Original Article
Baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation, oxidative stress and P38MAPK pathway in rat

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Abstract: Baicalin is one of the active ingredients in the skullcap, with a variety of pharmacological effects, such as blood pressure reduction, sedation, liver-protection, gallbladder-protection, anti-bacteria, anti-inflammation, etc. The aim of this study was to investigate the potential cardioprotective effects of baicalin ameliorates isoproterenol-induced acute myocardial infarction (AMI) through inducible nitric oxide synthase (iNOS), inflammation, oxidative stress and P38MAPK passageway in rat. Rat model of AMI was induced by isoproterenol (100 mg/kg) and then treated baicalin (various does of baicalin: 1 mg/kg, 10 mg/kg and 100 mg/kg, respectively) for 24 h. Infarct size, the heart weight to body weight ratio and creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT) of rats with AMI induced by isoproterenol were used to evaluate curative effect of baicalin on AMI. Meanwhile, iNOS and phosphorylation-p38 MAPK (p-p38) protein expressions, inflammatory factor and oxidative stress were inspected using western blot and commercial kits, respectively. In the present study, pre-treatment with baicalin (10 or 100 mg/kg) significantly ameliorated infarct size, the heart weight to body weight ratio and CK, CK-MB, LDH and cTnT levels in rats with AMI induced by isoproterenol. iNOS protein expression, the serum TNF-α, IL-6, MDA and SOD levels and p-38 protein expressions were significantly suppressed by treatment with baicalin (10 or 100 mg/kg). These results suggest that acute treatment with baicalin ameliorates AMI, iNOS, inflammation, oxidative stress and P38MAPK pathway in rat with AMI induced by isoproterenol.

Keywords: Baicalin, isoproterenol, acute myocardial infarction, iNOS, inflammation, oxidative stress

Introduction
Ischemic heart disease (IHD) is a major cause of cardiovascular disease, account for a large proportion of coronary atherosclerotic heart disease in ischemic heart disease, in which acute myocardial infarction (AMI) is a serious threat to human health [1]. Myocardial infarction that is caused by myocardial ischemia and coronary circulation disorder is a common cause of cardiovascular disease mortality; in addition, the incidence of arrhythmia caused by AMI is very high, of which the mortality rate is also high [2]. The annual number of death from cardiovascular disease accounts for about 20% of global mortality [3]. World Health Organization (WHO) estimates that 17 million people die of cardiovascular disease each year, and predicts that the deaths caused by cardiovascular disease will quadruple from 1985 to 2015, and ischemic heart disease will become the most important and the most common threats for human life by 2020 [4].

In the case of coronary atherosclerotic heart disease, especially AMI as a threat to human health, we need to work out the best treatment plan for AMI, so we need an animal model very similar to human AMI [1]. Isoproterenol is a synthesized of β adrenergic agonists, which can be used to replicate an animal model of acute AMI [5]. The replication requires large dose of injection that can lead to myocardial necrosis, especially in the endocardium of left ventricle and ventricular septum ground [6]. Studies have shown that, various biochemical indicators of moderate cardiomyopathy induced by 15 mg/kg dose of isoproterenol are significantly
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changed [3]. The main mechanism of isoproterenol-induced myocardial ischemia is the generation of free radicals and reactive oxygen species, lipid peroxidation, oxidative stress, calcium overload, etc [7]. The generation of free radicals and reactive oxygen species, lipid peroxidation, oxidative stress, calcium overload, etc. can lead to the change in membrane permeability, causing apoptosis, necrosis, finally leading to the slowing of the conduction between myocardial cells and the reduced cascade ability of electrical conduction, affecting the normal electrical activity of the heart [5].

As a kind of traditional Chinese medicine with a long history, baicalin is widely distributed, of which the resource is abundant [8]. It has been found that the pharmacological effects, such as anti-arrhythmia, blood pressure reduction and other effects are applied clinically, and new pharmacological effects such as the protections of the brain and cardiac ischemia-reperfusion injury, and the protection of the liver injury induced by a variety of causes, are explored and discovered, which plays a role in the treatment of diseases better for traditional Chinese medicine [9, 10]. In this study, the current study was designed to investigate the potential cardioprotective effects of baicalin ameliorates isoproterenol-induced AMI through inducible nitric oxide synthase (iNOS), inflammation, oxidative stress in rat.

Materials and methods

Animals

Adult male Wistar rat with body weight ranging from 240 and 260 g were used in the present study. Rats were housed under the same standard environmental conditions of light (a 12/12 h light/dark cycle), temperature (22 ± 2°C), and ambient humidity of 50 ± 10% with free access to water. Therefore, the experiment was performed in accordance with the Guide for the Care and Use of Laboratory Animals of The Second Hospital Affiliated to Zhengzhou University.

Induction of AMI

Firstly, isoproterenol was dissolved in normal saline, which was injected subcutaneously (100 mg/kg, S.C) into rats for 3 consecutive days at an interval of 24 h to induce AMI. All rats were sacrificed after injection 72 h.

Experimental group

All rats were randomly allocated into 5 groups. Rats in group 1 (control group) received injection of normal saline (0.1 ml/100 g, S.C) and were left untreated for the whole period of the experiment. In group 2 (Model group) rats were induced by isoproterenol. In group 3-5 (Baicalin group), AMI model rats were treated baicalin (various does of baicalin: 1 mg/kg, 10 mg/kg and 100 mg/kg, respectively) for 24 h [11].
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**Figure 4.** The effect of baicalin on myocardial enzymes in rat. The effect of baicalin on the CK (A), CK-MB (B), LDH (C) and cTnT (D) expression levels in rat. **P < 0.01 compared with control group; ##P < 0.01 compared with model group.

**Figure 5.** The effect of baicalin on iNOS in rat. The effect of baicalin on iNOS (A) protein using Western blot analysis, statistical analysis of iNOS (B) protein in rat. **P < 0.01 compared with control group; ##P < 0.01 compared with model group.

**Figure 6.** The effect of baicalin on inflammatory factor in rat. The effect of baicalin on the serum levels of TNF-α (A) and IL-6 (B) in rat. **P < 0.01 compared with control group; ##P < 0.01 compared with model group.

**Infarct size measurement**

All rats were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg body). Then, heart samples were immediately measured through the aorta and physiological saline was used to wash. The coronary artery was ligated after 6 h and the
left ventricle was placed at -80°C for 10-20 minutes. Heart samples were sliced into 2 mm thick sections. Infarct size of heart sample was measured with 1% 2,3,5-triphenyltetrazolium chloride (1.5%, Sigma Co; USA) for 30 min in the dark [12, 13].

**Tissue weights**

All rats were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg body). Then, the hearts were immediately removed and homogenized with 10 volumes of issue lysis buffer. Miscible liquids were centrifuged at 12,000 g for 10 minutes at 4°C. The protein concentration was determined Bicinchoninic Acid (BCA) protein kit (Menzel Gläser, Braunschweig, Germany). Equal protein was ionophoresis into a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to polyvinyl difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with phosphate-buffered saline (PBS) with 5% non-fat milk to block nonspecific binding sites. After blocked, the membranes were incubated with anti-iNOS (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA), anti-p38 (1:2000, Santa Cruz Biotechnology, Inc, Calif, USA) and anti-β-actin (1:500, Sangon Biotech, Shanghai, China) overnight at 4°C. The membrane was washed with Tris buffered saline Tween (TBST) for 2 h, and then was incubated with second antibody (anti-sheep) conjugated with horseradish peroxidase for 2 h.

**Western blot of iNOS and p38**

All rats were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg body). Then, the hearts were immediately removed and homogenized with 10 volumes of issue lysis buffer. Miscible liquids were centrifuged at 12,000 g for 10 minutes at 4°C. The protein concentration was determined Bicinchoninic Acid (BCA) protein kit (Menzel Gläser, Braunschweig, Germany). Equal protein was ionophoresis into a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to polyvinyl difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with phosphate-buffered saline (PBS) with 5% non-fat milk to block nonspecific binding sites. After blocked, the membranes were incubated with anti-iNOS (1:1000, Santa Cruz Biotechnology, Inc, Calif, USA), anti-p38 (1:2000, Santa Cruz Biotechnology, Inc, Calif, USA) and anti-β-actin (1:500, Sangon Biotech, Shanghai, China) overnight at 4°C. The membrane was washed with Tris buffered saline Tween (TBST) for 2 h, and then was incubated with second antibody (anti-sheep) conjugated with horseradish peroxidase for 2 h.

**Measurement of myocardial enzymes**

Vena cava blood samples were extracted after the occlusion of the coronary artery 6 h. Then, serum samples centrifuged at 3000 rpm for 25 min and were saved for measurement at -80°C. Creatine kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT) activities were measured using commercial ELISA kits according to the manufacture’s protocols (Nanjing Jiancheng Bioengineering Institute, China).
Determination of oxidative stress

Vena cava blood samples were extracted after the occlusion of the coronary artery 6 h. Then, serum samples centrifuged at 3000 rmp for 25 min and were saved for measurement at -80°C. Methane Dicarboxylic Aldehyde (MDA) and superoxide dismutase (SOD) activities were performed using a series of commercial kits according to the manufacture’s protocols (Sangong Biotech, Shanghai, China).

Caspase-3 activity assay

All rats were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg body). Then, the hearts were immediately removed and homogenized with 10 volumes of issue lysis buffer. Miscible liquids were centrifuged at 12,000 g for 10 minutes at 4°C. The protein concentration was determined Bicinchoninic Acid (BCA) protein kit (Menzel Gläser, Braunschweig, Germany). Equal protein was used to determine caspase-3 activity, incubated in a solution buffer at 37°C for 30 min and then initiated by adding Ac-DEVD-pNA (2 mM) and incubated at 37°C for 4 h. Caspase-3 activity was detected at the wavelength of 405 nm.

Statistical analysis

Data analysis was expressed as mean ± S.D. and performed using SPSS version. Statistical comparison between groups was carried out using one way ANOVA or Student’s t test. A P value of less than 0.05 was considered statistically significant.

Results

Effect of baicalin on infarct size in rat

The chemical structure of baicalin was indicated in Figure 1. The current work showed that the effect of baicalin (10 or 100 mg/kg) significantly reduced isoproterenol-induced infarct size in rat with AMI, compared to model group (Figure 2). But administrate of baicalin (1 mg/kg) could also reduce infarct size without statistical significance (Figure 2).

Effect of baicalin on the heart weight to body weight ratio in rat

Figure 3 showed that isoproterenol induced the heart weight to body weight ratio in rat with AMI. Pretreatment with baicalin (10 or 100 mg/kg) resulted in a dramatically significant reduction of increased the heart weight to body weight ratio in rat with isoproterenol-induced AMI in comparison with model group (Figure 3). However, no statistically significant difference was detected between administrate of baicalin (1 mg/kg) group and isoproterenol induced AMI model group (Figure 3).

Effect of baicalin on myocardial enzymes in rat

Figure 4 indicated that isoproterenol significantly induced the CK, CK-MB, LDH and cTnT expression levels in rat with AMI. Interestingly, the isoproterenol-induced CK, CK-MB, LDH and cTnT expression levels were significantly reduced by administrate of baicalin (10 or 100 mg/kg) in rat with isoproterenol induced AMI in comparison with model group (Figure 4).
Effect of baicalin on iNOS in rat

Figure 5A, 5B displayed that isoproterenol promoted the iNOS protein expression in isoproterenol induced AMI rat. However, no statistically significant difference was discovered between administrate of baicalin (1 mg/kg) group and isoproterenol induced AMI model group (Figure 5A, 5B). Pre-treatment of rats with baicalin (10 or 100 mg/kg) led to a more statistically significant decrease of iNOS protein expression than administrate of baicalin (1 mg/kg) group or isoproterenol induced AMI model group (Figure 5A, 5B).

Effect of baicalin on inflammatory factor in rat

Figure 6A, 6B disclosed that isoproterenol enhanced the serum levels of TNF-α and IL-6 in rat with AMI (Figure 6A, 6B). In addition, baicalin (1 mg/kg) pretreatment did not lead to a statistically significant reduction in serum TNF-α and IL-6 levels in rat with isoproterenol induced AMI (Figure 6A, 6B). Administrate of baicalin (10 or 100 mg/kg) observably receded the serum TNF-α and IL-6 levels in rat with isoproterenol induced AMI (Figure 6A, 6B).

Effect of baicalin on oxidative stress in rat

This study revealed that isoproterenol enhanced the serum levels of MDA and SOD in rat with AMI (Figure 7A, 7B). Nevertheless, the serum levels of MDA and SOD by treatment with baicalin (1 mg/kg) was similar to AMI model group (Figure 7A, 7B). Administrate of baicalin (10 or 100 mg/kg) markedly suppressed the serum MDA and SOD levels in rat with isoproterenol induced AMI (Figure 7A, 7B).

Effect of baicalin on caspase-3 activity in rat

The present study revealed that isoproterenol induced caspase-3 activity in rat with AMI (Figure 8). Meanwhile, as shown in Figure 8, the increase caspase-3 activity of baicalin by the dose of 1 mg/kg was very similar to the isoproterenol induced AMI group (P > 0.05). In addition, the increase caspase-3 activity was significantly decreased by treatment with baicalin (10 or 100 mg/kg) in rat with isoproterenol induced AMI group (Figure 8).

Effect of baicalin on p38 protein expression in rat

We further examined that the anti-apoptotic effect of baicalin on AMI, we inspected the p38 protein expression using western blot. As shown in Figure 9, the p38 protein expression in isoproterenol induced AMI group was significantly higher than that of control group. Meanwhile, there was no significant difference between isoproterenol induced AMI group and baicalin (1 mg/kg) group (P > 0.05). However, treatment with baicalin (10 or 100 mg/kg) significantly suppressed the p38 protein expression in isoproterenol induced AMI rats.

Discussion

Myocardial fibrosis and scar tissue formation after AMI is an important pathological change to induce heart failure [1]. Myocardial scar tissue formation is related to myocardial ischemia, size and location of infarct-related artery, the presence or absence of reperfusion and collateral circulation [10]. It is a long time since the studies showed nitric oxide (NO) was one of the most important signaling molecules regulating cardiovascular function, which could regulate blood pressure, dilate blood vessels, inhibit platelet aggregation and leukocyte adhesion, and inhibits cell proliferation in vascular smooth muscle, thereby regulating myocardial scar formation [2]. In cardiac ischemia state after AMI, iNOS begins to express and generate a large number of NO, and endogenous NO is massively released into regional damaged myocardial tissue in the repairing process of AMI, which directly react or interact with other factors, indirectly involved in scar formation and evolution process by adjusting fibroblasts, endothelial cells and other functions [14]. In recent years, the role of iNOS in AMI obtains more attention. In the present study, the therapeutic efficacy of baicalin weakened infarct size, the heart weight to body weight ratio and iNOS protein expression in rats with AMI induced by isoproterenol. Woo et al. reported that baicalein protects against cardiomyocytes from hypoxia/reoxygenation damage [15]. Tu et al. suggested that the neuroprotective effect of baicalin attenuates the mRNA expression of iNOS in a rat model of permanent focal cerebral ischemia [16].

After AMI, various mechanisms activate the immune system, causing inflammation [17]. During the infiltration of inflammatory cells, large amounts of cytokines are generated, involved in the inflammatory reaction after
myocardial infarction, including cell death, cell infiltration and extracellular remodeling stimulated by cell factors. Inflammation participates in the whole process of AMI pathogenesis [17]. The level of inflammatory cytokines in serum is related to the severity degree and duration; monitoring of concentrations of inflammatory cytokines in serum is of great value for determining the severity degree and prognosis for the patients with acute AMI. Appropriate inflammation can promote the repair of cardiac tissue and angiogenesis, but overreaction will lead to the formation of a lot of scar tissue and fibrosis, causing ventricular remodeling, and ultimately affecting heart function [18]. The results of the present study demonstrated that baicalin reduced the serum TNF-α and IL-6 levels in rat with AMI induced by isoproterenol. Cui et al. indicated that the anti-inflammation effect of baicalin significantly decreased TNF-α and IL-1β protein expression in colon tissues [19]. Lin et al. showed that baicalin attenuates renal ischemia-reperfusion injury through suppression of proinflammatory responses [11].

Domestic and foreign scholars have found that the myocardia are in oxidative stress status after AMI [20]. The studies of recent years show oxidative stress status is another important initiating and precipitating factors for AMI [20]. Foreign study has confirmed that oxidative stress induce cardiomyocyte apoptosis by damaging DNA, attacking proteins with enzymatic activity, oxidizing the proteins associated with transcription and inducing lipid peroxidation of cytomembrane [20, 21]. Experimental results show that after AMI, myocardial antioxidant ability is reduced, the level of oxidative stress is increased, myocardial apoptosis is aggravated, and it is obvious that apoptosis index and SOD/MDA ratio is negatively correlated [22, 23]. In this study, rat with AMI induced by isoproterenol, the serum MDA and SOD levels were significantly restrained by treatment with baicalin. Cao et al. suggested that the neuroprotection of baicalin attenuates oxidative of global cerebral ischemia/reperfusion injury [24] and SOD and MDA levels of reperfusion-induced damage in isolated rat hearts [25].

Apoptosis, also known as programmed cell death, is a form of cell death controlled initiatively by genes [26]. Under normal circumstances, when the individual organisms are mature, cardiomyocyte apoptosis is very rare [26]. But when the heart is under certain pathological condition, such as cardiac overload or myocardial ischemia, myocardial apoptosis occurs to a great extent [26]. The present study demonstrates that apoptosis is an important form of cardiac cell death after AMI, with great significance for myocardial infarction evolution, prognosis and outcome [27]. Caspase family proteases are a group of aspartate-specific cysteine proteases, activated by proteolytic cascade sequence, which plays a key role in the execution process of apoptosis, in which Caspase-3 is one of the important apoptosis executors in Caspase family, which is usually present in the cytoplasm as non-activated proenzyme after being synthesized, and activated into an activated form with various apoptotic stimuli proteolytically [27, 28]. And the main mechanism of action is to digest and destroy intracellular proteasome, to activate the nuclear endonuclease, causing DNA cleavage, so that DNA fragments are formed to destruct intracellular calcium pump function, leading to intracellular calcium overload [29]. Research suggests that up-regulating the expression of caspase-3 gene promotes AMI ischemic myocardium apoptosis [30]. Therefore, in our study, pretreatment with baicalin significantly decreased the increase caspase-3 activity in rat with isoproterenol induced AMI group. Zhou et al. reported that baicalin protects human skin fibroblasts through suppression of oxidative damage and caspase-3 detection [31]. Zhou et al. informed that baicalin attenuates focal cerebral ischemic reperfusion injury through suppression of caspase-3 [32].

P38MAPK is an important intracellular signaling enzyme, involved in a variety of intracellular information transfer processes, which can react to a wide range of extracellular stimuli [33]. It is mainly involved in cellular inflammatory response and apoptosis under the condition of stress [34]. Myocardial ischemia and hypoxia leads to the activation of P38MAPK, nuclear translocation starting TNF-α gene transcription and translation, and the secretion of TNF-α; and TNF-α can activate p38MAPK to induce apoptosis, which constitutes a positive feedback path, resulting in impaired cardiac function, amplifying the inflammatory cascade and thus causing the death of heart cells, so as to achieve cell death. In myocardial cells, IL-1β...
can induce P38MAPK activation and increase its activity, thereby inducing cardiac endothelial cell death and stimulating neutrophil function, leading to the increase of TNF-α and the accumulation of neutrophils in myocardial tissue, which causes damage to myocardial tissue [34, 35]. In our study, we found that baicalin significantly down-regulated P38MAPK protein expression in rat with isoproterenol induced AMI group. Wang et al. concluded that treatment with baicalin suppresses migration, invasion and metastasis through p38MAPK signaling pathway in breast cancer cell [36].

In conclusion, cardioprotective effects of baicalin ameliorates infarct size, the heart weight to body weight ratio in rat with AMI induced by isoproterenol and suppresses iNOS protein expression, inflammation and oxidative stress. Baicalin may be an effective therapeutic agent for the treatment of isoproterenol-induced AMI.

Disclosure of conflict of interest

None.

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