

Original Article

Betanin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation, oxidative stress-myeloperoxidase/low-density lipoprotein in rat

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Abstract: The present study aimed to investigate betanin ameliorates isoproterenol-induced AMI through iNOS, inflammation, oxidative stress-myeloperoxidase (MPO)/low-density lipoprotein (LDL) in rat. Cardiovascular diseases CVD is considered as the first killer of human beings. The major pathogenesis of malignant cardiovascular events is chronic heart failure caused by acute myocardial infarction (AMI). Adult male Sprague-Dawley (SD) rat was induced using 100 mg/kg of isoproterenol and used for proving the effect of betanin on AMI. Betanin improves cardiac function and inhibits infarct size in isoproterenol-induced AMI. Next, betanin inhibits inducible nitric oxide synthase (iNOS) and nuclear factor-kappa (NF- κ B) protein expressions of isoproterenol-induced AMI. Betanin reduced oxidative damage (such as superoxide dismutase (SOD), methane dicarboxylic aldehyde (MDA), catalase (CAT) and glutathione (GSH)) and Reactive Oxygen Species (ROS) production in isoproterenol-induced AMI. Interesting, betanin weakens the MPO activity and LDL level in isoproterenol-induced AMI. We firstly demonstrated that betanin ameliorates isoproterenol-induced AMI through iNOS, inflammation, oxidative stress-MPO/LDL in rat.

Keywords: Betanin, isoproterenol-induced acute myocardial infarction, iNOS, inflammation oxidative stress

Introduction

Acute myocardial infarction (AMI) is a kind of local myocardial ischemic necrosis caused by acute coronary artery occlusion and its consequences of blood flow interruption [1]. Based on coronary artery atherosclerosis, coronary thrombosis and/or coronary artery spasm lead to the vast majority of AMI [1]. It is easy for coronary artery atherosclerosis to form atheromatous plaque, which leads to the stricture of vascular lumen of one or more coronary branches [2]. The artery stenoses are exacerbated to occlusion because of atheromatous plaque rupture and coronary thrombosis, result in less blood flow [3]. Therefore, myocardial cells will be damaged by ischemia. The continuous and more severe ischemia leads to irreversible myocardial damage, namely myocardial infarction (MI) [4]. As a serious threat to human life and health, AMI has a mortality of 25% in all

kinds of deaths from diseases. The AMI mortality during hospitalization in Europe is 10-15% [3]. At the same time, cardiovascular diseases (CVD) have become serious threats to the people's health in our country in recent years, with the improving living level and extended life. Among them, AMI has increasing morbidity and mortality, and the death situations of which during hospitalization and post-discharge are similar to the situations abroad [5].

Oxidative stress is a pathological process that the balance between oxidative system and antioxidant system is broken because reactive oxygen species are produced too much and/or antioxidant capacity is decreased [3, 6]. Large amount of recent researches have shown that oxidative stress was one of the major mechanisms of CVD occurrence and development [6].

As a kind of natural edible pigments widely used around the world, Betanin exists in differ-

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ent kinds of plants such as amaranthaceae, chenopodiaceae, nyctaginaceae, cactaceae and phytolaccaceae [7]. Betanin, a kind of water-soluble nitrogen pigment, was found in beetroot first, hence the name [8]. Recent researches showed that betanin had several kinds of functions, such as sterilization, hypolipidemic, anti-atherosclerosis and anticancer [9, 10]. However, it remains unknown whether the betanin ameliorates isoproterenol-induced AMI. Therefore, the aim of present study was to investigate the effect of betanin ameliorates isoproterenol-induced AMI and explore its possible mechanism.

Materials and methods

Animal model and experimental group

Adult male Sprague-Dawley (SD) rat, weighing 260-300 g, were acclimatized and housed under the same standard environmental conditions of light (a 12/12 h light/dark cycle), $23 \pm 1^\circ\text{C}$, and $50 \pm 10\%$ humidity with free access to water. This experiment was approved by the Institutional Animal Care and Use Committee of Chinese PLA General Hospital and conformed to Guide for the Care and Use of Laboratory Animals from National Institutes of Health. All SD rats were randomly allocated into 5 groups: (1) control group, (2) AMI model group, (3-4) Betanin-treated groups (25 and 100 mg/kg/d). In control group, normal rats were received injection of normal saline (0.1 ml/100 g, S.C). In AMI model group and two Betanin-treated groups, all rats were established AMI rat model. We dissolved isoproterenol (Invitrogen, Carlsbad, CA, USA) into normal saline and ultimate density was 100 mg/kg. Then, mixture solution was injected subcutaneously into rats for 3 consecutive days at an interval of 24 h to establish AMI rat model. In AMI model group, AMI rats were received injection of normal saline (0.1 ml/100 g, S.C). In two Betanin-treated groups, AMI rats were received injection of 25 and 100 mg/kg/d of betanin for 3 days.

Measurement of cardiac function

Prior to sacrifice, fasting blood samples were extracted from vena cava. The blood samples were centrifuged at 2000 rpm for 20 min, and the plasma was stored at -80°C . The activity of

creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), cardiac troponin T (cTnT) and lactate dehydrogenase (LDH) of each rat were measured using commercial ELISA kits (Abcam, Shanghai, China).

Measurement of infarct size

The rats were sacrificed using overdose of pentobarbital sodium after blood sampling. Heart samples were immediately measured through the aorta and physiological saline. The coronary artery was ligated after 6 h and snap-frozen in liquid nitrogen and stored at -80°C . Heart samples were parceled into 2 mm tissue fragments and stained with 1.5% of 2,3,5-triphenyltetrazolium chloride (Sigma Co; USA) for 30 min in the dark and measured the infarct size of heart sample.

Measurement of iNOS and NF- κ B by Western blot

Heart samples were extracted and used to measure protein concentration using Bicinchoninic Acid (BCA) protein kit (Beyotime, Nanjing, China). 20 μg total protein was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred into 0.22 mm nitrocellulose membrane (Bio-Rad, Munich, Germany). The nitrocellulose membrane was incubated with phosphate-buffered saline (PBS) containing 5% non-fat dry milk with primary antibody: iNOS, NF- κ B (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA) and β -actin (1:5000, Cell Signaling Technology, USA) overnight at 4°C . The western blot was washed with goat anti-mouse IgG conjugated to peroxidase (Cell Signaling Technology). The membrane was incubated with chemoluminescence staining using an ECL detection kit (Bio-Rad, Hercules, USA) and quantified using the Gel Doc XR system (Bio-Rad).

Measurement of SOD, MDA, CAT and GSH

Blood samples were homogenized using 0.01 M sodium, 1.15% KCl and potassium phosphate buffer solution (pH 7.4) and centrifuged at 2000 rpm for 20 min at 4°C . Then, the supernatant was collected to measure the superoxide dismutase (SOD), methanedicarboxylic aldehyde (MDA), catalase (CAT) and glutathione

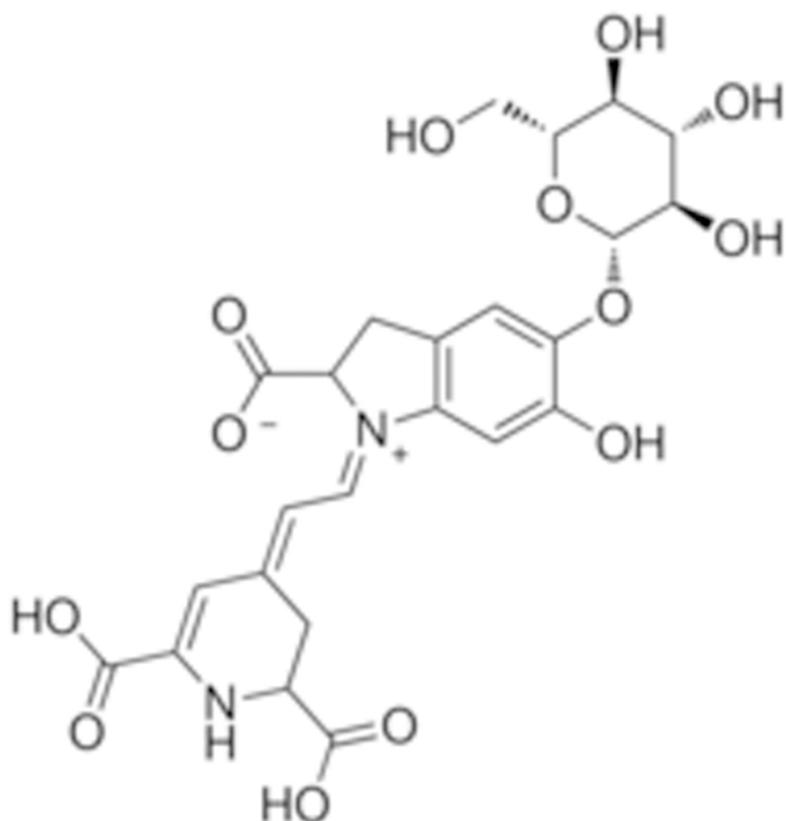


Figure 1. The chemical structure of betanin.

(GSH) contents were detected using diagnostic kits (Abcam, Shanghai, China).

Measurement of ROS production

Heart samples were homogenized using 0.01 M sodium, 1.15% KCl and potassium phosphate buffer solution (pH 7.4) and centrifuged at 2000 rpm for 20 min at 4°C. Then, the supernatant was extracted to determine ROS production using ROS assay kit (Abcam, Shanghai, China) according to the manufacturer's protocol. Miscible liquids were incubated with 2, 7-dichlorofluorescein-diacetate (DCFH-DA) at 37°C for 6 h and placed in wells and scanned to visualize the color development.

Measurement of myeloperoxidase (MPO)

Heart samples were homogenized using 0.01 M sodium, 1.15% KCl and potassium phosphate buffer solution (pH 7.4) and centrifuged at 2000 rpm for 20 min at 4°C. Then, the supernatant was extracted to measure the MPO contents were detected using diagnostic kits (Abcam, Shanghai, China).

Measurement of low-density lipoprotein (LDL)

Heart samples were homogenized using 0.01 M sodium, 1.15% KCl and potassium phosphate buffer solution (pH 7.4) and centrifuged at 2000 rpm for 20 min at 4°C. Then, the supernatant was extracted to measure the oxidation of LDL using the formation of conjugated diene (CD) lipid hydroperoxides at 234 nm.

Statistical analysis

Continuous variables were expressed as mean \pm standard deviation (SD) and compared using one-way ANOVA with the LSD multiple comparison test. The SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

A *P* value of less than 0.05 was considered statistically significant.

Results

Betanin improves cardiac function in isoproterenol-induced AMI

The chemical structure of betanin (CDS000584-1G, Sigma-Aldrich Co. LLC, Germany) was shown as **Figure 1**. To determine the effect of betanin on isoproterenol-induced AMI, the activity of CK, CK-MB, cTnT and LDH were measured. As shown in **Figure 2**, AMI effectively induced the CK, CK-MB, cTnT and LDH activities in rats, compared with control group. The AMI-induced CK, CK-MB, cTnT and LDH activities were effectively inhibited by treatment with betanin (25 and 100 mg/kg) in isoproterenol-induced AMI (**Figure 2**).

Betanin inhibits infarct size in isoproterenol-induced AMI

To identify the effect of betanin on isoproterenol-induced AMI, we measured infarct size of

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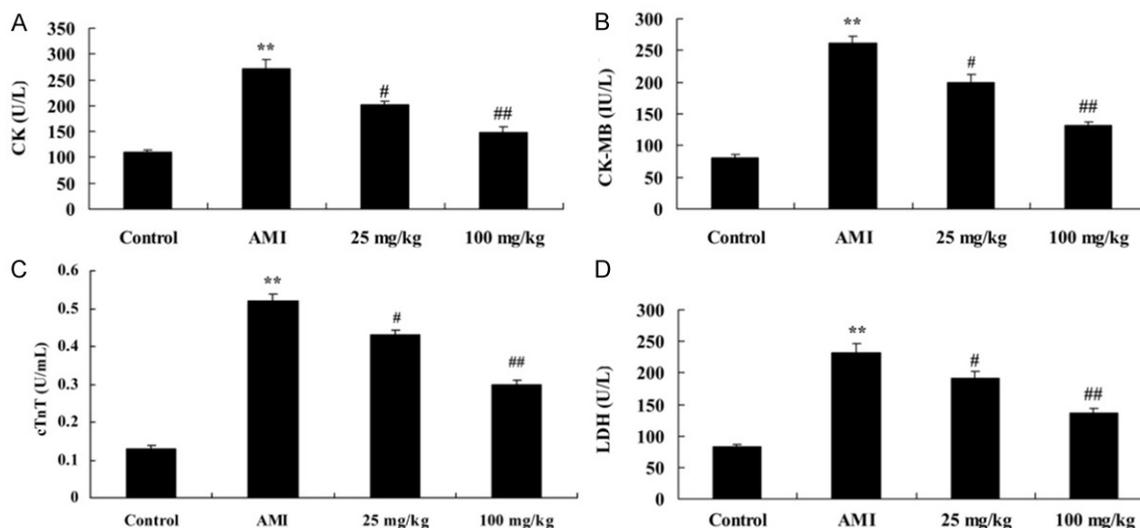


Figure 2. Betanin improves cardiac function in isoproterenol-induced AMI. Effect of betanin on the CK (A), CK-MB (B), cTnT (C) and LDH (D) activities in isoproterenol-induced AMI rats. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

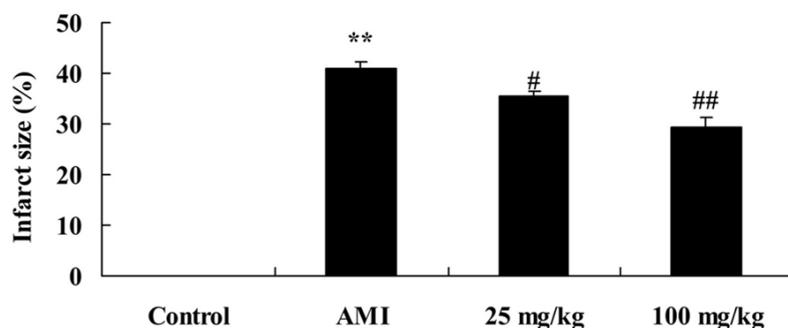


Figure 3. Betanin inhibits infarct size in isoproterenol-induced AMI. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

isoproterenol-induced AMI rats. As shown in **Figure 3**, there was a significant increase in infarct size of isoproterenol-induced AMI rats, compared with control group. However, betanin (25 and 100 mg/kg) effectively reduced the isoproterenol-induced infarct size in isoproterenol-induced AMI rats (**Figure 3**).

Betanin inhibits iNOS in isoproterenol-induced AMI

To recognize the effect of betanin on isoproterenol-induced AMI, the protein of iNOS expression was detected using western blot. **Figure 4** showed that the protein of iNOS expression in

isoproterenol-induced AMI rats was higher than that of no induced control rat. Nevertheless, pretreatment with betanin (25 and 100 mg/kg) effectively weakened the isoproterenol-induced iNOS protein expression in isoproterenol-induced AMI rats (**Figure 4**).

Betanin inhibits NF- κ B in isoproterenol-induced AMI

To identify the effect of betanin on isoproterenol-induced AMI, the protein of NF- κ B expression was detected using western blot. These results from Western blot showed that AMI-induced NF- κ B protein expression was observed, compared with control group (**Figure 5**). However, administration with betanin (25 and 100 mg/kg) effectively eliminated the AMI-induced NF- κ B protein expression in isoproterenol-induced AMI rats (**Figure 5**).

Betanin affects the SOD, MDA, CAT and GSH activities in isoproterenol-induced AMI

To ascertain the effect of betanin on isoproterenol-induced AMI, the SOD, MDA, CAT and GSH

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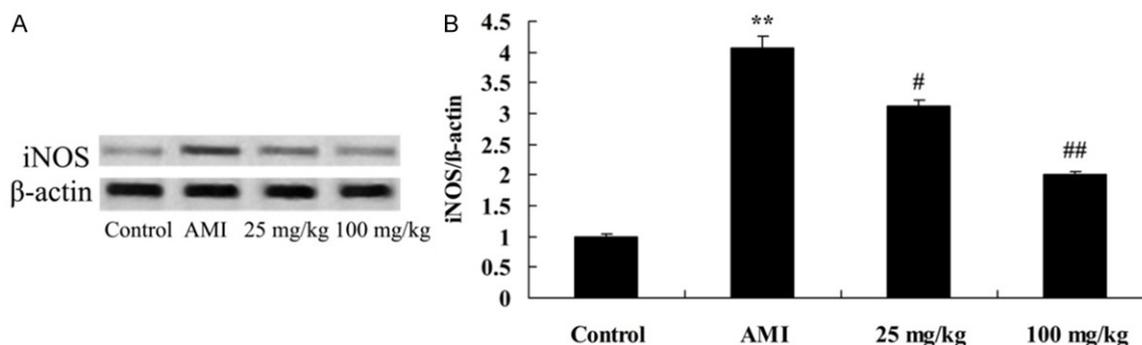


Figure 4. Betanin inhibits iNOS in isoproterenol-induced AMI. Betanin inhibits iNOS protein expression using Western blotting analysis (A) and statistical analysis of iNOS protein expression (B) in isoproterenol-induced AMI rats. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

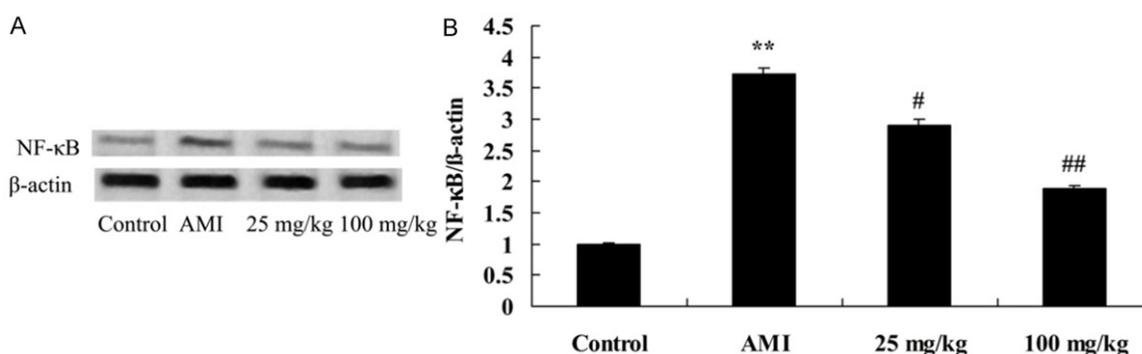


Figure 5. Betanin inhibits NF-κB in isoproterenol-induced AMI. Betanin inhibits NF-κB protein expression using western blotting analysis (A) and statistical analysis of NF-κB protein expression (B) in isoproterenol-induced AMI rats. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

activities were explored in this study. As shown in **Figure 6**, the SOD, CAT and GSH activities were markedly inhibited and the MDA activity was markedly increased in isoproterenol-induced AMI rats, compared with control group (**Figure 6**). Grotesquely, betanin (25 and 100 mg/kg) markedly reversed these changes in isoproterenol-induced AMI rats (**Figure 6**).

Betanin affect on the ROS production in isoproterenol-induced AMI

To identify the mechanism of betanin on isoproterenol-induced AMI, the ROS production in isoproterenol-induced AMI was measured in our study. AMI significantly increased the ROS production in isoproterenol-induced AMI rats, compared with control group (**Figure 7**). However, treatment with betanin (25 and 100 mg/kg) significantly suppressed the increase

ROS production in isoproterenol-induced AMI rats (**Figure 7**).

Betanin affect on the MPO activities in isoproterenol-induced AMI

To further recognize the mechanism of betanin on isoproterenol-induced AMI, the MPO activity in isoproterenol-induced AMI was inspected in our study. The MPO activity in isoproterenol-induced AMI was higher than that of control group (**Figure 8**). Interesting, the elevation of MPO activity was significantly inhibited by treatment with 25 and 100 mg/kg of betanin in isoproterenol-induced AMI rats (**Figure 8**).

Betanin affect on the LDL level in isoproterenol-induced AMI

To further determine the mechanism of betanin on isoproterenol-induced AMI, the LDL level in

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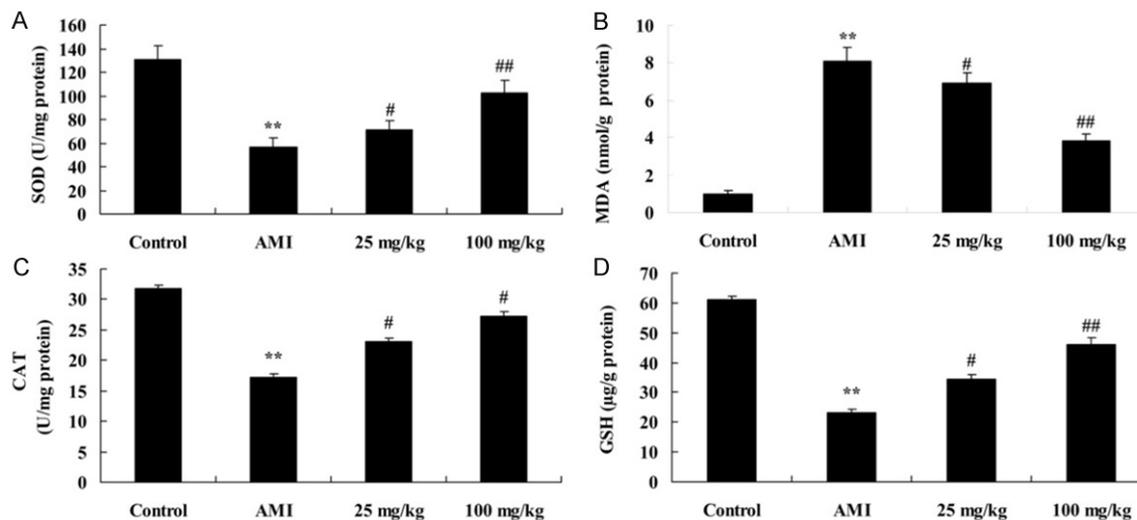


Figure 6. Betanin affect on the SOD (A), MDA (B), CAT (C) and GSH (D) activities in isoproterenol-induced AMI rats. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

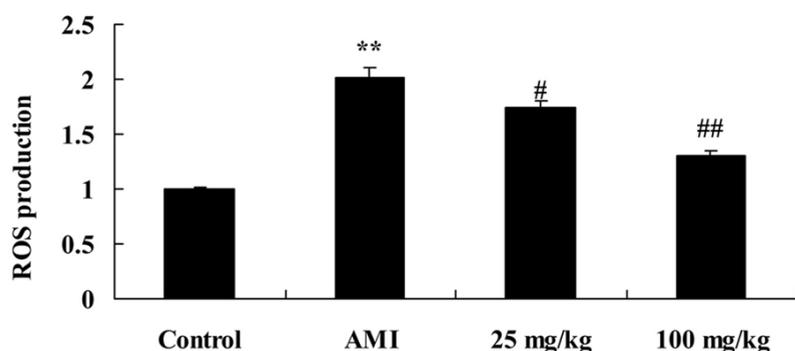


Figure 7. Betanin affect on the ROS production in isoproterenol-induced AMI. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

isoproterenol-induced AMI was examined in our study. There was a significant increase the LDL level in isoproterenol-induced AMI, compared with control group (Figure 9). As expected, treatment with 25 and 100 mg/kg of betanin significantly recedes the isoproterenol-induced LDL level in isoproterenol-induced AMI rats (Figure 9).

Discussion

According to the WHO report in 2000, the number of patients died on CVD is 17 million (that is

1 in 3 deaths is caused by CVD), which will be increased by 50% in 2020, 80% of which will be distributed in low and middle income countries [2]. MI will move up to the first place among different deaths in 2020, from the 5th place in 2000 [3]. At the same time, in 2004, there are 160 million hypertenics in China and 1 billion hypertenics in the world [11]. As a result, hypertension has become the first killer in China and the world, and there is 1

patient dead on disease of Cardiovascular System caused by hypertension in per 12 seconds [12]. Our study for the firstly time showed that betanin improved cardiac function and inhibited infarct size in isoproterenol-induced AMI.

Researches on AMI have become focused issues for experts and scholars inside and outside [13]. The nerve-endocrine-immune system of heart plays a key role in the adjustment of heart function and the occurrence and development of CVD. As an important bioactivator in

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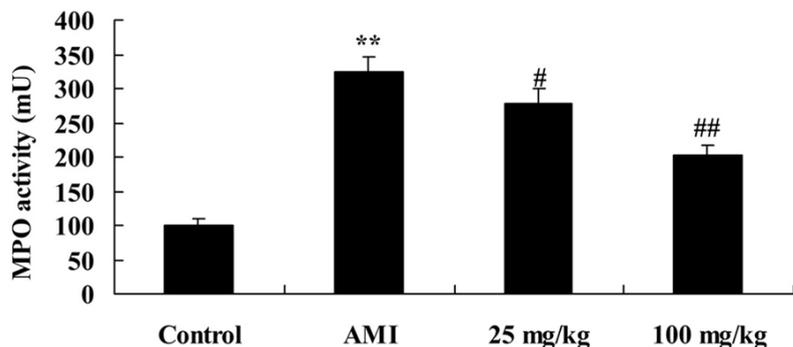


Figure 8. Betanin affect on the MPO activities in isoproterenol-induced AMI. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

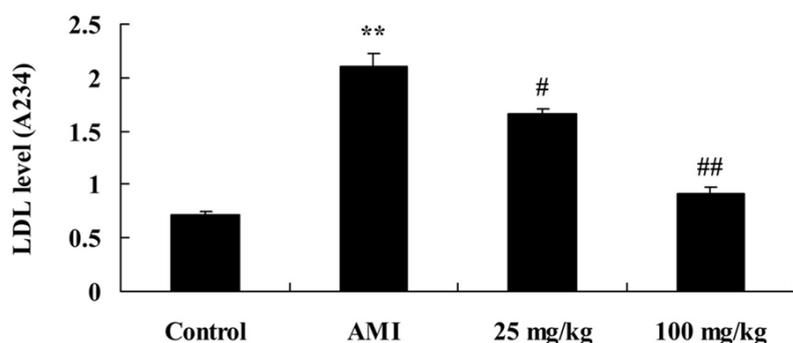


Figure 9. Betanin affect on the LDL level in isoproterenol-induced AMI. Control, control group; AMI, AMI model group; 25 mg/kg, 25 mg/kg/d Betanin-treated groups; 100 mg/kg, 100 mg/kg/d Betanin-treated groups. ** $P < 0.01$ compared with Control group; # $P < 0.01$ compared with AMI group; ## $P < 0.01$ compared with AMI group.

this system, nitric oxide (NO) has taken more and more attention [3]. It is proved by researches that the expression changes of endothelial NO synthase (eNOS) and induced NO synthase (iNOS) are the key factors of the heart failure developed from deteriorations of cardiac function and myocardial remodeling [14]. Our study indicated that pretreatment with betanin (25 and 100 mg/kg) effectively weakened the isoproterenol-induced iNOS protein expression in isoproterenol-induced AMI rats. Tan et al. reported that betanin protects against paraquat-induced acute kidney damage through the inhibition of iNOS [15].

AMI activates immune system and causes inflammatory response by many mechanisms [16]. A large number of cytokines, which are produced along with inflammatory cell infiltration, have participated in the inflammatory

response processes of AMI, including cell death, cellular infiltration and extracellular refactoring. Inflammation has taken part in the pathogenesis of AMI entirely [4]. The cytokines levels in serum are closely related to disease severity and disease course, as a result, the cytokine level detection plays a certain role in judging disease severity and prognosis [6]. Proper inflammation can promote myocardium restoration and angiogenesis, while excessive inflammation can lead to scar tissue formation and fibrosis greatly, resulting in ventricular remodeling and final declined heart function [5]. In present study, administrate with betanin significantly eliminated the AMI-induced NF- κ B protein expression in isoproterenol-induced AMI rats. Tan et al. reported that betanin protects against paraquat-induced acute kidney damage through the inhibition of oxidative stress and inflammation [15].

Along with the continuous in-depth studies on oxidative stress influence on AMI, oxidative stress has been considered as another initiated mechanism of VRM [6]. More and more researches have indicated that oxidative stress plays an important role in the occurring and development of ventricular remodeling and congestive heart-failure (CHF) after AMI [13]. It is proved abroad that oxidative stress can damage DNA, attack proteins with enzymatic activity, oxidate proteins associated with transcription and induce membranes lipid peroxidation, leading to myocardial apoptosis [17]. This experimental result have revealed that, after AMI, the myocardial antioxidant capacity was reduced, oxidative stress level was increased, myocardial apoptosis was increased and the myocardial apoptosis index was significantly negatively related with SOD/MDA [13]. In our

study, betanin significantly inhibited oxidative stress damage and ROS production in isoproterenol-induced AMI rats. Han et al. reported that betanin protects against paraquat-induced liver damage through suppression of oxidative stress and ROS in rats [15]. Zielińska-Przyjemska et al. indicated that betanin suppresses ROS production and apoptosis in human polymorphonuclear neutrophils [18].

A variety of inflammatory mediators, such as myeloperoxidase (MPO), are released by inflammatory cells infiltrated in ischemic myocardial tissue [19]. Oxidative stress was induced by the interaction between MPO in myocardium and oxygen radical produced by neutrophil, leading to damaged endothelial cell function [20]. As a result, VEC is disabled to produce NO so that dilatation function of vascular smooth muscle is affected, leading to reduced blood supply for local myocardium [21]. As a result, cell apoptosis and necrosis are increased, leading to increased collagen fibers in myocardium, cardiac wall hypokinesis, myocardial systolic and diastolic dysfunction [19]. Our study showed that treatment with 25 and 100 mg/kg of betanin significantly the elevation of MPO activity in isoproterenol-induced AMI rats. Han et al. reported that betanin protects against paraquat-induced liver damage through suppression of MPO [22]. Allegra et al. reported that betanin inhibits the MPO/nitrite-induced oxidation of human low-density lipoproteins [23].

LDL can entry arterial wall cells with a large amount of cholesterol, leading to increased platelet adhesiveness and aggregation, increased blood viscosity, reduced erythrocyte deformability [21]. All the factors above can promote arteriosclerosis. More and more LDL which entered into blood vessels will exacerbate the damage of vascular endothelial function, leading to further vessel lumen stenosis and more serious atherosclerosis [24]. As a result, patients are put in the risk of AMI [24]. When atheromatous plaque was disrupted and block blood vessels, there will be an AMI [25]. In this study, we found that betanin significantly receded the isoproterenol-induced LDL level in isoproterenol-induced AMI rats. Allegra et al. reported that betanin inhibits the MPO/nitrite-induced oxidation of human low-density lipoproteins [23]. Esatbeyoglu et al. reported that betanin induces phase II enzymes and prevents LDL oxidation [26].

In summary, we demonstrated for the first time that betanin ameliorates isoproterenol-induced AMI through iNOS, inflammation, oxidative stress-MPO/LDL in rat. These results hinted that betanin may be a potential new drug for AMI and needed further study.

Acknowledgements

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Disclosure of conflict of interest

None.

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