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Original Article

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

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Abstract: Baicalin belongs to glucuronic acid glycosides and after hydrolysisbaicalein and glucuronic acid come into being. It has such effects as clearing heat and removing toxicity, anti-inflammation, choleresis, bringing high blood pressure down, diuresis, anti-allergic reaction and so on. In this study, we investigated whether baicalin ameliorates isoproterenol-induced acute myocardial infarction and its mechanism. Rat model of acute myocardial infarction was induced by isoproterenol. Casein kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH), cardiac troponin T (cTnT) and infarct size measurement were used to measure the protective effect of baicalin on isoproterenol-induced acute myocardial infarction. iNOS protein expression in rat was analyzed using western blot analysis. Tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), malondialdehyde (MDA) and superoxide dismutase (SOD) and caspase-3 activation levels were explored using commercial ELISA kits. In the acute myocardial infarction experiment, baicalin effectively ameliorates the level of CK, CK-MB, LDH and cTnT, reduced infarct size in acute myocardial infarction rat model. Meanwhile, treatment with baicalin effectively decreased the iNOS protein expression, inflammatory factors and oxidative stresses in a rat model of acute myocardial infarction. However, baicalin emerged that anti-apoptosis activity and suppressed the activation of caspase-3 in a rat model of acute myocardial infarction. The data suggest that the protective effect of baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat.

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

Introduction

Acute myocardial ischemia is caused mostly by the interruption and a sharp reduction of coronary ischemia [1]. As a result, myocardial necrosis happens because of an acute ischemia hypoxia severely and enduringly [2]. There is a high risk of death in the acute phase of myocardial infarction and the chronic phase is featured with ventricular remodeling and heart failure. Ventricular remodeling's clinical manifestations are the expansion of left ventricular, thinning of wall-thickness, decrease in the systolic and diastolic functions [3]. Ventricular remodeling is a pathological process which is closely related with hemodynamic disorder and if it goes for a long time, chronic heart failure will come into being involving a variety of biological signals' pathway regulation [4]. There is a potential pathophysiological mechanism in the process of ventricular remodeling and heart failure, that is, apoptosis.

To cause myocardial ischemia using isoproterenol is a reliable and simple test method. It is regarded that the mechanism of isoproterenol inducing myocardium damage is related to the overload of calcium in myocardial cells [5]. Large doses of isoproterenol lead to overexcitation of heart beta receptors, heart rate increasing, cardiac contractility enhancing and myocardial oxygen consumption increasing. To excite beta receptors, expand peripheral vessel, reduce its resistance, blood pressure comes down, especially diastolic blood pressure drops lead to myocardial ischemia and a large amount of oxygen free radical is produced [6]. Overexcitement of heart beta receptors contributes to the increase of local cardiovascular angiotensin, which promotes the overloading of

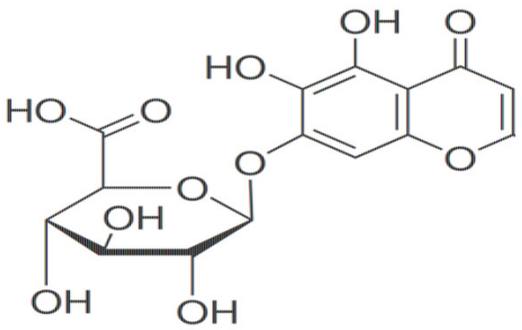

Figure 1. The chemical structure of baicalin.

Ca^{2+} inside cardiomyocytes. When myocardial ischemia happens, overloading of Ca^{2+} in cells can activate active phospholipase causing decomposition of membrane phospholipids; Increase in free radicals derived from xanthine oxidase can cause lipid peroxidation of cellular membrane which all can lead to destruction of cell membrane or even cell death [7].

Baicalin is one the effective compositions of scutellariabaicalensis, this paper, based on the researches on the pharmacological actions of baicalin at home and abroad in the past 10 years, demonstrates that the pharmacological activities of baicalin are multiple [8]. It has an effect on scavenging oxygen free radicals, alleviating ischemic reperfusion of the organizations, regulation of the immune, accelerating the apoptosis and so on. With the development of biotechnology and the progress in separating chemical components from traditional Chinese medicine, baicalin has a great potential value of development and application in terms of antioxidant [9], anti-tumor [10], anti-HIV [11], treating cardiovascular disease [12] and so on [13]. However, protective effect of baicalin on isoproterenol-induced acute myocardial infarction remains unknown. Therefore, these encourage studies have stimulated us to investigate whether the potential protective effect of baicalin on soproterenol-induced acute myocardial infarction and this curative effect is involved in iNOS, inflammation and oxidative stress in rat.

Materials and methods

Drug administration

The chemical structure of baicalin (Sigma, with a purity of 95%) was indicated in **Figure 1** and dissolved in physiological saline. Casein kinase (CK), the MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT) commercial kits, tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), malondialdehyde (MDA) and superoxide dismutase (SOD) commercial ELISA kits were purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Animals and experimental model

Health male Wistar rats (250-280 g) were purchased from animal center of Tongji University School of Medicine and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. These rats were had ad libitum access to water and rodent chow and housed under 07.00 to 19.00 h (12 h-12 h light-dark cycle) in a temperature controlled room. 100 mg/kg of isoproterenol was dissolved in normal saline and injected subcutaneously to rats for two consecutive days to induce acute myocardial infarction.

Experimental groups

All rats were randomly divided into five groups: (i) control group (n = 8), (i) isoflurane group (n = 8), isoflurane + low-dose baicalin group (1 mg/mg n = 8), isoflurane + medium-dose baicalin group (10 mg/mg n = 8), isoflurane + high-dose baicalin group (100 mg/mg n = 8). Isoproterenol-induced acute myocardial infarction rats were induced by clamping the left renal artery for 45 min plus a right nephrectomy.

Enzyme linked immunosorbent assay (ELISA) of the CK, CK-MB, LDH activities and the level of cTnT

After treated with baicalin, whole bloods samples were collected from blood circum after the reperfusion. Immediately, bloods samples were centrifuged at 12,000 g for 10 minutes at 4°C and then the supernatant were collected for measurement at -20°C. Activities of CK, CK-MB, LDH and the level of cTnT were performed using a suite of commercial kits, in according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Nanjing, China).

Infarct size measurement

After treated with baicalin, the aorta was cannulated and 1% 2,3,5-triphenyltetrazolium

Table 1. Baicalin ameliorates the level of CK, CK-MB, LDH and cTnT in a rat model of acute myocardial infarction

Groups	CK (U/mL)	CK-MB (IU/L)	LDH (U/L)	cTnT (U/mL)
Control	0.21 ± 0.04	81.23 ± 5.38	1668.34 ± 314.69	0.07 ± 0.05
Isoflurane	0.98 ± 0.07*	218.44 ± 9.11*	5913.33 ± 502.11*	0.62 ± 0.07*
Low-dose	0.81 ± 0.05	177.55 ± 8.84	4773.93 ± 446.55	0.56 ± 0.06
Medium-dose	0.66 ± 0.03#	141.11 ± 7.31#	4071.44 ± 411.92#	0.38 ± 0.05#
High-dose	0.47 ± 0.06#	121.28 ± 5.78#	3371.93 ± 331.41#	0.26 ± 0.03#

*$P < 0.01$ vs. control group, #$P < 0.01$ vs. isoflurane group. Control, Control group; Isoflurane, isoflurane-induced group; Low-dose baicalin, baicalin (1 mg/kg)-treated; Medium-dose baicalin, baicalin (10 mg/kg)-treated; High -dose baicalin, baicalin (100 mg/kg)-treated.

chloride (1.5%; Sigma-Aldrich Co.) was perfused into the aorta and coronary arteries after the reperfusion. Infarct size of the heart was incubated at 37°C for 30 min in the dark [14, 15]. Then the ligature around the left main coronary artery was retightened and the area of the myocardium was stained with 2 ml of 2% Evans blue dye through aorta by the patent coronary arteries. The infarct size area was measured by a blue staining and the area at risk was determined by negative staining. The heart area with brick red was regarded as normal myocardium, but the area without color was regarded as the ischemic heart muscles.

Western blot analysis

After treated with baicalin, cardiac cytosolic samples were collected from each group after the reperfusion. The frozen tissues were homogenized with lysis buffer on ice. Then, the homogenate were centrifuged at 12,000 g for 10 minutes at 4°C and were saved for measurement at -20°C. The concentration of protein was measured by bicinchoninic acid protein assay (Sangon Biotech, Shanghai, China). An equal amount of total protein was subjected on SDS-PAGE, and transferred electrophoretically onto polyvinylidene fluoride membranes. After blocking, the membranes were incubated with antibodies, anti-iNOS (1:1500, Thermo Scientific, IL, USA) and anti-β-actin (1:5000, Badrilla, UK) overnight at 4°C. After washing, the membranes were incubated with anti-mouse (1:5000, ZSGB-BIO, Beijing, China) immunoglobulin antibodies conjugated to horseradish peroxidase for 1 hour. The relative band intensity was determined by a gel image analysis system (Bio-Rad, USA).

ELISA of TNF-α and IL-6

After treated with baicalin, whole bloods samples were collected from blood circum after the reperfusion. Immediately, bloods samples were centrifuged at 12,000 g for 10 minutes at 4°C and then the supernatant were collected for measurement at -20°C. Level of TNF-α and IL-6 were performed using a suite of commercial kits, in according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Nanjing, China).

ELISA of MDA and SOD

After treated with baicalin, whole bloods samples were collected from blood circum after the reperfusion. Immediately, bloods samples were centrifuged at 12,000 g for 10 minutes at 4°C and then the supernatant were collected for measurement at -20°C. Level of MDA and SOD were performed using a suite of commercial kits, in according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Nanjing, China).

Caspase-3 activation assay

Approximately, 50 μg cardiac cytosolic proteins were incubated in a solution buffer at 37°C for 30 min. Briefly, caspase-3 and caspase-9 activations were measured by Caspase-3 and Caspase-9 Activities Assay Kit (Sangong Biotech, Shanghai, China). 10 μL protein cell lysate per sample were added 80 μL reaction buffer with 10 μL substrate (Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) and incubated at 37°C for 4-6 h. Caspase-3 activation was measured with a Microplate Reader (Bio-Rad, Hercules, CA) at an absorbance of 405 nm. The change in fluorescence (excitation at 400 nm) was detected, at the wavelength of 405 nm.

Statistical analysis

Data were analyzed by SPSS 17.0 software and expressed as the means ± SEM. Differences

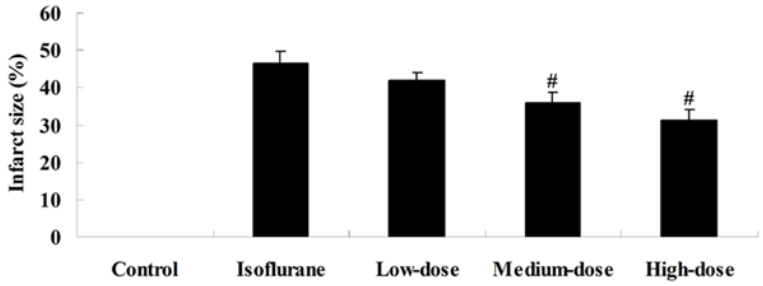

Figure 2. Baicalin ameliorates infarct size in acute myocardial infarction rat model. Control, control group; Isoflurane, isoflurane group; Low-dose, isoflurane + low-dose baicalin group (1 mg/mg); Medium-dose, isoflurane + medium-dose baicalin group (10 mg/mg); High-dose, isoflurane + high-dose baicalin group (100 mg/mg). $^{\#}P < 0.01$ compared with isoflurane group.

were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for individual comparisons between each group mean. A *P*-value, 0.05 was considered statistically significant.

Results

Baicalin ameliorates the level of CK, CK-MB, LDH and cTnT in a rat model of acute myocardial infarction

To study whether the effects of baicalin ameliorates cardiac function in a rat model of acute myocardial infarction, the level of CK, CK-MB, LDH and cTnT were tested in this study. In isoproterenol-induced acute myocardial infarction group, the measurement level of CK, CK-MB, LDH and cTnT significantly increased compared with the control group (**Table 1**). Pretreatment with baicalin at the dose of 10 mg/kg and 100 mg/kg significantly reduced the measurement level of CK, CK-MB, LDH and cTnT in acute myocardial infarction rats, compared to that in the isoproterenol-induced acute myocardial infarction group (**Table 1**).

Baicalin ameliorates infarct size in acute myocardial infarction rat model

In order to examine whether the effects of baicalin on infarct size in acute myocardial infarction rat model, infarct size was inspected in our study. As shown in **Figure 2**, isoproterenol significantly induced infarct size in acute myocardial infarction rat model compared with the control group. After treatment with baicalin (10 mg/kg and 100 mg/kg), infarct size was significantly declined in acute myocardial infarction rats, compared to that in the isoproterenol-induced acute myocardial infarction group (**Figure 2**).

Baicalin ameliorates iNOS protein expression in a rat model of acute myocardial infarction

To imitate whether the effect of baicalin on acute myocardial infarction was associated with changes in NO release, we evaluated iNOS protein expression in heart samples. According to Western blot analysis, isoproterenol significantly promoted the protein expression of iNOS in acute myocardial infarction rats compared with the control group (**Figure 3A**, **3B**). Change in iNOS expression was significantly reduced by treatment with baicalin at the dose of 10 mg/kg and 100 mg/kg, compared to that in the isoproterenol-induced acute myocardial infarction group (**Figure 3A**, **3B**).

Baicalin ameliorates inflammation in a rat model of acute myocardial infarction

To establish whether the mechanisms of baicalin on acute myocardial infarction were associated with inflammatory factors, we examined the levels of TNF-α and IL-6 in heart samples. When compared with control group, the levels of TNF-α and IL-6 were significantly increased by treatment of isoproterenol in acute myocardial infarction rats (**Figure 4A**, **4B**). However, these changes were significantly receded by administrate of baicalin at the dose of 10 mg/kg and 100 mg/kg, compared to that in the isoproterenol-induced acute myocardial infarction group (**Figure 4A**, **4B**).

Baicalin ameliorates oxidative stress in a rat model of acute myocardial infarction

To investigate whether the mechanisms of baicalin on acute myocardial infarction were associated with oxidative stress, we verified the levels of MDA and SOD in heart samples. As shown in **Figure 5A**, **5B**, isoproterenol significantly activated the levels of MDA and suppressed the levels of SOD in acute myocardial infarction rats (**Figure 5A**, **5B**). Interesting, baicalin administrate significantly reversed

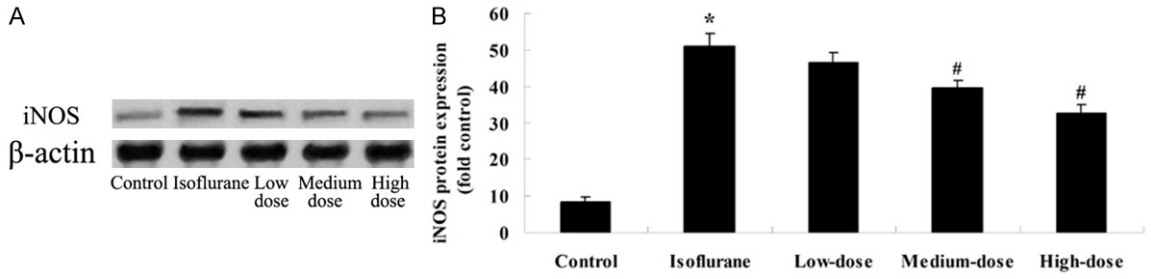

Figure 3. Baicalin ameliorates iNOS protein expression in a rat model of acute myocardial infarction. Baicalin ameliorates iNOS protein expression (A) and statistical analysis of iNOS protein expression (B) in a rat model of acute myocardial infarction. Control, control group; Isoflurane, isoflurane group; Low-dose, isoflurane + low-dose baicalin group (1 mg/mg); Medium-dose, isoflurane + medium-dose baicalin group (10 mg/mg); High-dose, isoflurane + high-dose baicalin group (100 mg/mg). $^{\#}P < 0.01$ compared with isoflurane group.

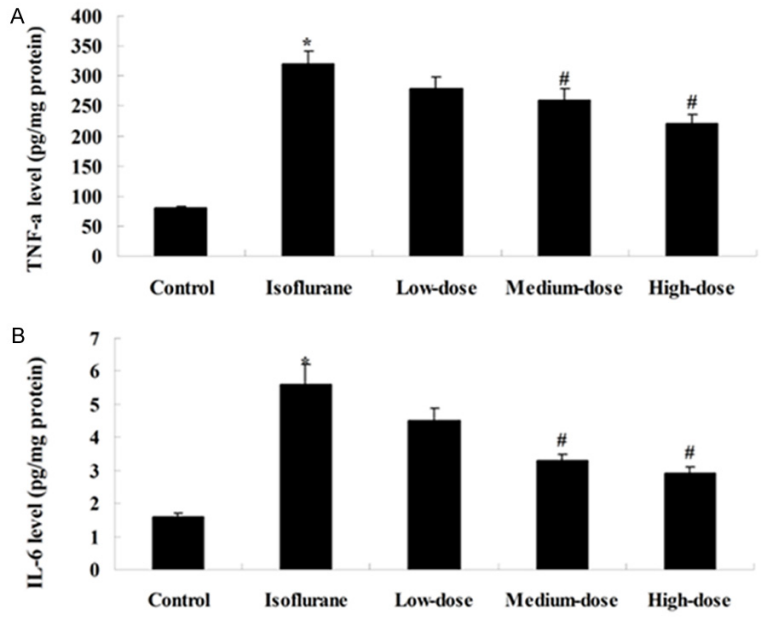

Figure 4. Baicalin ameliorates inflammation in a rat model of acute myocardial infarction. Baicalin ameliorates the TNF-α and IL-6 levels in a rat model of acute myocardial infarction. Control, control group; Isoflurane, isoflurane group; Low-dose, isoflurane + low-dose baicalin group (1 mg/mg); Medium-dose, isoflurane + medium-dose baicalin group (10 mg/mg); High-dose, isoflurane + high-dose baicalin group (100 mg/mg). $^{\#}P < 0.01$ compared with isoflurane group.

these changes, compared to that in the isoproterenol-induced acute myocardial infarction group (**Figure 5A**, **5B**).

Baicalin ameliorates cellular apoptosis in a rat model of acute myocardial infarction

To provide whether the mechanisms of baicalin on acute myocardial infarction were associated with cellular apoptosis in a rat model of acute myocardial infarction, caspase-3 activation was inspected in our study. When compared with control group, isoproterenol significantly promoted the activation of caspase-3 in isoproterenol-induced acute myocardial infarction rats (**Figure 6**). In contrast, baicalin significantly weakened caspase-3 and cellular apoptosis in a rat model of acute myocardial infarction, compared to that in the isoproterenol-induced acute myocardial infarction group (**Figure 6**).

Discussion

Myocardial ischemia refers to the pathological state of reduced oxygen supply and residual metabolites caused by reduced blood perfusion, which is an imbalance between myocardial oxygen supply and demand. In many circumstances, myocardial ischemia is resulted from the combined effect of the one with increased oxygen demand and the one with reduced amount of oxygen. The mechanism of making myocardial ischemia model of mice by the use of ISO is to act on β receptor of the heart, accelerate the heart rate, increase cardiac contractility, and increase myocardial oxygen consumption significantly, thus causing myocardial ischemia. Compared with other ani-

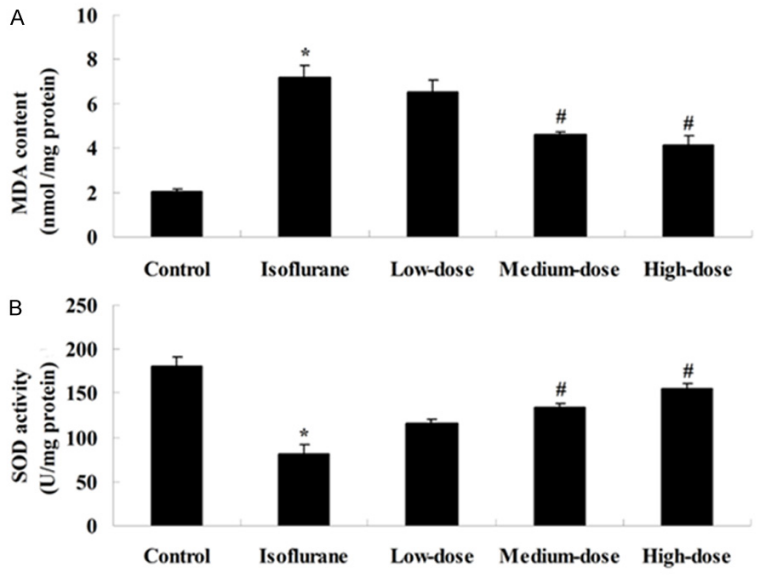

Figure 5. Baicalin ameliorates oxidative stress in a rat model of acute myocardial infarction. Baicalin ameliorates the MDA and SOD levels in a rat model of acute myocardial infarction. Control, control group; Isoflurane, isoflurane group; Low-dose, isoflurane + low-dose baicalin group (1 mg/mg); Medium-dose, isoflurane + medium-dose baicalin group (10 mg/mg); High-dose, isoflurane + high-dose baicalin group (100 mg/mg). $^{\#}P < 0.01$ compared with isoflurane group.

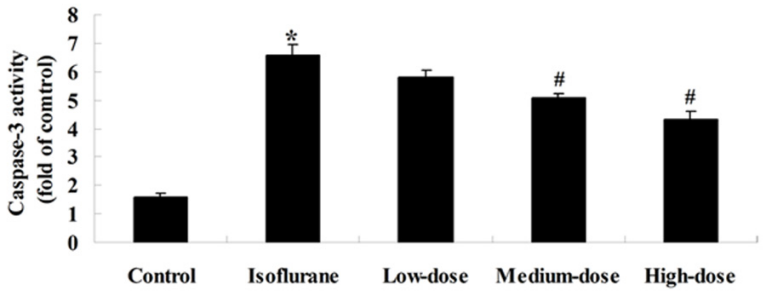

Figure 6. Baicalin ameliorates cellular apoptosis in a rat model of acute myocardial infarction. Control, control group; Isoflurane, isoflurane group; Low-dose, isoflurane + low-dose baicalin group (1 mg/mg); Medium-dose, isoflurane + medium-dose baicalin group (10 mg/mg); High-dose, isoflurane + high-dose baicalin group (100 mg/mg). $^{\#}P < 0.01$ compared with isoflurane group.

mal models of myocardial ischemia, this model has the advantages of low cost, easy operation, good repeatability and so on. The results from this study revealed the treatment with baicalin ameliorated infarct size and the level of CK, CK-MB, LDH and cTnT in acute myocardial infarction rat model. Kong et al. demonstrated that the efficacy of baicalin protects the myocardium through antioxidant in reperfusion-induced damage rat [16]. The results of the present study hinted that baicalin may be a potential cardioprotection drug for clinical application.

Nitric oxide (NO) comes into being under the catalysis of Nitric oxide synthase (NOS), and it is a messenger molecule with many biological effects. It plays a significant role in the treatment of cardiovascular disease [17]. At present NOS has three effects: I type is called central nervous system type or types of nervous system, mainly distributing in the brain and nerve cells; II type is called iNOS, mainly distributing in macrophage and epithelial cells around the inflammation area, and it expresses itself induced by some motivation. III type is called endothelial NOS, mainly locating on endothelial cell [18]. Under the circumstances of myocardial infarction, iNOS expresses motivated by cardiac ischemia stress and produces much NO used for immune system and cardiovascular system. The macrophage in the immune system, the fibroblast in the cardiovascular system, vascular endothelial cell and the like take part in the important process of formation of myocardial scar after myocardial infarction [19]. Our data from this study disclosed that the effect of baicalin ameliorated iNOS protein expression in a rat model of acute myocardial infarction. Tu et al. suggested that treatment with baicalin decreases the iNOS expression and inhibits TLR2/4 signaling pathway in rat brain with permanent cerebral ischemia [20]. The results of our study displayed that the effect of baicalin on acute myocardial infarction may be connected with suppression of the iNOS expression in isoproterenol-induced acute myocardial infarction rats.

Inflammatory reaction is the main pathological process in the early period of myocardial infarction. Studies suggest: inflammatory factors can expand the Infarct size, increase left ventricular end-diastolic volume, reduce cardiac systolic potential and the cardiac output, and act an important role in ventricular remodeling, the occurrence and development of heart failure [21]. After myocardial infarction, myocardial remodeling is an important cause for the occurrence and development of heart failure [22]. In terms of the effects of inflammatory factor on myocardial remodeling and heart failure process, the heart failure process is related to toxic effect that endogenesis cell factor has on heart and peripheral circulation. Cell factor doesn't cause heart failure directly, but its overexpression can lead to the process of heart failure [23]. In the present study, baicalin ameliorated the TNF-α and IL-6 levels in a rat model of acute myocardial infarction. Dong et al. reported that baicalin significantly inhibits the IL-6 and TNF-α mRNA expressions in HBE16 airway epithelial cells [24]. Zhang et al. displayed that baicalin suppresses the TNF-α, IL-1β and IL-6 levels of brain microvascular endothelial cells injured in rat [25]. Our study represented that the anti-inflammatory action of baicalin exist an important role in treatment of myocardial infarction.

Hypoxia is a pessimal stimulus to organism, and it affects many kinds of metabolism of organism, especially the energy supply to oxidation of organism and finally leading to apoptosis of myocardial cells which could contribute to depression of contraction function and cardiac pump function in further [26]. One point has also been approved in studies that the apoptosis of myocardial cells even local ones can lead to disorders of the cellular structure and electrical activity. The myocardial cell apoptosis is related with left ventricular remodeling in the end-stage and dysfunction contributing to ventricular remodeling after acute myocardial infarction. After acute myocardial infarction, for a rat, myocardial necrosis appears in infarct zone and scar forms, and meanwhile, apoptosis of myocardial cells happens in infarct size, scar area, infarction border zone and non-infarcted zone [27]. In our study, baicalin ameliorates oxidative stress and cellular apoptosis in a rat model of acute myocardial infarction. Wen et al. demonstrated that baicalin prevents cadmium through suppression of oxidative stress [28]. Jiang et al. indicated that baicalin inhibits colistin sulfate-induced apoptosis through reducing caspase-3 activity in PC12 cells [29]. The present study expressed the anti-oxidative and anti-apoptosis effect of baicalin displays a protective effect on acute myocardial infarction.

In conclusion, results of the present study indicate that the baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat.

Disclosure of conflict of interest

None.

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