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Full Length Research Paper

The effect of hydroxy safflower yellow A on inflammatory reaction in myocardium of the rats after acute myocardial infarction

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This study was designed to investigate the effect of hydroxy safflower yellow A (HSYA) on inflammatory reaction in ratmyocardium after acute myocardial infarction (AMI). 138 male Wistar rats were randomly divided into six groups: normal group, sham group, control group, injecting SY positive control group (SY group, 90 mg/kg), HSYA high-dose group (HSYA-H group, 40 mg/kg), and HSYA low-dose group (HSYA-L group, 20 mg/kg), n = 23. The AMI injury of rats was induced by ligating the anterior descending coronary artery. After the treatment of drugs, the concentrations of IL-1β and IL-6 in serum of the rats on HSYA-H, HSYA-L and SY groups significantly decreased when compared with those of the rats in the control group (P<0.05). The percentage of cells with the positive expressions of NF-κB and CRP in the drug treatment groups significantly decreased when compared with those of cells in the control group (P<0.05). Moreover, when compared with those of NF-κB in the control group, mRNA and protein expressions of NF-κB in myocardium in SY, HSY-L and HSY-H groups significantly decreased (P<0.05). In addition, the mRNA and protein expressions of NF-κB in myocardium in HSY-H group significantly decreased (P<0.05).HSY-A and SY can reduce the levels of hs-CRP, IL-1β and IL-6 in serum of the AMI rats. The inhibitory effect of HSY-A on inflammation is the main mechanism to improve AMI rats.

Key words: hydroxy safflower yellow A; acute myocardial infarction; inflammation

INTRODUCTION

Acute myocardial infarction (AMI) is the leading cause of morbidity and mortality among all the cardiovascular pathologies, including embolic vascular occlusions, angina pectoris, peripheral vascular insufficiency, cardiac surgery, and cardiogenic shock (Mann and Nolan, 2006). This is despite controlling some risk factors such as arteriosclerosis and treatments via surgical intervention. AMI is a circumstance characterized by two events: ischemia and reperfusion of myocardium, leading to myocardium injury and loss of its function. Furthermore, an acute loss of myocardium following MI results in increased loading conditions that induces ventricular remodeling of infarcted border zone and remote non-infarcted myocardium (Wu et al., 2011). Currently, AMI and unstable angina have progressively become a major concern, because of their high prevalence and mortality as well as their related high treatment costs (Robert et al., 2005).

Inflammatory process plays an important role in the myocardial healing process after an acute ischaemic

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event (Luigi et al., 2007). Important cytokines like tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6 are the starting promoters of the humoral post-MI healing process. They directly interfere with the myocardial contractility, the vascular endothelial function, and the recruitment of other inflammatory cells.

One of the major therapeutic goals for AMI is to alleviate myocardial necrosis and optimize cardiac repair following myocardial infarction. Hydroxy safflower yellow pigment A (HSYA) is the active ingredient of the safflower plant which has been demonstrated to antagonize platelet-activating factor receptor binding, and thus is used to treat several ischemic diseases, including myocardial ischemia, cerebral ischemia, coronary heart disease, and cerebral thrombosis (Liu et al., 2008; Zhu et al., 2003, 2005). According to recent studies, HSYA is a hydrophilic drug with low oral bioavailability, belonging to the biopharmaceutics classification system (BCS) III class of drugs (Wang et al., 2008). Recently, HSYA has been found to alleviate carbon tetrachloride (CCl_4)-induced liver fibrosis in rats (Zhang et al., 2011). Also, HSYA was demonstrated to prevent cerebral ischemia-reperfusion injury by inhibition of thrombin generation (Sun et al., 2010). Yan et al. (2011) found that HSYA could significantly alleviate bleomycin-induced early pulmonary inflammation by suppressing the activation of nuclear factor-kappa B (NF-κB), phosphorylation of p38 mitogen-activated protein kinase (MAPK) and inhibiting the augmentation of pro-inflammatory and pro-fibrogenic cytokines expression (Yan et al., 2012). But it is still unknown whether HSYA can prevent AMI by anti-inflammatory effect. In this study, we designed to investigate the effect of HSYA on inflammatory factors and nuclear transcription factors during the prevention AMI.

MATERIALS AND METHODS

Animals and reagents

Male Wistar rats (138; aged 5 to 7 weeks, 200 ± 20 g) were purchased from Chinese Academy of Medical Sciences, Institute of Experimental Animals. IL-1β, IL-6 and IL-10 kits were purchased from American Rapid Bio Lab Company; high sensitivity C-reactive protein (hs-CRP) kit was purchased from American Adlitteram Diagnostic Laboratories (ADL). Monoclonal antibody rabbit anti-CRP antibody, concentrated polyclonal antibody of NF-kB p65 antibody, versatility secondary antibody, and diaminobenzidine (DAB) staining kit were purchased from Beijing Boaosen Biotechnology Co., Ltd. Reverse transcriptase polymerase chain reaction (RT-PCR) kit was purchased from Sigma, USA; NF-κB p65 primers were provided by Invitrogen Corporation, USA.

Experimental drug

Injection safflower yellow pigment, (SY; 150 mg/branch, contains flavonoids (80 mg) and hydroxy safflower yellow pigment A (HSYA; 67 mg)) was provided by Shanxi Huahui Kai Tak Pharmaceutical Co., Ltd. 80302003; HSYA (98.1135%) from Taiyuan Hua Wei Pharmaceutical Co., Ltd., 20070910.

The preparation of rat AMI model

Chloral hydrate (0.8 ml/100 g) of 3.5% was intraperitoneally injected to anesthetize rats. After fixing on the back and after disinfection, the skin of the rat was cut open in 4,5 intercostal skin, and the thoracic cavity was open and the heart exposed. The pericardium of rat was cut and the heart was extruded, the root of left anterior descending coronary of the rat was ligated between pulmonary cone and the left atrial appendage, threaded and ligated on the bottom 2 to 3 mm of the left atrial appendage root. Then, the thoracic cavity of the rat was sutured. Electrocardiogram was recorded immediately before and after thoracotomy. The remaining operation of the rats on sham group was the same as rats on AMI group except for ligation of coronary artery.

Animal grouping and treatment

Male Wistar rats (138) were randomly divided into six groups: normal group, sham group, control group, injecting SY positive control group (SY group, 90 mg/kg), HSYA high-dose group (HSYA-H group, 40 mg/kg), and HSYA low-dose group (HSYA-L group, 20 mg/kg), n = 23. 360 min after the ligation, two rats were dead in the control, HSYA-H, and HSYA-L groups and one rat was dead in SY group.

Administration of dose

According to the results of pharmacodynamics study before injecting SY in clinic, the rats on the normal, model and sham groups were injected with saline at the same volume. The aforementioned drugs were injected just after and 120 min after induction of AMI.

Experimental animal

Rats (138) were treated with euthanasia 360 min after ligation, and about 5 ml blood was collected from abdominal aorta of rats. After centrifugation, the serum was collected and frozen on -80°C. Six hearts from each group were removed, and fixed with formalin. Then 6 hearts were routinely embedded with paraffin for pathological examination and immunohistochemistry. The other 5 hearts from each group were removed to store for molecular biology.

Preparation of pathological specimens of hearts

After anesthesia, the rat hearts were removed and rinsed cleanly with normal saline. According to surgical marker of infarction location, myocardial tissue was excised below the ligation site. Then, the myocardial tissue was fixed in 4% paraformaldehyde for 24 h, and was embedded in paraffin. 4-μm slices were cut along the long axis of the left ventricular at interval of 1 mm cross-section, and stained with hematoxylin-eosin (HE) staining. The pathological change of myocardial tissues was observed in optical microscope.

Immunohistochemistry method and measurement

Serial 4-μm paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. After blocking sections with 20% (v/v) goat serum in phosphate-buffered saline, sections were incubated overnight at 4°C with NF-κB antibody (Scant Cruz, 1:100) and CRP (1:100, Scant Cruz). Sections were then incubated with the

appropriate secondary antibodies. Positive areas were counted and expressed as a percentage of the myocardial tissue. A negative control, where the primary antibody was replaced with either mouse or rat IgG at the same dilution, was always included. Blinded analysis of positive immunostained sections was performed with the image-analysis program (Image Pro Plus, Media Cybernetics).

Western-blotting

Total protein was isolated from rat myocardial tissues. Tissues were collected and homogenized in protein extraction buffer [50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% protease inhibitor cocktail]. The lysate was centrifuged at 12,000 ×g for 10 min, and the supernatant was collected. The supernatant (30 ×g of protein) was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk, the membranes were probed with the primary NF-κB antibody (1: 5000) for 1 h, followed by a secondary antibody (goat anti-rabbit immunoglobin G (IgG) horseradish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge, Beijing, China), or probed with the primary antibodies anti-actin (1:500; Sigma) and anti-β-actin (1:3000; Proteintech Group, Inc., Chicago, IL), followed by a secondary antibody (goat anti-mouse IgG horseradish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge). Protein expression was detected with an enhanced chemiluminescence detection system (Vigorous, Beijing, China).

RT-PCR

Total RNA was isolated from each specimen of frozen rat myocardial tissue using Trizol reagent according to the manufacturer's instructions. RNA concentration and purity were determined by the Thermo Scientific NanoDrop 2000 (Wilmington, U.S.A.). First-strand cDNA synthesis was performed with 2 μg of the total RNA in a reaction volume of 20 μl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. One microliter of cDNA was amplified in 20 μl reactions using SYBR® Premix Ex TaqTM on an iCycler iQ Real-time Detection System. The following gene-specific primers were used. β-actin primer: Sense, 5'-AACACCCCAGCCATGTACG-3'; Antisense, 5'-CG CT CAGGAGGAGCAATGA-3'; NF-κB primer: Sense, 5'-AAGATCAATGCTA CACAGG-3'; Antisense, 5'-CCTCAATGTCTTCTTTCTGC-3'; PPAR-γ primer: Sense, 5'-GACCACTCCCACTCCTTTGA-3′; Antisense 5'-CGAC ATTCAATTGCC ATGAG-3′. 30 μl Reaction system: RT-PCR enzyme mix, 2 μl; 20X buffer (Mg^{2+} free), 1.5 μl; $MgCl_2$ (25 mM), 1.5 μl; deoxyribonucleotide triphosphate (dNTP; 10 mM), 1.0 μl; CX1 sense primer, 1.0 μl; CX1 anti-sense primer, 1.0 μl; RNA, 2.0 μl; dH_2O, 20 μl. PCR amplification was carried out as follows: initial denaturation at 95°C for 15 s, 35 cycles with denaturation at 95°C for 5 s, annealing at 61°C for 15 s. Relative quantification was determined using the $2^{-\Delta Ct}$ method with data normalized to β-actin house keeping gene.

RESULTS

Effect of HSYA on pathological myocardial tissue of AMI rats

The results of HE staining from the rats in the normal group in light microscope showed that normal myocardial cells were arranged regularly and cellular nucleus was well-stacked, cardiac muscle fibers were arranged uniformly and myocardial structure was normal. While the results from AMI rats on the control group showed that myocardial cells were arranged in scattered manner and cardiac muscle fibers were broken. The intercellular space of myocardial cells in infarction zone was widened and infiltrated with some inflammatory cells including neutrophil granulocyte and monocytes, and red blood cells (Figure 1).

Effect of HSY-A on the concentrations of hs-CRP, IL-1β, IL-6, and IL-10 in serum of AMI rats

As shown in Figure 2, 360 min after AMI induction, the concentrations of IL-1*β* and IL-6 in serum of the rats in the control group significantly increased when compared with those of rats on the sham group ($P<0.05$), while the concentration of IL-10 in serum of the rats in the control group significantly decreased when compared with those of rats in the sham group ($P<0.05$). It indicates that inflammatory reaction obviously happened on acute ischemic stage during AMI. After the treatment of drugs, the concentrations of IL-1*β* and IL-6 in serum of the rats in HSY-high dose group, HSY-low dose group and SY group significantly decreased when compared with those of rats in the control group ($P<0.05$), while there was no significant difference in the concentration of IL-10 in serum of the rats between all the drug-treatment and control groups ($P > 0.05$).

The effect of HSY-A on the NF-κB and CRP protein expression in myocardium of AMI rats

As shown in Figure 3, the immunochemistrial results showed that small amounts of the cells with the positive expressions of NF-κB and CRP were detected in myocardium of the rats in the normal group and sham group. There were small amounts of yellow brown granulation in intracytoplasm. The percentages of the cells with the positive expressions of NF-κB and CRP in the control group were significantly increased when compared with that of the cells on the sham group ($P<0.05$), while the percentage of the cells with the positive expressions of NF-κB and CRP in the drug treatment groups significantly decreased when compared with that of cells in the control group ($P<0.05$). Compared with the drug-treatment group, there were no significant difference on the percentage of the cells with the positive expressions of NF-κB and CRP ($P >0.05$).

Effect of HSY-A on the NF-κB expression in myocardium of AMI rats

As shown in Figure 4, the results detected by RT-PCR

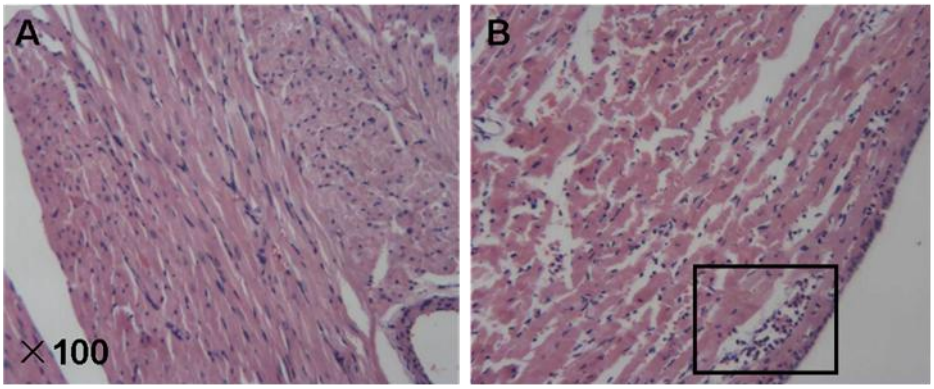

Figure 1. The pathological change of the infarction myocardiom of the rats after ligation treatment. A. normal group; B. control group. Black rectangle indicated the infiltration of inflammatory cells.

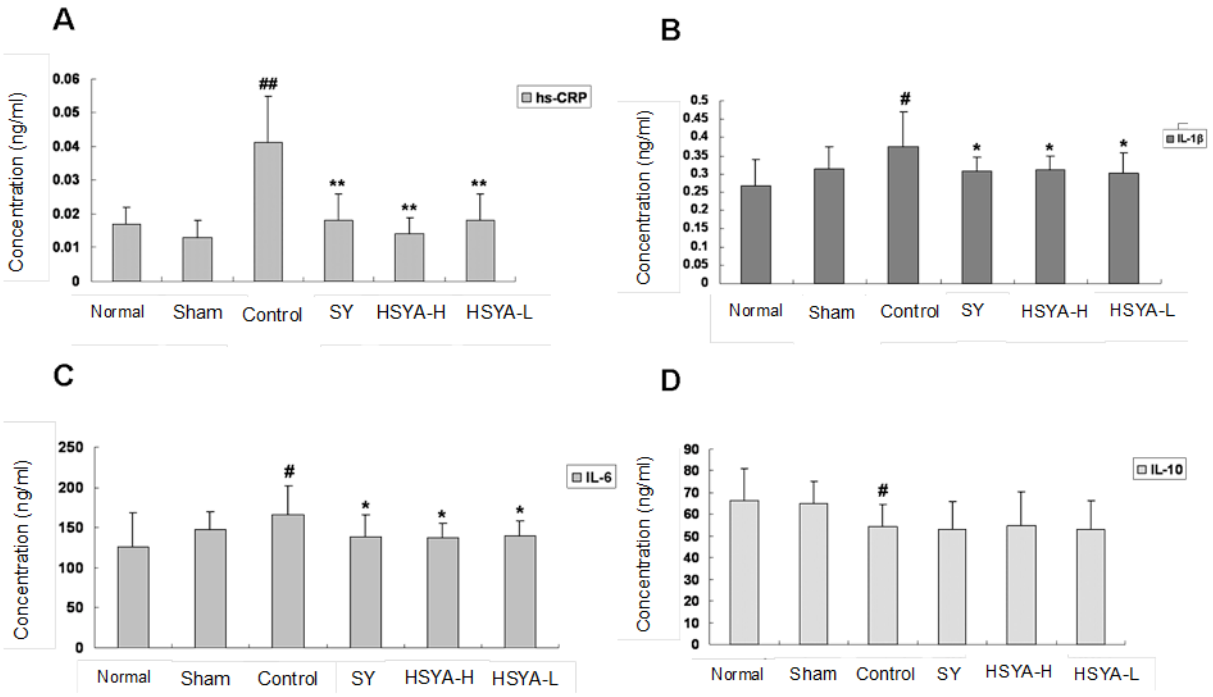

Figure 2. The effect of HSY-A on the concentrations of hs-CRP, IL-1β, IL-6, and IL-10 in serum of AMI rats. A. hs-CRP; B. IL-1β; C. IL-6; D. IL-10 (n=12).

and Western-blot showed that when compared with that of NF-κB on sham group, the mRNA and protein expression of NF-κB in myocardium on the control group significantly increased (P<0.05). Compared with that of NF-κB in the control group, the mRNA and protein expressions of NF-κB in myocardium of SY, HSY-L and HSY-H groups significantly decreased (P<0.05). In addition, compared with the other drug treatment group, the mRNA and protein expressions of NF-κB in myocardium in HSY-H group significantly decreased (P<0.05).

DISCUSSION

HSYA was isolated from the dried flower of *Carthamus tinctorius* L, which was extensively used in traditional Chinese medicine (TCM) to treat cirrhosis. In our previous study, the data showed that HSYA can protect myocardium from ischemia and reduce the levels of cardiac troponin T (cTn-T) and creatine kinase-MB (CK-MB) in serum of AMI rats (Fu et al., 2011). In this study, based on the key role of inflammation in the process of AMI, our data showed that HSYA can inhibit significantly

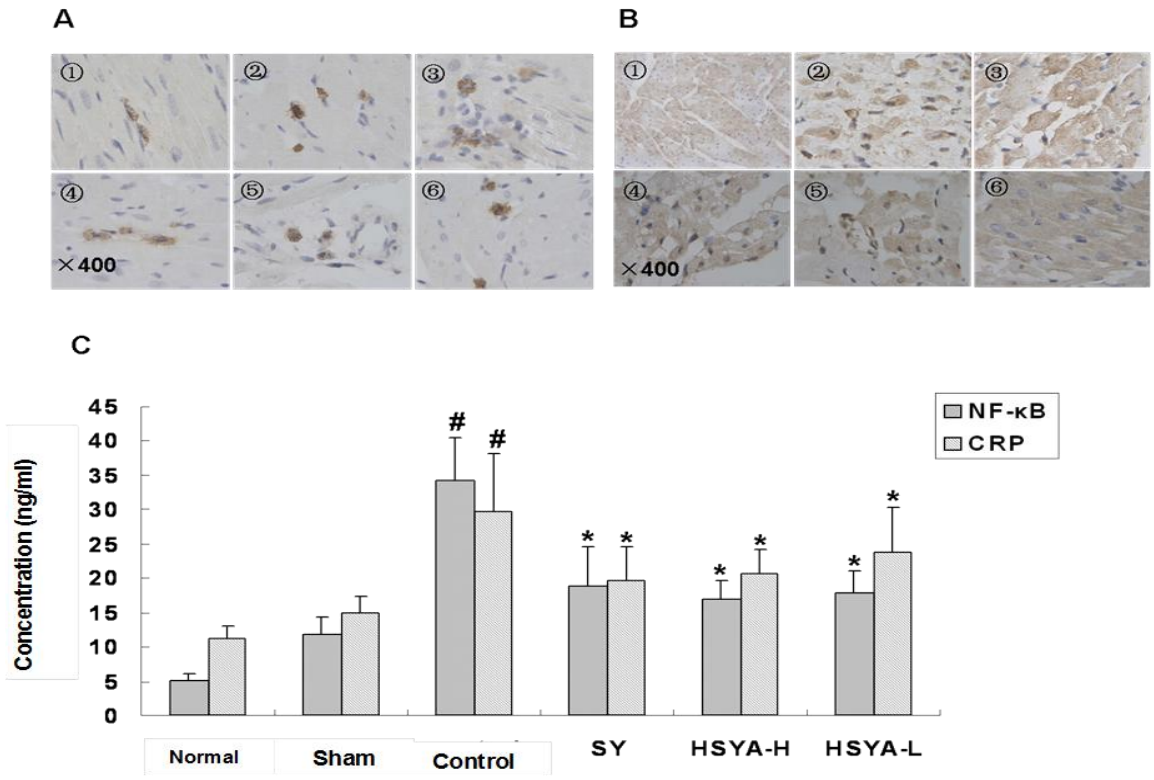

Figure 3. The effect of HSY-A on the NF-κB and CRP expression in myocardium of AMI rats. A. The immunochemical results of NF-KB of the rats on each group. B. The immunochemical results of CRP of the rats on each group. C. The statistical results of NF-κB and CRP expression in myocardium of AMI rats on each group.

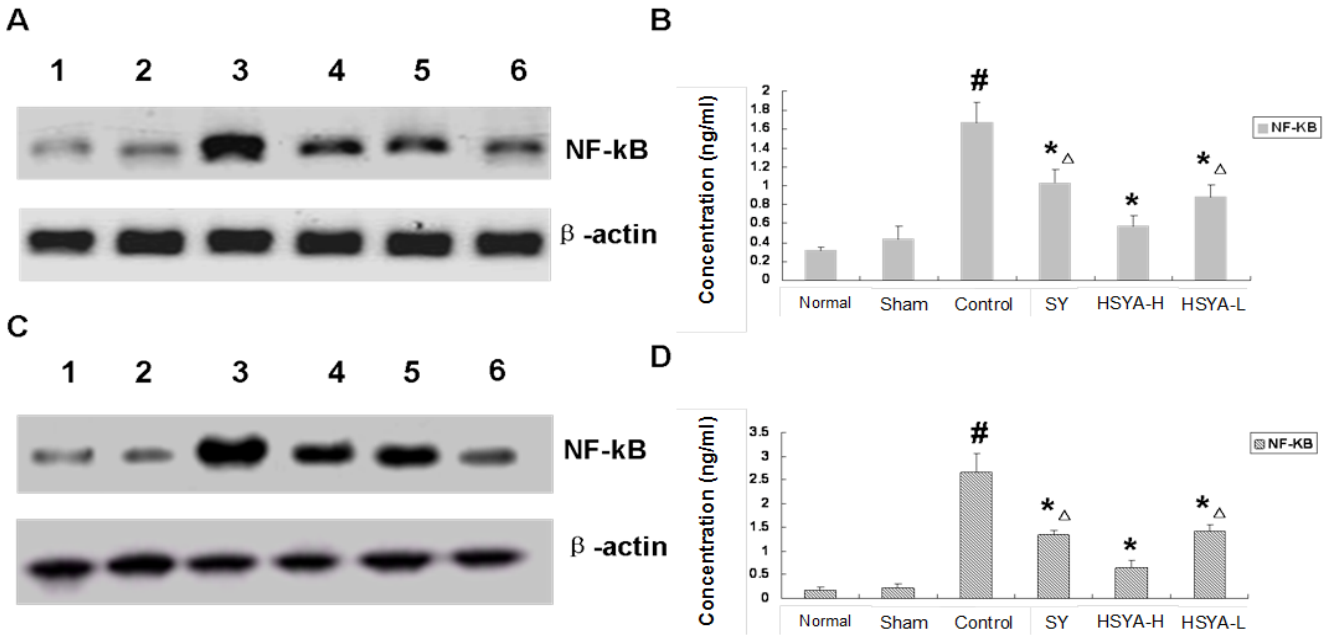

Figure 4. The effects of HSY-A on the expression of NF-κB in AMI cardium of the rats on each group. A. The results of the expression of NF-κB in AMI cardium of the rats on each group by RT-PCR. B. The histogram according to the results of the expression of NF-κB in AMI cardium of the rats on each group by RT-PCR. C. The results of the expression of NF-κB in AMI cardium of the rats on each group by Western-blotting. D. The histogram according to the results of the expression of NF-κB in AMI cardium of the rats on each group by Western-blotting.

inflammatory reaction by reducing the level of hs-CRP, IL-6 and IL-1β in serum and possibly by regulating the activation of NF-κB.

Myocardial infarction is associated with inflammatory reaction in a complex interaction between a variety of pleiotropic inflammatory mediators, which is a prerequisite for healing and scar formation. Among them, the elevation of interleukin, as an indicator that reflects immune system, is significantly related to cardiovascular events (Chamorro, 2004). IL-1β can not only reflect the intensity of inflammatory reaction, but also indirectly predict the extent of myocardial injury. IL-6, also known as a pro-inflammatory cytokine, is a central regulatory factor of inflammation and plays a key role in acute myocardial ischemia and vascular injury in coronary heart disease. IL-6 can enhance the adhesion of white blood cells and myocardial cells, and aggravate damage of myocardial cells (Liu et al., 2007). Anti-inflammatory cytokine IL-10 may inhibit inflammatory reaction and immune reaction (Sheng et al., 2004). When myocardial ischemia occurs, the levels of IL-1β and IL-6 are increased rapidly, and are positively correlated to the increased degree of myocardium damage. A large number of studies have shown that anti-inflammatory factors and pro-inflammatory cytokines are involved simultaneously in the process of ischemic injury. In the process of myocardial ischemia-induced inflammatory response, the increase of anti-inflammatory cytokine IL-10 can inhibit inflammatory response and injury (Prasanna et al., 2010). Monocyte-derived macrophages and mast cells may produce cytokines and growth factors necessary for fibroblast proliferation and neovascularization, leading to effective repair and scar formation. At this stage expression of inhibitory cytokines such as IL-10 may play a role in suppressing the acute inflammatory response and in regulating extracellular matrix metabolism.

The results suggested that after the treatment of injection SY and HSYA, the concentrations of pro-inflammatory cytokines IL-1β and IL-6 in serum were reduced when compared with the control group, but there was no significant change in anti-inflammatory cytokine IL-10 after the treatment of SY and HSYA. It suggested that the inhibitory effect of HSYA on myocardial ischemic injury induced by ligating coronary artery of rats was related to the inhibitory effect of inflammatory response in AMI.

Previous study showed that hs-CRP was one of the independent risk factors for coronary heart disease, and it is an accurate and objective indicator to reflect inflammation activation (Lin and Li, 2007). In the case of AMI, serum CRP increases following cytokines activation and binds to the damaged myocardial cells. Further, it stimulates the complement cascade, which may finally increase the MI size, worsening the overall post-MI outcomes (Barrett et al., 2002; Paoletti et al., 2004).

In this study, 360 min after AMI, the concentration of hs-CRP in the serum of rats in the control group significantly increased when compared with that of rats in the sham group, which suggested that inflammatory reaction is significant at acute phase of ischemia. After drug treatment, the levels of hs-CRP in the serum of rats in hydroxyl high-dose, low dose and safflower yellow pigment groups significantly reduced when compared with that of rats in the control group. It indicated that HSYA can inhibit inflammatory reaction during myocardial infarction. CRP is one of the most powerful predictors of myocardial infarction, stroke, and vascular death currently known. NF-κB activation may be responsible for the synergistic effect of IL-1β on IL-6-induced CRP expression. In this study, the immunohistochemical results indicated that 360 min after myocardial ischemia, inflammatory reaction is significant in ischemic myocardium and the protein expression of CRP in myocardium is significantly decreased, which corresponds with the results of serum determination. The percentage of the cells with the positive expressions of NF-κB and CRP after the treatment of HSYA and SY significantly decreased when compared with those of cells in the control group.

NF-κB is considered as one of the most important transcription factors, and is the central link of regulating immune response, stress response, apoptosis, and inflammation. It can be activated by a variety of different stimuli and it participates in expression and regulation of a variety of genes, especially genes involved in defense functions of the body. In recent years, studies have shown that myocardial ischemia at early stage can induce activation of NF-κB to regulate gene transcription (Xiyuan et al., 2009) and NF-κB plays an important role in myocardial ischemic preconditioning, ischemia, hypoxia, reperfusion injury and apoptosis, especially in the process of inducing myocardial inflammatory reaction, and increasing the release of TNF-α, IL-1β and IL-6 (Lu et al, 2009; Yao et al., 2011), and aggravating myocardial injury. At present, a variety of NF-κB inhibitors have been used to relieve myocardial ischemia and reperfusion injury. NF-κB is activated by a vast number of agents, including cytokines such as TNF-α and IL-1β and free radicals. The genes regulated by the NF-κB family of transcription factors are diverse and include those involved in the inflammatory response, cell adhesion and growth control (Martine et al., 2011). NF-κB activation has been demonstrated in various models of experimental myocardial ischemia and reperfusion (Kupatt et al., 1999; Shimizu et al., 1998).

According to the results from RT-PCR and Western blotting, after AMI, compared with that of rats in normal and sham groups, the mRNA and protein expression of NF-κB on the AMI control group is significantly increased, while the expressions of NF-κB on HSYA-L and HSYA-H groups, especially on HSYA-L group, significantly reduced when compared with that of rats in the AMI control group. It suggested that HSYA can inhibit the

release of downstream inflammatory factors to protect ischemic myocardium by inhibiting the activation of NF-κB in the process of myocardium ischemia.

The immunohistochemical results indicated that 360 min after myocardial ischemia, inflammatory reaction is significant in ischemic myocardium and the protein expression of CRP in myocardium is significantly decreased, which corresponds with the results of serum determination.

Previous studies showed that treatment with HSYA also alleviated bleomycin-induced increase of mRNA level of TNF-α, IL-1 and transforming growth factor (TGF-β1) in lung homogenates. Moreover, HSYA inhibited the increased activation of NF-κB (Sun et al., 2010). HSYA decreased NF-κB p65 nuclear translocation, inhibited the mRNA expressions of pro-inflammatory cytokine TNF-α, IL-1β and IL-6 in mice with acute lung injury (Sun et al., 2010). The results also were corresponding with our experimental results in this study.

In summary, it can be suggested that HSY-A and SY can reduce the levels of hs-CRP, IL-1β and IL-6 in serum of the AMI rats. HSYA is the main component of SY to reduce myocardial ischemia. The inhibitory effect of HSY-A on inflammation after AMI is the main mechanism to improve AMI of rats.

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