

Arctigenin exerts protective effects against myocardial infarction via regulation of iNOS, COX-2, ERK1/2 and HO-1 in rats

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Abstract. The present study aimed to determine the protective effects of arctigenin against myocardial infarction (MI), and its effects on oxidative stress and inflammation in rats. Left anterior coronary arteries of Sprague-Dawley rats were ligated, in order to generate an acute MI (AMI) model. Arctigenin was administered to AMI rats at 0, 50, 100 or 200 μ mol/kg. Western blotting and ELISAs were performed to analyze protein expression and enzyme activity. Arctigenin was demonstrated to effectively inhibit the levels of alanine transaminase, creatine kinase-MB and lactate dehydrogenase, and to reduce infarct size in AMI rats. In addition, the activity levels of malondialdehyde, interleukin (IL)-1 β and IL-6 were significantly suppressed, and the levels of glutathione peroxidase, catalase and superoxide dismutase were significantly increased by arctigenin treatment. Arctigenin treatment also suppressed the protein expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and heme oxygenase 1 (HO-1), and increased the protein expression levels of phosphorylated-extracellular signal-regulated kinase 1/2 (p-ERK1/2) in AMI rats. Overall, the results of the present study suggest that arctigenin may inhibit MI, and exhibits antioxidative and anti-inflammatory effects through regulation of the iNOS, COX-2, ERK1/2 and HO-1 pathways in a rat model of AMI.

Introduction

Acute myocardial infarction (AMI) may cause irreversible damage to functional myocardial cells, negative myocardial remodeling and progressive deterioration of cardiac function (1). In addition, it remains the primary cause of disease

incidence and mortality in China (1). According to the Heart Disease and Stroke Statistics report by the American Heart Association (2), ~2,200 patients succumbed to cardiovascular diseases every day in 2008. In addition, an average of 785,000 cases of coronary atherosclerosis (3) and an average of 195,000 cases of MI are reported annually in China. AMI is the most serious type of coronary heart disease, which involves cardiomyocyte necrosis due to long-term ischemia and oxygen deficit. In addition, oxidative stress has significant effects on the pathological alterations associated with MI (4).

AMI may cause aerobic metabolic disorders due to long-term ischemia and hypoxia caused by coronary occlusion, which may lead to myocardial cell interstitial hyperemia, edema and degenerative necrosis, accompanied by increased inflammatory cell infiltration (5). Oxidative damage, caused by the excessive generation of free radicals and oxygen free radicals in cardiac tissue, results in damage to the structure and function of myocardial cell membranes, mitochondrial damage and autolysis (6). Myocardium under accelerated ischemia may progress from reversible injury to irreversible degenerative necrosis (7). Malignant arrhythmia may also occur, which may lead to further ventricular remodeling and cardiac dysfunction. Revascularization and reperfusion therapy are currently considered the most effective therapeutic methods; however, ischemia reperfusion can cause further damage to the remaining myocardium. The important mechanism underlying this damage may be oxidative stress (7). The longer the duration of ischemic and hypoxic conditions, the more evident the oxidative stress response will be in myocardial cells. In accordance, the higher the degree of myocardial injury, the heavier the state of illness will be (8).

Great Burdock (*Arctium lappa*) Achene extract is used in traditional Chinese medicine to treat anemopyretic cold, measles, carbuncles, ingested poison and other diseases (9). Modern pharmacological research has demonstrated that Great Burdock Achene extract possesses antibiotic, antitumor and hypoglycemic effects (10). Arctigenin (Fig. 1) is one of the active ingredients extracted from Great Burdock Achene, which has numerous pharmacological effects, including anti-tumor and neuroprotective activities; arctigenin also has strong anti-inflammatory, immunoregulatory and antiviral activity, and inhibits the heat-shock response (11,12). The present study aimed to evaluate the protective effects of arctigenin against MI and the potential underlying mechanisms.

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Materials and methods

AMI model rats. The present study was approved by the Ethics Committee of Liaocheng People's Hospital of Shandong Province (Liaocheng, China). A total of 40 male Sprague-Dawley rats (weight, between 250 and 300 g; age, 10-12 weeks), were purchased from Jinan Jinfeng Experimental Animal Co., Ltd. (Shandong, China) and housed at 22-24°C and 55% humidity in an animal room under a 12-h light/dark cycle with free access to water and food. The present study was performed in accordance with the recommendations from the Guide for Animal Management Rules from the Ministry of Health of Liaocheng People's Hospital of Shandong Province. To generate the AMI model, rats were anesthetized by peritoneal injection with 1% pentobarbital (40 mg/kg). Subsequently, coronary arteries were exposed and the descending branch of the left anterior coronary artery was marked with silk and ligated for 1 h with hemostatic forceps; the wound was sutured (1-2 cm) following ligation in order to generate the AMI model.

Treatment groups. A total of 40 male Sprague-Dawley rats were randomly assigned into the following subgroups (n=8 rats/group): i) Sham group, which was used as a control (treated with normal saline); ii) AMI model group (vehicle control; AMI rats were treated with normal saline following the generation of the AMI model; iii) AMI rats treated with 50 μ mol/kg arctigenin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) following the generation of the AMI model; iv) AMI rats treated with 100 μ mol/kg arctigenin following the generation of the AMI model; and v) AMI rats treated with 200 μ mol/kg arctigenin following the generation of the AMI model. Rats were sacrificed following treatment with arctigenin for 1 week.

Measurement of alanine transaminase (ALT), creatine kinase (CK)-MB, lactate dehydrogenase (LDH), malondialdehyde (MDA), glutathione peroxidase (GSH-PX), catalase (CAT), superoxide dismutase (SOD), interleukin (IL)-1 β and IL-6 activity. Following arctigenin treatment, blood was immediately collected from the abdominal aorta and used to analyze the serum levels of ALT (cat. no. C009-2), CK-MB (cat. no. H197), MDA (cat. no. A003-1), GSH-PX (cat. no. A005), CAT (cat. no. A007-1), SOD (cat. no. A001-1), IL-1 β (cat. no. H002) and IL-6 (cat. no. H007) using Commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Measurement of caspase-3 activity. Following arctigenin treatment, mice were sacrificed and their hearts were removed and homogenized in ice-cold radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were measured using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (10 μ g) were then incubated with the caspase-3 substrate, Ac-DEVD-pNA (cat. no. C1115; Beyotime Institute of Biotechnology) for 2 h at 37°C in order to measure caspase-3 activity, according to the manufacturer's protocol.

Evaluation of infarct size. Following arctigenin treatment, rats were sacrificed and the hearts were removed. The hearts were

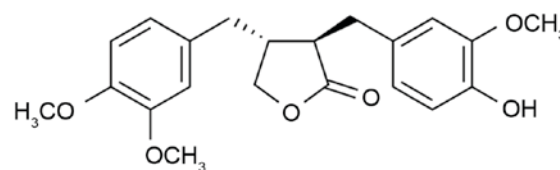


Figure 1. Chemical structure of arctigenin.

sliced into five sections (1.0 mm) perpendicular to the long axis, and the sections were incubated with 1% 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich; Merck KGaA) in phosphate solution at 37°C for 10 min. Infarct sizes were determined by computer morphometry using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis. Following arctigenin treatment, mice were sacrificed and their hearts were removed and homogenized in ice-cold radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were measured using the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (80 μ g/lane) were separated by 10% SDS-PAGE and were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk powder for 1 h at 37°C and probed with antibodies against inducible nitric oxide synthase (iNOS; cat. no. sc-649; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cyclooxygenase 2 (COX-2; cat. no. sc-7951; 1:500; Santa Cruz Biotechnology, Inc.), phosphorylated-extracellular signal-regulated kinase 1/2 (p-ERK1/2; cat. no. sc-101761; 1:500; Santa Cruz Biotechnology, Inc.), ERK1/2 (cat. no. 4695; 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), heme oxygenase-1 (HO-1; cat. no. sc-10789; 1:500; Santa Cruz Biotechnology, Inc.) and β -actin (cat. no. sc-7210; 1:500; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature, and the immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology, Inc.). The optical densities of immunopositive bands were determined by Gene Tools image analysis (Syngene, Frederick, MD, USA) and Bio-Rad Quantity One software v3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation of 3 independent experiments. Analysis was performed using SPSS v17.0 software (SPSS, Inc., Chicago, IL, USA). Statistical comparisons between groups were conducted using one-way analysis of variance and the Tukey post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Arctigenin reduces CK-MB, ALT and LDH levels in AMI rats. The present study evaluated the protective effects of arctigenin by analyzing the levels of biochemical markers of AMI in the rat model, including CK-MB, ALT and LDH

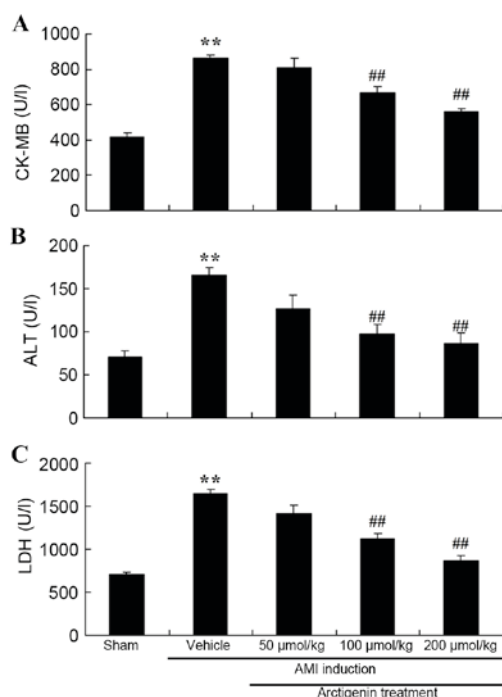


Figure 2. Arctigenin decreases the levels of CK-MB, ALT and LDH in AMI model rats. Arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) decreases the AMI-induced activity of (A) CK-MB, (B) ALT (C) and LDH in AMI rats. ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the untreated AMI model group. ALT, alanine transaminase; AMI, acute myocardial infarction; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase.

(Fig. 2). The levels of CK-MB, ALT and LDH were higher in the AMI model rats compared with the Sham group. However, treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) significantly reduced the levels of CK-MB, ALT and LDH compared with in the untreated AMI control group (Fig. 2).

Arctigenin treatment reduces infarct size in AMI rats. To confirm the protective effects of arctigenin on AMI, the infarct size in AMI rats was examined. As presented in Fig. 3, the infarct size in AMI rats was significantly increased compared with the Sham group, whereas treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) markedly reduced the infarct size compared with untreated AMI rats (Fig. 3).

Arctigenin reduces inflammation in AMI rats. The effects of arctigenin on AMI-induced inflammation were investigated by examining the activity of IL-1 β and IL-6 (Fig. 4). Untreated AMI model rats exhibited a significant increase in IL-1 β and IL-6 activity compared with the Sham group. Conversely, treatment with 100 or 200 $\mu\text{mol/kg}$ arctigenin significantly reduced the activity of IL-1 β and IL-6 compared with the untreated AMI group (Fig. 4).

Arctigenin decreases oxidative stress in AMI rats. The effects of arctigenin on AMI-induced oxidative stress in AMI rats were examined. As shown in Fig. 5A-C, AMI resulted in decreased GSH-PX, CAT and SOD activity compared with the Sham group. However, treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) significantly increased the activity of GSH-PX, CAT and SOD in AMI rats (Fig. 5A-C). Conversely, MDA

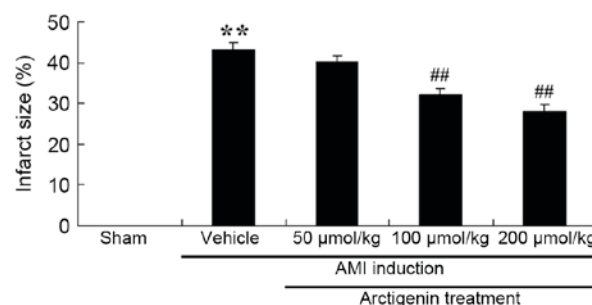


Figure 3. Arctigenin reduces infarct size in AMI rats. Arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) reduces infarct size in AMI-induced rats. ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the AMI model group. AMI, acute myocardial infarction.

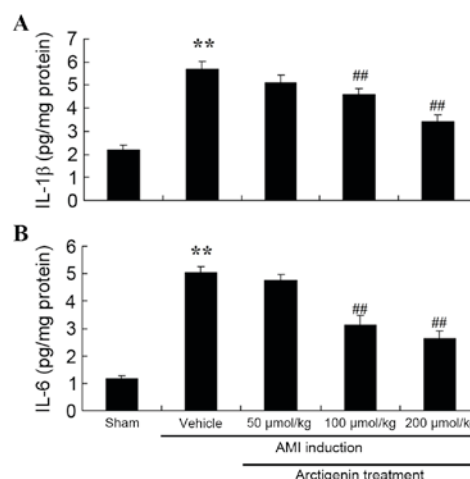


Figure 4. Arctigenin decreases inflammation in AMI rats. Arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) reduces the activity of (A) IL-1 β and (B) IL-6 in AMI-induced rats. ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the AMI model group. AMI, acute myocardial infarction; IL, interleukin.

activity was higher in AMI rats compared with the Sham group (Fig. 5D); MDA activity was subsequently reduced in AMI rats upon treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) (Fig. 5D). These results demonstrated that arctigenin may possess antioxidative effects in AMI, and thus may be effective as a clinical treatment for AMI.

Arctigenin reduces iNOS protein expression in AMI rats. Protein expression levels of iNOS were detected by western blot analysis (Fig. 6). iNOS protein expression was significantly increased in AMI rats, compared with the Sham group, and was lowered in AMI rats treated with arctigenin (100 or 200 $\mu\text{mol/kg}$).

Arctigenin reduces the protein expression levels of COX-2 in AMI rats. The effects of arctigenin on the protein expression levels of COX-2 in AMI rats were determined by western blot analysis (Fig. 7). COX-2 protein expression was significantly higher in AMI model rats compared with the Sham group. However, treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) significantly reduced the protein expression levels of COX-2 in AMI rats.

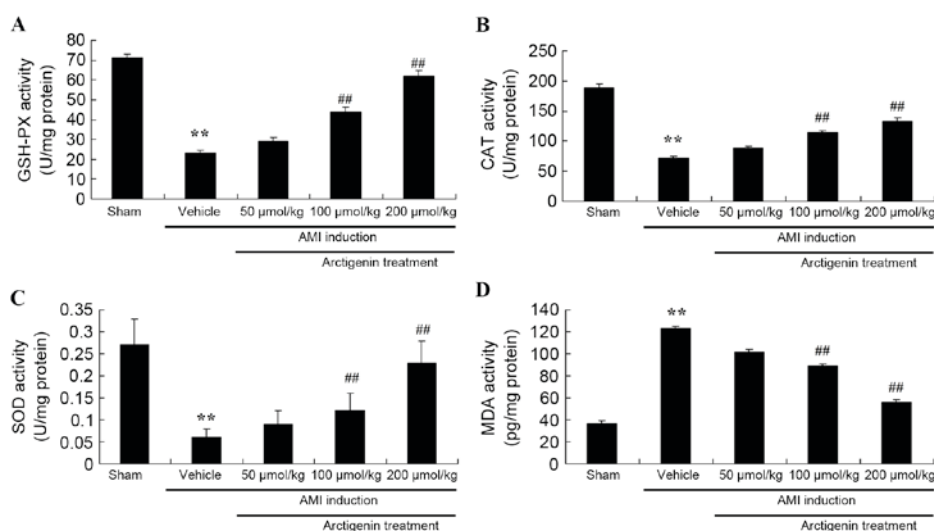


Figure 5. Arctigenin reduces oxidative stress in AMI rats. Arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) reduces the activity of (A) GSH-PX, (B) CAT, (C) SOD and (D) MDA in AMI rats ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the AMI model group. AMI, acute myocardial infarction; CAT, catalase; MDA, malondialdehyde; GSH-PX, glutathione peroxidase; SOD, superoxide dismutase.

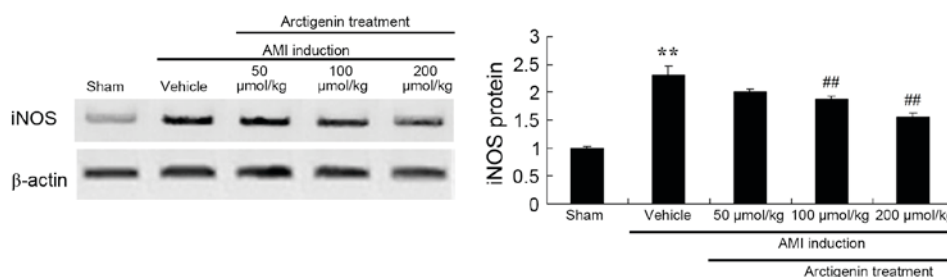


Figure 6. Arctigenin reduces the expression of iNOS in AMI rats. Western blotting and densitometric analysis were conducted to determine the effects of arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) on the protein expression levels of iNOS in AMI-induced rats. ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the AMI model group. AMI, acute myocardial infarction; iNOS, inducible nitric oxide synthase.

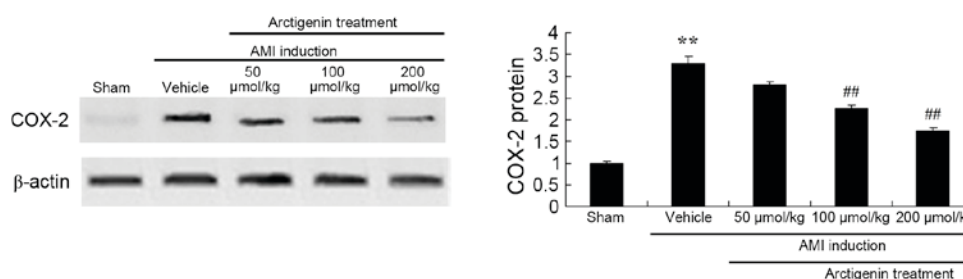


Figure 7. Arctigenin reduces COX-2 expression in AMI rats. Western blotting and densitometric analysis were conducted to determine the effects of arctigenin treatment (50, 100 and 200 $\mu\text{mol/kg}$) on the protein expression levels of COX-2 in AMI-induced rats. ** $P < 0.01$ compared with the Sham group; ## $P < 0.01$ compared with the AMI model group. AMI, acute myocardial infarction; cyclooxygenase 2.

Arctigenin decreases p-ERK1/2 protein expression in AMI rats. The protein expression levels of p-ERK1/2 were analyzed by western blotting, in order to examine the underlying mechanisms involved in AMI (Fig. 8). The protein expression of p-ERK1/2 was induced in the AMI model group when compared with the Sham group (Fig. 8). Treatment with arctigenin (100 or 200 $\mu\text{mol/kg}$) significantly decreased the protein expression of p-ERK1/2 in AMI rats (Fig. 8).

Arctigenin decreases the protein expression levels of HO-1 in AMI rats. To confirm the underlying mechanisms of arctigenin treatment in AMI rats, HO-1 protein expression was examined by western blotting. As shown in Fig. 9, AMI induction led to an increase in the protein expression levels of HO-1 in AMI rats compared with the Sham group. Elevated HO-1 protein expression was significantly reduced in AMI rats treated with arctigenin (100 or 200 $\mu\text{mol/kg}$) compared with untreated AMI rats.

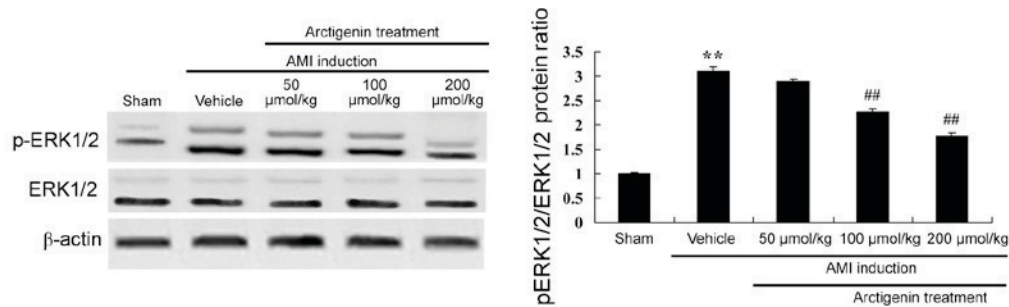


Figure 8. Arctigenin decreases p-ERK1/2 expression in AMI rats. Western blotting and densitometric analysis were conducted to determine the effects of arctigenin treatment (50, 100 and 200 μ mol/kg) on the protein expression levels of p-ERK1/2 in AMI-induced rats. ** P <0.01 compared with the Sham group; ## P <0.01 compared with the AMI model group (vehicle). AMI, acute myocardial infarction; p-ERK1/2, phosphorylated-extracellular signal-regulated kinase 1/2.

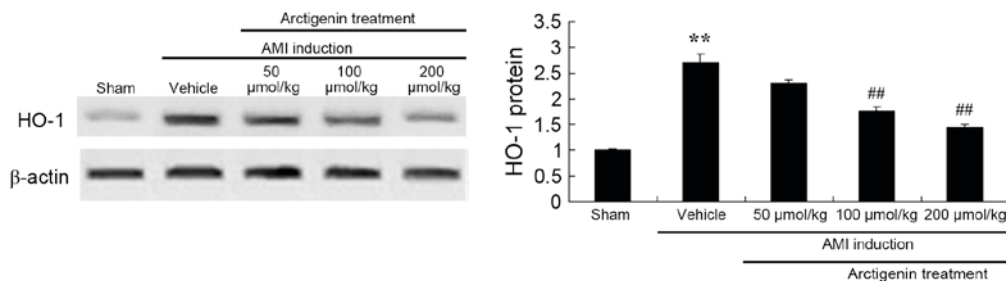


Figure 9. Arctigenin reduces the expression of HO-1 in AMI rats. Western blotting and densitometric analysis were conducted to determine the effects of arctigenin treatment (50, 100 and 200 μ mol/kg) on the protein expression levels of HO-1 in AMI-induced rats. ** P <0.01 compared with the Sham group; ## P <0.01 compared with the AMI model group. AMI, acute myocardial infarction; HO-1, heme oxygenase-1.

Arctigenin reduces caspase-3 activity in AMI rats. The present study analyzed the effects of arctigenin on AMI-induced apoptosis in rat heart cells by measuring caspase-3 activity by ELISA. As shown in Fig. 10, caspase-3 activity was significantly increased in AMI rats, compared with the Sham group, whereas treatment with arctigenin (100 or 200 μ mol/kg) significantly reduced caspase-3 activity in AMI rats (Fig. 10).

Discussion

AMI is an important pathological and lethal syndrome worldwide. Following occurrence of AMI, dredging the blocked coronary artery in a timely manner is the only effective therapeutic strategy to save ischemic myocardial tissue and to restore cardiac function (2). However, reperfusion injury may lead to damage or death of ischemic cardiac muscle cells (13). Effective methods for the treatment of myocardial reperfusion injury have been investigated; however, there is currently no definite strategy or drug available (14). Despite the common therapeutic strategy of timely ischemic myocardial perfusion recovery, 25% of patients with AMI will develop chronic cardiac failure as a result of reperfusion injury (15). Reperfusion injury is a key factor for myocardial injury associated with myocardial infarction recanalization; therefore, reducing reperfusion injury is conducive to myocardial cell survival, reducing the loss of myocardial function and reducing the probability of developing chronic cardiac failure (16). In the present study, treatment with arctigenin significantly lowered the AMI-induced levels of

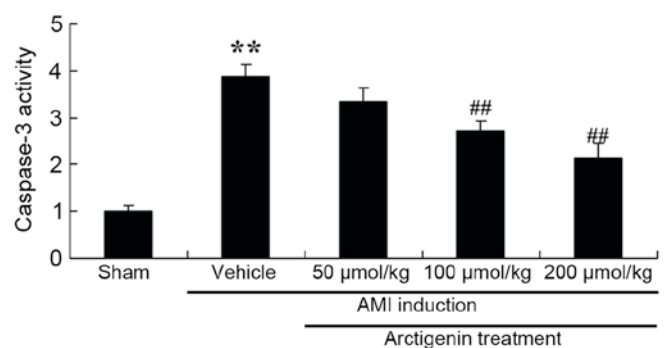


Figure 10. Arctigenin decreases caspase-3 activity in AMI rats. ** P <0.01 compared with the Sham group; ## P <0.01 compared with the AMI model group. AMI, acute myocardial infarction.

ALT, CK-MB and LDH, and reduced the infarct size in AMI model rats.

Oxidative stress refers to an imbalance of oxidation and antioxidation in the body (17). Oxidation may lead to granulocyte inflammatory infiltration, increased protease secretion and the production of a large amount of intermediate products (18). The degree of myocardial injury due to the increased levels of oxygen radicals in peripheral circulation can be evaluated through the change in activity of endogenous antioxidant enzymes, such as SOD, in the peripheral blood (17). Reduced glutathione is a tripeptide that is composed of glutamic acid, cysteine and glycine, and is expressed in various organs. It is a coenzyme that is involved in the activation of numerous

other enzymes, participates in the Krebs cycle and sugar metabolism, and helps maintain physiological functions of the cell (19). Results from the present study revealed that the protective effects of arctigenin reduced oxidative stress and iNOS expression in AMI model rats. Kou *et al* (20) demonstrated that arctigenin decreased COX-2 expression and inhibited STAT1/3 expression, which led to a decrease in iNOS expression. In addition, Zhang *et al* (11) reported that arctigenin exerts protective effects against lipopolysaccharide (LPS)-induced oxidative stress and inflammation by suppressing HO-1 and iNOS signaling in mice.

Tumor necrosis factor- α (TNF- α) is a multifunctional protein that is mainly produced by the activated scavenger/mononuclear cell system. Normal myocardial cells cannot produce TNF- α (21); however, in the case of AMI pump failure, TNF- α expression increases significantly and becomes a reliable indicator by which to evaluate the clinical prognosis of AMI (22). IL-6 is a proinflammatory cytokine with numerous functions that is secreted by activated monocytes, macrophages, T lymphocytes, endothelial cells and fibroblasts (23). A previous study reported that IL-6 may be the most powerful predictor of mortality caused by cardiogenic shock in patients with AMI within 30 days (24). In the present study, AMI rats treated with arctigenin exhibited significantly decreased IL-1 β and IL-6 activity.

Decreased coronary blood flow resulting from arterial thrombosis or atherosclerosis may lead to myocardial anoxia (25). In myocardial cells of neonatal rats cultivated *in vitro*, hypoxia led to an increase in the mRNA and protein expression levels of HO-1, whereas under normoxic conditions, HO-1 expression was reduced (26). Environment-induced hypoxia may lead to pulmonary hypertension and may induce right ventricular hypertrophy (27). HO-1-knockout mice under normoxic conditions exhibit normal behavior, whereas under hypoxic conditions, despite having a similar degree of pulmonary hypertension, HO-1 knockout mice were subject to more serious right ventricular dilation and infarction accompanied by atherothrombosis (28). In the present study, treatment with arctigenin significantly reduced the protein expression levels of HO-1 in AMI rats. Zhang *et al* (11) reported that arctigenin protects against LPS-induced oxidative stress and inflammation through suppression of HO-1 and iNOS signaling in mice.

Ischemic preconditioning is a procedure that prepares the myocardium to tolerate long-term ischemic damage by subjecting myocytes to transitory periods of ischemia (29). A previous study demonstrated that COX-2 may be involved in the preadaptation process of myocardial ischemia and serves a key protective role (30). Conversely, COX-2 inhibitors prevent the protective effects of the ischemia preadaptation process by inhibiting the expression of prostaglandin I₂ receptor (30). In addition, the delayed ischemic preconditioning protection mediated by COX-2-regulated opioid-type receptors of rat myocardium may be suppressed following treatment with the COX-2 inhibitor NS-398 (31). It has also been demonstrated that activation of opioid receptors may induce the delayed protective effects of ischemic preconditioning, which is dependent on COX-2 expression, to mitigate myocardial suppression and reduce myocardial infarct size (32). The present study demonstrated that treatment with arctigenin significantly reduced the protein expression levels of COX-2 in AMI model rats.

ERK1 and ERK2 are important members of the mitogen-activated protein kinase signaling pathway superfamily (33). They may be activated by growth stimulating factors, such as growth factors, cytokines and stretching, and are able to mediate various cellular responses, including cell growth, differentiation and apoptosis (34). In cell culture experiments, ERK1/2 agonists significantly enhance hypertrophy and hyperplasia of myocytes (35). Furthermore, hypertrophic myocardial cells also exhibit increased functional ERK1/2 expression (36). *In vivo* experiments also demonstrated that ventricular hypertrophy due to acute or chronic pressure load may lead to varying degrees of increased ERK activity, whereas ERK activity decreases during periods of heart failure (36). The results of the present study demonstrated that treatment with arctigenin significantly decreased the protein expression levels of p-ERK1/2 in AMI rats. Li *et al* (37) reported that arctigenin may suppress transforming growth factor- β 1 in renal tubular epithelial cells through the reactive oxygen species-dependent ERK/nuclear factor- κ B signaling pathway.

In conclusion, the results of the present study revealed that the protective effects of arctigenin reduced the levels of ALT, CK-MB and LDH, and inhibited infarct size in AMI rats. Arctigenin also exhibited antioxidative and anti-inflammatory activities by suppressing the expression levels of iNOS, COX-2 and HO-1, and activating ERK1/2 signaling. These findings suggested that arctigenin may prove clinically useful in treating AMI.

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