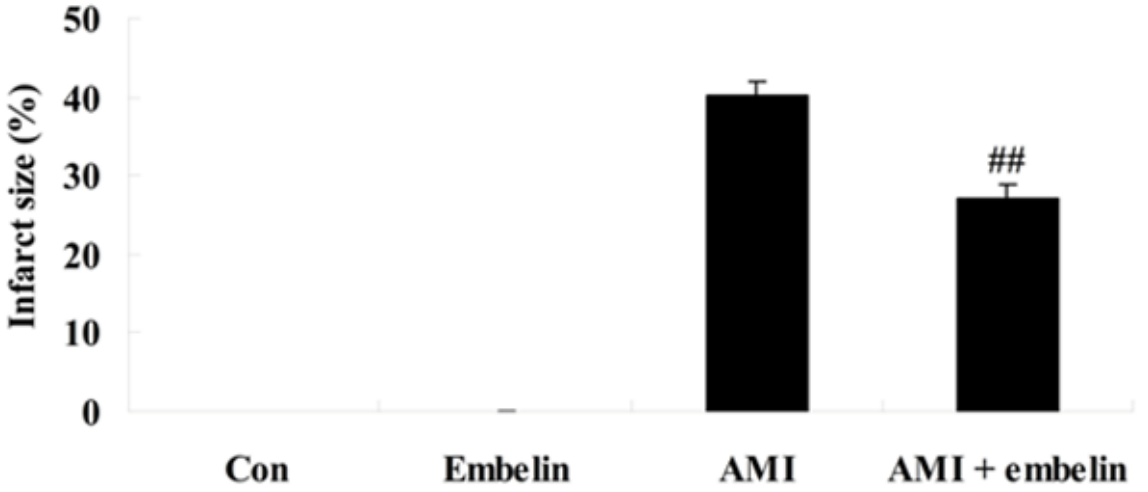

Figure 3. Embelin suppresses infarct size in AMI rat model. $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

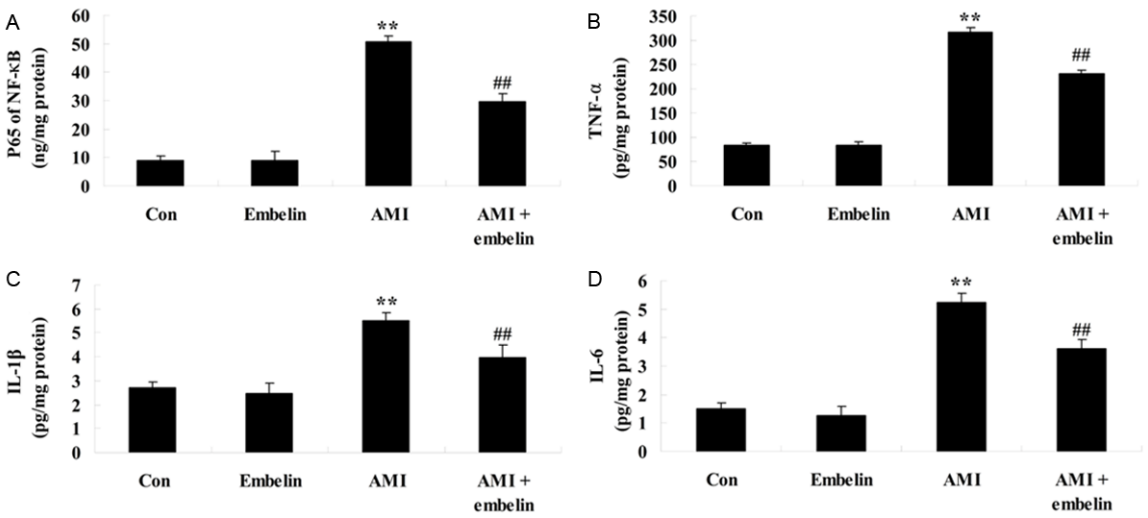

Figure 4. Embelin suppresses inflammatory in AMI rat model. Embelin suppresses NF-κB (A), TNF-α (B), IL-1β (C) and IL-6 (D) levels in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

polyvinylidene fluoride membranes (Pierce, Rockford, USA). The membranes were blocked with TBS-0.05% Tween 20 (TBST) containing 5% non-fat milk to block nonspecific binding sites for 2 h at room temperature. Then, the membranes were incubated with anti-cytochrome c (1:1000, Cell Signaling Technology, Boston, MA), anti-Bcl-2 (1:1000, Cell Signaling Technology, Boston, MA), anti-Bax (1:1000, Cell Signaling Technology, Boston, MA), anti-PARP (1:1000, Cell Signaling Technology, Boston, MA) and anti-β-actin (1:1000, Cell Signaling Technology, Boston, MA) overnight at 4°C. The membranes were washed with TBST solution thrice and incubated with anti-mouse IgG (1:1000, Beijing Applygen Technologies, Inc., Beijing, China) conjugated for 2 h at room temperature. The analysis of the bands was resolved by a gel image analysis system (Pierce Biotechnology, Rockford, USA).

Statistical analysis

The data were performed with SPSS 17.0 software and repeated at least three times. The

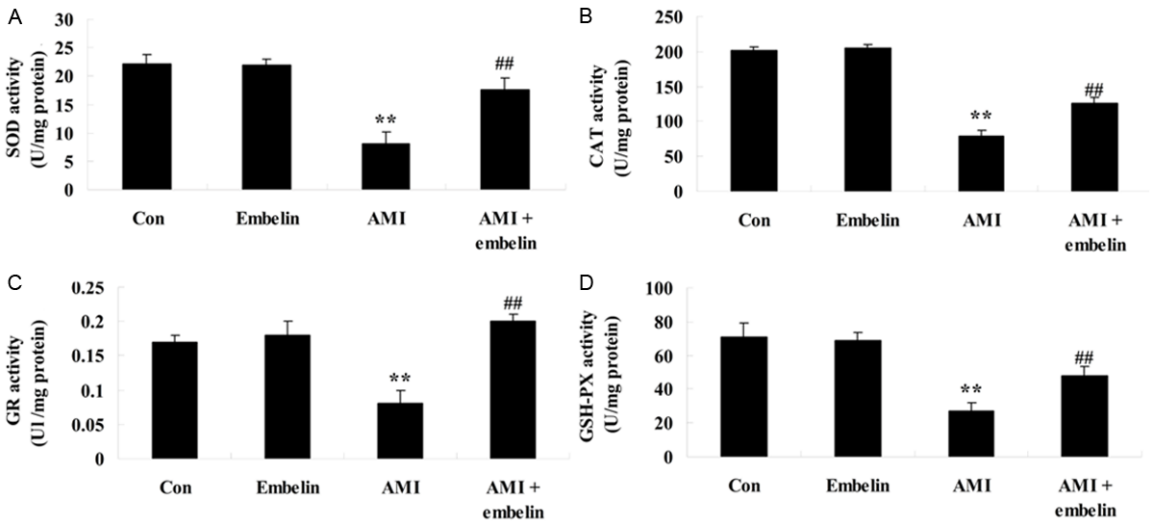

Figure 5. Embelin suppresses oxidative stress in AMI rat model. Embelin suppresses the SOD (A), CAT (B), GR (C) and GSH-PX (D) activities in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

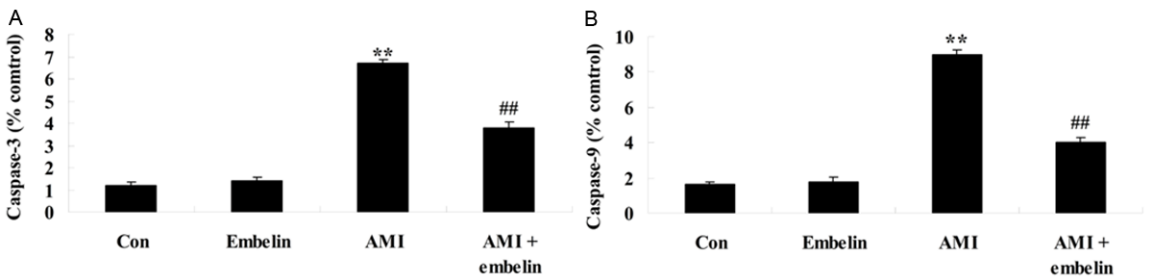

Figure 6. Embelin suppresses caspase-3 & 9 in AMI rat model. Embelin suppresses caspase-3 (A) and 9 (B) activities in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

data are shown as the mean ± SEM unless otherwise noted; Differences were assessed by two-tailed Student's t-test. In all cases, a $P < 0.05$ was considered statistically significant.

Results

Embelin suppresses serum levels of CK-MB, LDH and AST in a rat model of AMI

The chemical structure of embelin (Sigma, with a purity > 98%) was showed in **Figures 1** and **2** illustrated that after treatment with embelin, there was a gradual increase of serum CK-MB, LDH and AST levels in the AMI rats group compared with the control group. Embelin reduced the serum CK-MB, LDH and AST levels of AMI rats, compared to the AMI rats group (**Figure 2**).

Embelin suppresses infarct size in AMI rat model

As shown in **Figure 3**, infarct size in AMI rat model was increased compared with the control group. After administration of embelin significant reduction of infarct size, compared to the AMI rats group (**Figure 3**).

Embelin suppresses inflammatory in AMI rat model

As shown in **Figure 4A-D**, NF-κB, TNF-α, IL-1β and IL-6 levels of AMI rats were advanced compared with the control group. When pretreated with embelin, the anti-inflammatory effect of embelin was markedly attenuated the NF-κB, TNF-α, IL-1β and IL-6 levels of AMI rats, compared to the AMI rats group (**Figure 4A-D**).

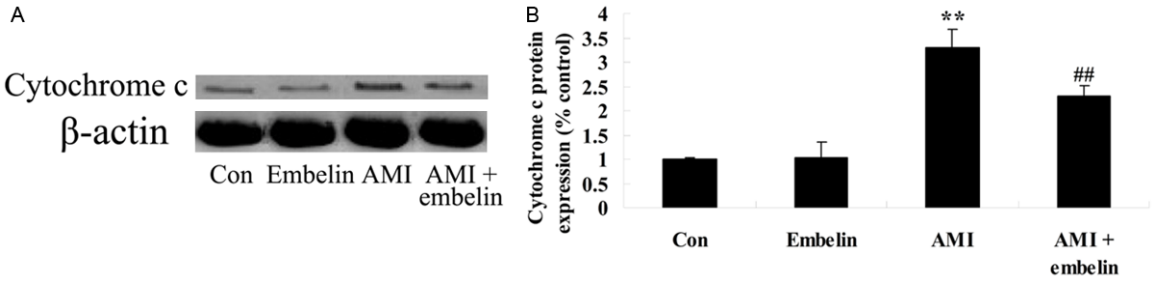

Figure 7. Embelin regulates cytochrome c in AMI rat model. Embelin regulates cytochrome c protein expression using Western blot analysis (A), statistical analysis of cytochrome c protein expression level (B) in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

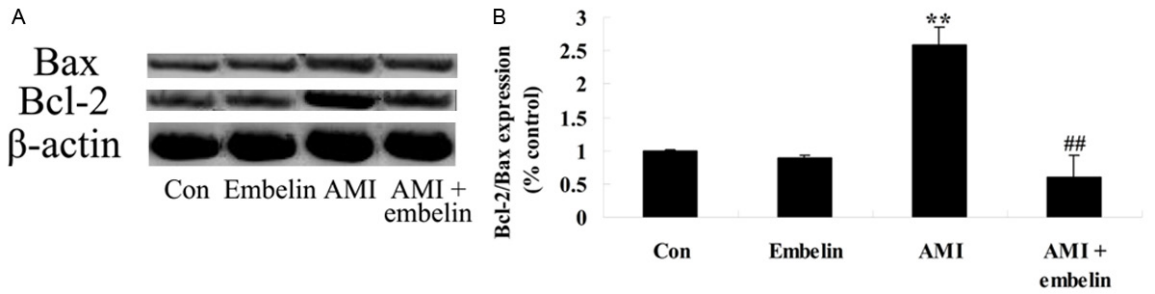

Figure 8. Embelin regulates Bcl-2/Bax in AMI rat model. Embelin regulates Bcl-2 and Bax protein expression using Western blot analysis (A), statistical analysis of Bcl-2 and Bax protein expression level (B) in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

Embelin suppresses oxidative stress in AMI rat model

As shown in **Figure 5A-D**, SOD, CAT, GR and GSH-PX activities of AMI rats were depressed compared with the control group. Embelin observably raised the SOD, CAT, GR and GSH-PX activities of AMI rats, compared to the AMI rats group (**Figure 5A-D**).

Embelin suppresses caspase-3 & 9 in AMI rat model

To further analysis the anti-apoptosis effect of embelin on AMI rats, caspase-3 and caspase-9 activities were detected. As shown in **Figure 6A**, **6B**, the caspase-3 and caspase-9 activities were boosted in AMI rats compared with the control group. The caspase-3 and caspase-9 activities were restrained by the treatment with embelin, compared to the AMI rats group (**Figure 6A**, **6B**).

Embelin regulates cytochrome c in AMI rat model

To further detect the cardioprotective effect of embelin on AMI rats, the cytochrome c protein expression of AMI rats was measured by western blot analysis. As shown in **Figure 7A**, **7B**, the cytochrome c protein expression was promoted in AMI rats compared with the control group. When pretreated with embelin, the cytochrome c protein expression was downturn, compared to the AMI rats group (**Figure 7A**, **7B**).

Embelin regulates Bcl-2/Bax in AMI rat model

To further explore the cardioprotective effect of embelin on AMI rats, Bcl-2 and Bax protein expressions of AMI rats were explored by western blot analysis. As shown in **Figure 8A**, **8B**, Bcl-2 protein expression was reduced, Bax protein expression was increased, and Bcl-2/Bax protein expression was augmented in AMI rats

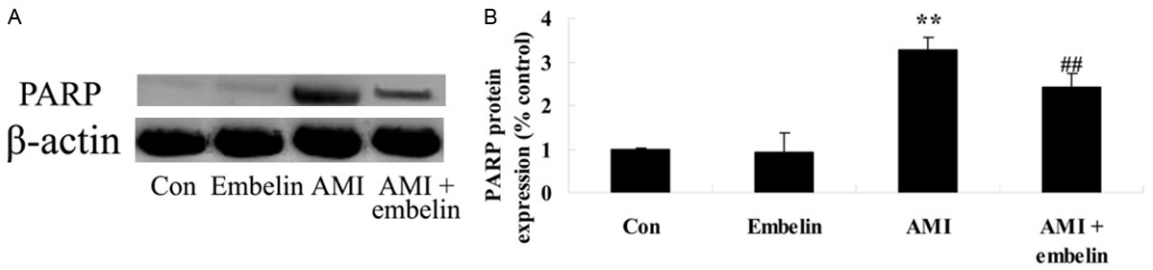

Figure 9. Embelin regulates PARP in AMI rat model. Embelin regulates PARP protein expression using Western blot analysis (A), statistical analysis of PARP protein expression level (B) in a rat model of AMI. $^{**}P < 0.01$ versus control group, $^{\#\#}P < 0.01$ versus AMI group. Con, control-group; Embelin, embelin (50 mg/kg)-treated group; AMI, AMI group; AMI + embelin-treated group.

compared with the control group. Bcl-2/Bax protein expression was reversed by the treatment with embelin, compared to the AMI rats group (**Figure 8A**, **8B**).

Embelin regulates PARP in AMI rat model

To further analyze the cardioprotective effect of embelin on AMI rats, PARP protein expression of AMI rats were checked by western blot analysis. As shown in **Figure 9A**, **9B**, PARP protein expression of AMI rats was hoisted compared with the control group. After administration of embelin significantly lessened PARP protein expression, compared to the AMI rats group (**Figure 9A**, **9B**).

Discussion

For AMI, mostly due to the absolute or relative lack of coronary blood supply, myocardial metabolism shifts from aerobic metabolism to anaerobic glycolysis, high-energy phosphate compounds are quickly exhausted and myocardial energy drops, which result in serious damage to cardiac function, pumping barriers and shutoff, and these changes occur in a few minutes after acute coronary occlusion [16]. The study presented here has identified administering embelin reduced the serum CK-MB, LDH and AST levels, and infarct size of AMI rats. Therefore, embelin might be a better potential development drug for AMI or other cardiovascular diseases.

Domestic and international studies have confirmed that the occurrence of AMI is secondary to myocardial necrosis, and peripheral blood neutrophils and monocytes are aggregated in the damaged and necrosis myocardial tissues in a short time [3]. In acute phase of myocardial infarction, outstanding performance of tissue microenvironment is the significant increase of a large number of neutrophils, monocytes and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [17]. In this study we provide further evidence for previously and newly identified that embelin suppressed the NF-κB, TNF-α, IL-1β and IL-6 levels of AMI rats. These findings suggested that the pro-inflammatory effect of embelin decreased TNF-α, IL-1β, and IL-6 to levels, and the expression of NF-κB p65 in myocardial ischemia-reperfusion injury rats [18].

During AMI, due to acute ischemia and hypoxia, myocardial oxygen metabolism is hindered, resulting in a large amount of free radicals generated in cardiac tissue to damage cell membranes, causing changes membrane integrity and permeability and dysfunction of ion channels, then myocardial ischemia is developed from reversible damage into irreversible necrosis and severe ventricular arrhythmias, thereby causing cardiac structure and dysfunction [19]. On the basis of reperfusion, improving the oxidative stress of myocardial cells as soon as possible can save the myocardial injury better, reduce the disease and improve prognosis. The studies abroad have confirmed that oxidative stress can induce cardiomyocyte apoptosis by damaging DNA, attacking proteins with enzymatic activity or oxidizing proteins that are related to transcription and inducing lipid peroxidation of cell membrane [20]. This study showed that embelin advanced the SOD, CAT, GR and GSH-PX activities of AMI rats. Radhakrishnan et al. suggested that embelin prevents oxidative stress induced by Ultraviolet B irradiation through its antioxidant property [21]. Thippeswamy et al. reported that the protective effect of embelin might be antioxidant

and anti-inflammatory activities against acetic acid induced ulcerative colitis in rats [22]. Thus, we presume that treatment with embelin inhibited the caspase-3 and caspase-9 activities of AMI rats. Thippeswamy et al. suggested that the protective effect of embelin reduced the caspase-3 and caspase-9 activities against transient global ischemia-induced brain damage in rats [23].

Cytochrome-oxidase (CcO) takes part in the respiration oxidation of mitochondria to provide energy for the cells. In cell respiration CcO is in the end of cytochrome systems, involved in the oxidation of mitochondrial respiration in mitochondria surface, and reacting with CcO to catalyze indophenol, which transfers electrons of respiratory substrate through the cytochrome system directly to molecular oxygen (with automatic oxidation), to provide energy for the cells [24, 25]. Previous studies in the protection mechanisms of ischemic preconditioning delay relative to myocardial ischemia-reperfusion injury show ischemic preconditioning can promote the enhanced expression of the proteins CcO related to mitochondrial respiratory chain, improve oxygen metabolism in cells. If the expression of cell CcO is inhibited, the cardioprotective effect of ischemic preconditioning can be canceled [26]. Result of our study showed that the cytochrome c protein in AMI rats was decreased by the treatment with embelin. Radhakrishnan et al. suggested that embelin prevents oxidative stress induced by Ultraviolet B irradiation through suppression of cytochrome c [21].

Myocardial apoptosis is a major reason for the loss of AMI cardiac myocytes and the cardiac failure. Most cells require the expression of new genes and the synthesis of protein during apoptosis, as there have been reported a large number of genes may promote or inhibit the occurrence of apoptosis. In the studies on the regulation of genes, Bcl-2-related genes are indicated associated with apoptosis in more studies. Overexpression of Bcl-2 genes can block apoptosis, as apoptotic suppressor genes [27]. Bax is apoptosis-promoting gene, primarily expressed in the mitochondria and endoplasmic reticulum membrane. Bax and Bcl-2 have a high homology, and prone to apoptosis by inhibiting the activity of Bcl-2, of which the different levels of expressions lead to different cell fate; overexpression of Bcl-2 prevents the occurrence of apoptosis, and overexpression of bax tend to promote apoptosis [28]. Embelin reduced the reduced Bcl-2/Bax protein expression of AMI rats. Thippeswamy et al. reported that the protective effect of embelin against transient global ischemia-induced brain damage in rats through reduction of Bcl-2/Bax signal pathway [29].

Poly (ADP-ribose) polymerase I (PARPI) is a protease present in all eukaryotic cells except yeast with the molecular weight 113~116KD, which plays an important role in maintaining chromosome stability, DNA repair and replication, cell death and apoptosis and gene transcription. ARPI regulates the expressions of a variety of cytokines such as TNF-α and IL-6 by the transfection ADP ribosylation modification of histones and the interactions with multiple transcription factors that regulate inflammatory cells (e.g., NF-kappaB, API), which is one of the important mechanisms of the occurrence and development of the immune response after the participation of PARPI in myocardial infarction [30]. Further experimental studies have shown that in the process of acute myocardial ischemia-reperfusion injury, enzymatic activity of PARPI is increased significantly, inhibiting PARPI activity can significantly reduce myocardial necrosis area, and removing PARPI gene can significantly reduce cardiac ischemia-reperfusion-induced damage; in myocardial tissue of the rat that has ventricular remodeling, PARPI activity is increased significantly [31]. Inhibiting PARPI activity can significantly improve cardiac function and vascular relaxation ability of the rat. Embelin suppressed PARP protein expression of AMI rats in our study. Sahu et al. reported that embelin cleaved PARP protein expression in rats of isoproterenol-induced injury [32].

Taken together, these experiments provide detailed evidence that the cardioprotective effect of embelin on AMI rat. Pretreatment with embelin inhibits inflammatory reactions, oxidative stress and associated cardiomyocyte apoptosis in AMI rat. The results of the present study also demonstrate that the effect of embelin on AMI was involved with cytochrome c, Bcl-2/Bax, PARP signal pathway. Hence, it may be concluded that embelin pre-treatment might be beneficial for prevention of AMI or other heart disease.

Disclosure of conflict of interest

None.

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