Anti-Inflammatory Effect of Guan-Xin-Er-Hao via the Nuclear Factor-Kappa B Signaling Pathway in Rats with Acute Myocardial Infarction

Xue-Ya ZHANG\(^1\), Xi HUANG\(^{1,2}\), Feng QIN\(^2\), and Ping REN\(^2\)

\(^1\)Department of Integrated Chinese and Western Medicine, West China Hospital, Sichuan University, Chengdu 610041, P.R. China and \(^2\)Laboratory of Ethnopharmacology, and Institute of Integrated Traditional Chinese and Western Medicine, Xiangya Hospital, Central South University, Changsha 410008, P.R. China

Abstract: A traditional Chinese medicine, Guan-Xin-Er-Hao (GXEH), is a famous multiple target therapeutic polypharmaceutical. Our aim was to evaluate whether or not oral administration of GXEH has an anti-inflammatory effect associated with blockade of nuclear factor-kappa B (NF-κB), and to investigate the NF-κB-mediated pro-inflammatory cytokines expression pathway during acute myocardial infarction (AMI) in rats. Sprague-Dawley rats were randomly assigned to four groups: oral GXEH administered at 15 or 5 g/kg, the vehicle control and sham-operated groups. Thirty minutes after giving GXEH or 0.9% NaCl (p.o.) once, coronary arteries were occluded except for the sham-operated rats. We measured 24-h infarct size, 3-h expression of NF-κB protein in the myocardial left ventricular tissues and serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP). Compared with rats receiving vehicle, rats administered 15 g/kg GXEH had significantly reduced 24-h infarct size, expression of NF-κB protein and serum concentrations of TNF-α, IL-6, and CRP. GXEH at 5 g/kg did not have a significant effect on these parameters. In conclusion, GXEH exhibited an anti-inflammatory effect through inhibition of the NF-κB-mediated signaling pathway leading to downregulation of pro-inflammatory cytokine expression. These findings provide new evidence of the cardioprotective effect of GXEH through reduction of infarct size by mediating lots of endogenous materials via multiple pathways to act on myocardial cells in the treatment of cardiovascular disease.

Key words: acute myocardial infarction, anti-inflammatory, Guan-Xin-Er-Hao, NF-κB pathway, traditional Chinese medicine

Introduction

It has been proposed that a better way of treating complex diseases such as cardiovascular disease (CVD) may be to aim at several targets. Polypharmacology may provide a solution in this field [5]. Traditional Chinese medicines (TCMs) consist of several types of medicinal herb or mineral, and multiple components can hit multiple targets and exert synergistic therapeutic effects [27]. Traditional Chinese formula Guan-Xin-Er-Hao (GXEH)
is a well-known, multiple-target, therapeutic polypharmaceutical which is used in China and other Asian countries to treat CVD.

In previous studies, GXEH formula showed anti-anginal effects, attenuated ST-segment depression, and lowered total cholesterol and low-density lipoprotein cholesterol [2, 11, 30]. Recently, it has been demonstrated that GXEH protects the heart from ischemic injury by inhibiting myocardial apoptosis [7, 8, 12, 28, 36, 37], increasing coronary flow velocity [34, 37] and suppressing oxidative stress [24]. However, to the best of our knowledge, no research has reported on the anti-inflammatory effect of GXEH during the acute phase of myocardial infarction.

It is well known that atherosclerosis is an inflammatory disease [25], and that inflammation plays an important role throughout the development and progression of CVD [10]. A recent study indicated that nuclear factor-kappa B (NF-κB) was a transcription factor involved in important biological processes, and appeared to be the master regulator in the inflammatory process [3, 20]. In the NF-κB-mediated pathway, pro-inflammatory cytokine secretion is regulated by NF-κB activation [9, 14]. The use of anti-inflammatory drugs as a new anti-inflammatory strategy has been applied to CVD therapy [15, 16]. It has been demonstrated that some TCMs have anti-inflammatory effects in CVD treatment [18, 29, 35].

In our previous studies, GXEH reduced infarct size by mediating lots of endogenous materials via multiple pathways to act on myocardial cells during acute myocardial infarction (AMI) in rats [12, 24, 36, 37]. Accordingly, we speculated that GXEH might have an anti-inflammatory effect and that the anti-inflammatory effect might be one of the cardioprotective mechanisms.

Our aim was to evaluate whether GXEH has an anti-inflammatory effect associated with blockade of NF-κB, and to investigate the NF-κB-mediated pro-inflammatory cytokine expression pathway in AMI rats.

**Materials and Methods**

**Composition and preparation of GXEH formula**

GXEH contains *Salvia miltiorrhiza* Bge., *Carthamus tinctorius* L., *Paeonia lactiflora* Pall., *Ligusticum chuanxiong* Hort., and *Dalbergia odorifera* T. Chen in a ratio of 2:1:1:1:1 [32]. Formula extracts were prepared as previously described [36, 37]. All of the herbs were purchased from West China Hospital (Chengdu, China). They were also identified by the herbal medicine botanist Professor Z. H. Hu, Department of Botanical Anatomy of Northwest University, Xi’an, China. GXEH was boiled twice in distilled water (1:12, w/v) for 30 min. The blended supernatants were then lyophilized, and stored at 4°C until use. The extraction yield was approximately 28% (w/w, dried extract/crude herb). In this study, the dose of GXEH given to the rats was that of the dried extract, which was administered to the rats in a reconstituted solution.

According to high-performance liquid chromatography (HPLC) [24, 37], the contents of each component in the crude herb (mg/g, n=3) were: tanshinol 0.704 ± 0.007, protocatechualdehyde 0.015 ± 0.0005, peoniflorin 3.715 ± 0.131, hydroxysafflor yellow A 2.067 ± 0.017, and ferulic acid 0.129 ± 0.001. The chromatogram of GXEH is shown in Fig. 1. Each column represents the mean ± standard deviation (SD). Chromatography was used for quality control of GXEH.

**Experimental protocol**

Six-week-old male Sprague-Dawley rats (200–250 g) originated from Shanghai Laboratory Animal Center (Shanghai, China) was conformed to the Regulations for the Administration of Affairs Concerning Experimental Animals (1988), and was approved by the Animal Experimental Center for West China Hospital, Sichuan University (Chengdu, China). The rats were administered and housed under standardized environmental conditions (temperature 22°C, 12-h light-dark cycle),
and had unlimited access to food and water. They were randomly assigned to four groups of 20: (i) a myocardial infarction (MI) + vehicle group given 0.9% NaCl (20 ml/kg); (ii) a MI + GXEH 15 g/kg group given GXEH (15 g/kg); (iii) a MI + GXEH 5 g/kg group given GXEH (5 g/kg); and (iv) sham MI group given 0.9% NaCl (20 ml/kg). Administration was via the oral route. The rat dose of GXEH in the present study was converted according to our previous study. The high dose used in rats was 15 g/kg, which is equivalent to a 2.5 g/kg dose administered to healthy humans [37] and clinical patients [30] without causing significant side effects. The dosage in the rat was 6.7 times the dose given to humans, which is consistent with the findings of others [12, 23]. The experiments were performed 30 min after administration of GXEH (15 or 5 g/kg) or 0.9% NaCl 20 ml/kg.

Surgical preparation

The studies were carried out in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Sichuan University. Rats were anesthetized with sodium pentobarbital at a dose of 40 mg/kg (i.p.), then intubated through a tracheotomy and ventilated with room air (tidal volume, 3 ml/100 g; respiratory rate, 68 cycles/min) using a small animal respirator (DH-150, Taimeng, Chengdu, China). Additional anesthesia was given during the experiment, when necessary. A thoracotomy was done in the fourth intercostal space on the left chest side and the heart was exposed. The pericardium was opened and a 1.0 silk suture placed around the left anterior descending coronary artery (LCA) close to its origin without externalization of the heart. The coronary artery was occluded, and the chest partially closed. Sham-operated rats were subjected to the same procedures without LCA ligation. Hearts and serum were harvested at the end of a 3-h ischemic period for NF-κB, C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) analysis, and at the end of 24 h for infarct size.

Determination of infarct size

The determination of infarct size was performed as previously described [36, 37]. At the end of the 24-h ischemic period, Evans blue dye was injected into the aortic root to stain the normally perfused region blue and outline the area at risk (Ar). Then the heart was removed, frozen at –20°C and sliced into 1-mm thick transverse sections. The Ar was separated from the non-ischemic zone and incubated at 37°C for 10 min in a 1% solution of triphenyltetrazolium chloride (TTC) (SCR, Shanghai, China) to differentiate necrotic (pale) from non-necrotic Ar. The Ar as a percentage of the left ventricle (Lv) (Ar/Lv), and the area of necrosis (An) as a percentage of the Ar (An/Ar) were calculated.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of CRP, IL-6, and TNF-α were determined by ELISA using a commercial kit (Usclfife, Wuhan, Hubei, China) with the recommended protocol. At the end of the 3-h ischemic period, we used a serum separator tube and allowed whole blood to clot for 30 min before centrifugation for 15 min at 1,000 × g. We then removed serum. According to the kit instructions, we added 100 µl of standard, blank, or sample per well, incubated for 2 h at 37°C and removed the liquid. We added 100 µl of Detection Reagent A to each well, incubated for 1 h at 37°C, and used wash buffer to wash each well. Then we added 100 µl of Detection Reagent B to each well, incubated for 1 h at 37°C, and washed each well again. After adding 90 µl of Substrate Solution, followed by incubation for 20 min, 50 µl of Stop Solution was added at room temperature. We determined the optical density of each well using a microplate spectrophotometer (BioTek, Winooski, VT, USA) at 450 nm wavelength. Finally, we created a standard curve to interpret the results.

Preparation of myocardial cytoplasm and nuclear protein

The method of extracting cytoplasm and nuclear protein was performed as described by Li et al. [18]. Myocardial cytoplasm and nuclear protein were isolated by a commercially available Nuclear and Cytoplasmic Protein Extraction Kit (BioTeke Corporation, Beijing, China) according to the manufacturer’s instructions. Briefly, the myocardial left ventricular tissues were minced in phosphate-buffered saline (PBS) and homogenized in ice-cold lysis buffer using a glass-glass homogenizer (DY89-1, Xinzhi, Chengdu, China). The
mixture was centrifuged at 500 \( \times \) g for 3 min and the supernatant was removed. Then the tissues were added to 100 \( \mu l \) of ice-cold CER extraction. We vortexed the tube vigorously for 5 s and incubated the tube on ice for 10 min; then, vortexed the tube for 5 s and centrifuged for 10 min (16,000 \( \times \) g) again. The supernatant containing the non-nuclear fraction (cytoplasm extract) was kept. The insoluble fraction in the tube, which contained the nuclei, was resuspended in 50 \( \mu l \) of ice-cold NER extraction. Samples were vortexed for 15 s and returned to ice every 5 min for 40 min. Then, the samples were centrifuged at 16,000 \( \times \) g for 10 min and the supernatants contain the nuclear fraction were saved. Protein concentrations were determined with a protein assay kit (Zhong Shan-Golden Bridge Biotechnology, Beijing, China).

**Western blot**

Equal amounts of cytoplasm or nuclear protein were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer at 90 V, then electroblotted to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA, USA). Membranes were blocked at 4°C with 5% non-fat milk and incubated overnight at 4°C with primary rabbit polyclonal anti-rat NF-\( \kappa \)B p65 antibody (dilution, 1:500; Abzoom, Dallas, TX, USA). Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (dilution, 1:2000; Zhong Shan-Golden Bridge Biotechnology). The proteins were visualized using an electrochemiluminescence detection system (Pierce, Rockford, IL, USA) with 5 to 10 min exposure after washing the membrane, and analyzed by Quantity One Analysis Software (Bio-Rad). Protein levels of NF-\( \kappa \)B were normalized to \( \beta \)-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

**Statistical analysis**

All data are expressed as mean \( \pm \) SD. In the animal study, one-way ANOVA was used to analyze differences in parameters. Values of \( P<0.05 \) were considered statistically significant.

---

**Results**

**Effect of GXEH on myocardial infarction**

Photographs of myocardial infarction in the vehicle control and GXEH-treated groups are shown in Fig. 2A. Ar (red staining and pale area) was expressed as the percentage of lv (Ar/lv), and An (pale area) was expressed as the percentage of Ar (An/Ar). As shown in Fig. 2B, there were no significant differences in Ar/lv among the groups. Administration of GXEH (15 g/kg) significantly reduced myocardial infarct size (An/Ar) compared with the vehicle group (28.45 \( \pm \) 7.76 vs. 49.24 \( \pm \) 11.65%, \( P<0.05 \)). There was also a significant difference between the 15 and 5 g/kg GXEH groups (28.45 \( \pm \) 7.76 vs. 46.15 \( \pm \) 11.10%, \( P<0.05 \)).

**Effect of GXEH on NF-\( \kappa \)B expression**

To investigate whether GXEH-mediated cytoprotection in cardiomyocytes is through the NF-\( \kappa \)B-dependent pathway, we determined the expression of NF-\( \kappa \)B protein (Fig. 3). Myocardial infarction resulted in a marked increase in the expression of NF-\( \kappa \)B compared with the
Treatment with GXEH (15 g/kg) was associated with a reduced expression of NF-κB compared with the vehicle group (0.43 ± 0.08 vs. 1.03 ± 0.30%, P<0.01; 0.17 ± 0.05 vs. 0.32 ± 0.06%, P<0.01). Its effect was seen in both the cytoplasm (Fig. 3A) and nucleus (Fig. 3B). There was a significant difference between the two GXEH groups (P<0.01).

**Effect of GXEH on serum levels of TNF-α, IL-6, and CRP**

To determine if the effect of GXEH is via the NF-κB-mediated pro-inflammatory cytokine expression pathway and verify that GXEH has an anti-inflammatory effect, serum levels of TNF-α, IL-6, and CRP were determined by ELISA (Fig. 4). Coronary occlusion obviously increased serum levels of TNF-α, IL-6, and CRP compared with the sham group. Treatment with GXEH (15 g/kg) significantly reduced serum TNF-α (9.89 ± 0.12 vs. 11.74 ± 1.33%, P<0.01), IL-6 (10.30 ± 0.56 vs. 11.48 ± 1.08%, P<0.05), and CRP (2.10 ± 0.04 vs. 2.27 ± 0.13%, P<0.01) levels relative to the vehicle group. There was a significant difference between the 15 and 5 g/kg GXEH groups (P<0.05). As shown in Fig. 4A and 4B, pro-inflammatory cytokine TNF-α and IL-6 serum levels reveal that GXEH downregulated the NF-κB-mediated pro-inflammatory cytokine expression pathway. As shown in Fig. 4C, CRP serum level provided further evidence that an acute pro-inflammatory cytokine-induced event was suppressed by GXEH. All of these results verify that GXEH has an anti-inflammatory effect via the NF-κB-mediated pro-inflammatory cytokine expression pathway.

**Discussion**

As polypharmaceuticals can effect multiple targets and provide efficacy [5], research into the anti-inflammatory mechanism of GXEH is important. In this study, our data showed for the first time the anti-inflammatory effect of GXEH. Infarct size was reduced by GXEH via the NF-κB-mediated pro-inflammatory cytokine expression pathway. This finding provides additional therapeutic evidence of the multiple-target effect of GXEH in the treatment of CVD, although 5 g/kg GXEH elicited no significant effects.

Infarct size is an important parameter in the evaluation of the effectiveness of cardiovascular drugs in the treatment of CVD [21]. GXEH reduced 24-h infarct size (Fig. 2), indicating that GXEH has a cardioprotective effect, a result consistent with our previous studies [24, 36, 37]. A recent study concluded that CRP is a specific inflammatory marker of CVD [1, 3], and that the serum concentration of CRP is associated with coronary
In the present study, occlusion of the coronary artery was associated with an increased serum CRP level (Fig. 4C). There are also other reports concluding that CRP is a specific inflammatory marker of AMI [10, 19]. Treatment with GXEH (15 g/kg) significantly reduced the serum CRP level (Fig. 4C), indicating that GXEH has an anti-inflammatory effect in AMI. Together with the reduced infarct size (Fig. 2), this indicates that the anti-inflammatory effect of GXEH also invokes a cardioprotective mechanism.

Recent investigations have shown that anti-inflammatory action is tightly associated with blockade of transcription factor NF-κB [33], which can be viewed as the master regulator of the inflammatory process [3, 20]. NF-κB activation can be induced by many extracellular stimuli such as oxidative stress during myocardial ischemia. Under such conditions, NF-κB is activated by the IKK/NFκB and Mitogen-Activated Protein Kinase (MAPK) pathways, leading to initiation of its down-regulation [3]. In our study, occlusion of the coronary artery was associated with increased cardiomyocyte expression of NF-κB (Fig. 3), indicating that NF-κB was activated by myocardial ischemia, as reported elsewhere in the literature [3]. Treatment with GXEH (15 g/kg) significantly reduced the expression of NF-κB (Fig. 3), indicating that GXEH has an anti-inflammatory effect in cardiac myocytes via the NF-κB signaling pathway in AMI.

The present study also showed the anti-inflammatory effect of GXEH via the NF-κB-mediated pro-inflammatory cytokine expression pathway (Fig. 4). Pro-inflammatory cytokines are believed to be the main regulators of the inflammatory reaction in the acute phase [6]. Higher levels of pro-inflammatory mediators, including IL-6 and TNF-α, are risk markers for coronary artery disease, and might be related to increased morbidity and mortality in cardiac disease [26]. IL-6 is a key inflammatory factor, and has been implicated in the pathogenesis and progression of CVD [13]. In particular, increased expression of IL-6 in cardiac tissue is associated with myocardial ischemia [13]. TNF-α is also an acute phase reactive protein and is one of the important pathological factors in inflammatory responses [4]. During the pathological progression of myocardial ischemia, and after activation of NF-κB, TNF-α is released from macrophages, monocytes and mast cells within minutes, and IL-6 is secreted from vascular endothelial cells in response to inflammatory mechanical stimuli [9, 14]. The released cytokines are activated by NF-κB in the liver, which is their principal target organ [20]. These inflammatory factors stimulate the liver and CRP is secreted by the liver in response to a variety of inflammatory cytokines. CRP further stimulates the release of IL-6 and TNF-α, accelerating injury to the blood vessels [17]. In our study, occlusion of the coronary artery was associated with increased serum levels of TNF-α and IL-6 (Fig. 4A and 4B), indicating that
pro-inflammatory cytokine expression pathway was upregulated, as reported in previous studies [9, 13, 14]. Treatment with GXEH (15 g/kg) significantly reduced serum levels of TNF-α and IL-6 (Fig. 4A and 4B) showing that NF-κB-mediated cardioprotection by GXEH reduced the expression of pro-inflammatory cytokines in AMI. Considering that GXEH reduced tissue expression of NF-κB (Fig. 3), as well as serum levels of TNF-α, IL-6, and CRP (Fig. 4), we conclude that the mechanism of the anti-inflammatory effect of GXEH downregulated pro-inflammatory cytokine expression via the NF-κB-mediated signaling pathway.

In addition, we observed that serum levels of CRP (ng/ml), IL-6 (pg/ml), and TNF-α (pg/ml) were all lowered by GXEH (15 g/kg) (Fig. 4). We consider that this result is related to the serum being collected at the end of 3-h ischemic period. Because the classic ischemic preconditioning is short-lived and fast-decaying, with anti-ischemic effects appearing within 3 h [31], we chose a 3-h ischemic period to determine the parameters. According to Gabriel et al. [6], the serum IL-6 level in AMI showed a curved time-course and was highly correlated to CRP; both of them peaked on days 1 and 2. Also, in another study, serum TNF-α level was elevated throughout the course of observation [23]. Thus, the lower concentration serum levels of CRP, IL-6, and TNF-α seen in the current study were in accordance with findings reported elsewhere in the literature.

In conclusion, the present study demonstrated that GXEH has an anti-inflammatory effect, and that modulation of the NF-κB signaling pathway might be one of the molecular mechanisms providing cardioprotection as well as downregulation of the release of inflammatory cytokines. These findings provide new evidence of the cardioprotective effect of GXEH, which reduces infarct size by mediating lots of endogenous materials via multiple pathways to act on myocardial cells in the treatment of CVD.

Acknowledgments

This work was supported by a grant (No. 30325045) from the National Science Fund for Distinguished Young Scholars of China and was partly supported by the Natural Science Foundation of China (No. 30572339).

References


