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Luhong formula inhibits myocardial fibrosis in a paracrine manner by activating the gp130/JAK2/STAT3 pathway in cardiomyocytes

Huiyan Qu[1], Yong Wang[1], Yingjie Wang, Tao Yang, Zhou Feng, Yang Qu, Hua Zhou*

Department of Cardiology, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

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ABSTRACT

Ethnopharmacological relevance: Luhong formula (LHF)—a traditional Chinese medicine containing *Cervus nippon Temminck, Carthamus tinctorius L., Cinnamomum cassia Presl, Codonopisis pilosula(Franch.) Nannf., Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao, Lepidium apetalum Willd*—is used in the treatment of heart failure.

Aim of the study: To investigate the antifibrotic efficacy of LHF in a myocardial infarction-induced rat model of heart failure and to determine its mechanism of action.

Material and methods: Myocardial infarction was induced in rats by coronary artery ligation, and cardiac fibroblasts were isolated. Neonatal rat cardiomyocytes (NRCMs) were isolated from 2 to 3-day-old Sprague-Dawley male rats, and cardiomyocyte hypertrophy was induced by isoprenaline. Histological examination was carried out to estimate the degree of myocardial fibrosis. Expression of gp130/JAK2/STAT3 pathway proteins was measured by western blot. The mRNA levels of downstream genes of gp130/JAK2/STAT3 pathway (i.e., *CTGF*, *TSP-1*, and *TIMP1*) were determined by RT-PCR; while CTGF, TSP-1, and TIMP1 protein levels were measured by ELISA. To investigate paracrine effects, cell proliferation and collagen synthesis was measured after treating cardiac fibroblasts with the conditioned media from isoprenaline-treated NRCMs.

Results: Histopathological changes showed that LHF inhibited myocardial fibrosis in heart failure rats. Treatment with LHF up-regulated gp130, JAK2, and STAT3 protein expression in heart tissue, and down-regulated *CTGF*, *TSP-1*, and *TIMP1* gene expression. Isoprenaline-treated NRCMs displayed lower expression of the gp130, JAK2, and STAT3 pathway proteins and higher secretion of its downstream signaling molecules (CTGF, TSP-1, TIMP1). LHF inhibited cardiac fibroblast proliferation and collagen synthesis after treatment with the conditioned media from isoprenaline-treated NRCMs.

Conclusion: LHF treatment attenuates myocardial fibrosis *in vivo*. LHF inhibits cardiac fibroblasts proliferation and collagen synthesis in a paracrine manner by activating the gp130/JAK2/STAT3 pathway in cardiomyocytes, thereby inhibiting the secretion of downstream profibrogenic cytokines.

1. Introduction

Heart failure (HF) afflicts millions of patients annually, and is the most common cause of cardiac morbidity and mortality worldwide (Ana and Frazier, 2014). Myocardial fibrosis is the main pathological change in HF, leading to distorted organ architecture and impaired cardiac function (Min et al., 2013). In the myocardium, cardiomyocytes (CMs) and cardiac fibroblasts (CFs) are spatially intermingled, with virtually every CM bordering one or more CF. Therefore, CMs can interact with CFs in a paracrine manner and via direct cell-to-cell contact (Zhang, 2012; Stephanie et al., 2010). A growing body of evidence indicates that the interaction between CMs and CFs determines myocardial fibrosis progression (Kalyani et al., 2012; Aibin et al., 2015). With the onset of HF, CMs become hypertrophic and can secret various cytokines, which can convert CFs into "activated" myofibroblasts and enhance collagen deposition, leading to fibrosis (Raghu

Abbreviations: LHF, Luhong formula; HF, heart failure; MI, myocardial infarction; CMs, cardiomyocytes; NRCMs, neonatal rat cardiomyocytes; NRCFs, neonatal rat cardiac fibroblasts; SD, Sprague-Dawley; ISO, isoprenaline; CTGF, connective tissue growth factor; TSP-1, thrombospondin-1; TIMP1, tissue inhibitor of metalloproteinase 1; CCK-8, cell counting kit-8; CFs, cardiac fibroblasts; gp130, glycoprotein 130; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3; TCM, traditional Chinese medicine; HPLC-MS, high performance liquid chromatography tandem mass spectrometry; LAD, left anterior descending coronary artery; ELISA, enzyme-linked immunosorbent assay; OD, optical density; COL1A1, Collagen type I alpha 1; COL3A1, Collagen type III alpha 1

* Corresponding author.
E-mail address: zhouhuam@hotmail.com (H. Zhou).
[1] Both authors contributed equally to this work.

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et al., 2014). However, no effective antifibrotic agents are currently available to interrupt specific aspects of this pathway.

Recent studies indicate that the glycoprotein 130 (gp130)/janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway plays a central role in the interaction between CMs and CFs (Arash et al., 2014). Binding of extracellular ligands, such as the interleukin-6 (IL-6) family, to gp130 activates JAK2. In turn, JAK2 recruits and activates STAT3, which translocates to the nucleus to transactivate target gene that promote cell survival and growth of CMs. Indeed, a previous study showed that treating neonatal rat cardiomyocytes (NRCMs) with isoprenaline (ISO) for 24 h inhibited STAT3 activation and significantly increased the surface area of CMs (Suresh et al., 2012). Further experiments showed that STAT3 knockout in CMs in mice resulted in the up-regulation of numerous profibrotic genes, such as connective tissue growth factor (CTGF), thrombospondin1 (TSP-1), and tissue inhibitor of metalloproteinase 1 (TIMP1), suggesting that STAT3 inhibition may enhance myocardial fibrosis in a paracrine manner (Denise et al., 2004).

In China, heart failure mainly belongs to the Traditional Chinese Medicine(TCM) category of “heart-kidney Yang deficiency, blood stasis” (Bi et al., 2013). Both the "heart-kidney interaction" theory and the "unsmooth blood circulation results in water retention" theory are academic theory of heart failure: the former explains the correlation between kidney yang deficiency and this disease, the latter explains the correlation between blood stasis and this disease (Fu and Wang, 2013; He, 2015). Luhong formula (Chinese national patent number ZL 2006 1 0147305.0) is an empirical compound prescription based on these theory. In recent decades, Luhong formula(LHF) has been used in the treatment of heart failure, such as heart failure caused by coronary heart disease, hypertension, rheumatic heart disease and dilated cardiomyopathy (Qu et al., 2011). Recent studies have demonstrated that LHF has better clinical effect especially in treating heart failure caused by coronary heart disease (Zhou et al., 2007; Xue et al., 2015; Lai et al., 2015; Liu et al., 2015a, 2015b). LHF is composed of six herbs: *Cervus nippon Temminck, horn which has ossified, water soaked and then Sun-dried(lu jiao, Antler);Carthamus tinctorius L., flos, stoving(hong hua, Safflower);Cinnamomum cassia Presl, twig, Sun-dried (gui zhi, Ramulus Cinnamomi);Codonopisis pilosula(Franch.) Nannf., root, Sun-dried (ming dang shen, Medicinal Changium Root);Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao, root, stir-fried (huang qi, Milkvetch Root);Lepidium apetalum Willd, seed, Sun-dried(ting li zi, Pepperweed Seed)* . According to Chinese medical theory, LHF can warm and tonify the heart and kidney, activate blood. LHF was prescribed to treat heart failure with these symptoms: palpitation, chest congestion, breathlessness, aversion to cold and cold extremities, edema especially of the legs and ankles, scanty clear urination. In order to evaluate the effect of LHF on heart failure, our group has registered a large- scale, multi-center, randomized, controlled clinical trial in Chinese Clinical Trial Registry: Multi-center clinical trial on Luhong Formula in intervening Coronary Artery Disease combined Heart Failure(Registration ID: ChiCTR-TRC-13003242). Our result has demonstrated that LHF improve heart function, TCM clinical symptoms, and increase quality of life and exercise tolerance without evident toxic effects (Liu et al., 2015a, 2015b; Li, 2016). LHF has also been shown to block and reverse ventricular remodeling process to a certain extent in patients with heart failure after myocardial infarction (Xue et al., 2015). Moreover, LHF could also up-regulate the expression of the angiotensin converting enzyme 2 (ACE2) and down-regulate the expression of angiotensin II in rats with HF, thereby inhibiting ventricular remodeling (Xu et al., 2015). However, whether LHF is effective against myocardial fibrosis remains unknown.

In this study, we first investigated the antifibrotic efficacy of LHF in myocardial infarction (MI)-induced HF rats. We then determined the mechanisms behind the antifibrotic action of LHF by examining its effects on the gp130/JAK2/STAT3 pathway in CMs and its downstream signaling molecules.

2. Materials and methods

2.1. Materials

Perindopril (an ACE inhibitor) was purchased from Servier (Tianjin) Pharmaceutical Co., Ltd (China). Isoprenaline (ISO) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd (China). Antibodies against phospho-gp130, phospho-JAK2, and phospho-STAT3 were obtained from Cell Signaling Technology Inc. (Boston, Massachusetts, USA). Reverse transcriptase (RT) kits were purchased from Thermo Fisher Scientific Inc (Waltham, Massachusetts, USA). The cell counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from USCN Life Science Inc (Wuhan, China).

2.2. Preparation of Luhong formula

The formula to create one dose of Luhong is presented in Table 1. Chinese medicines were purchased from Shanghai Hua-Yu Chinese Herbs Co., Ltd. (Shanghai, China), including *Cervus nippon Temminck* (lot#20121022), *Carthamus tinctorius L.* (lot#20120603), *Cinnamomum cassia Presl* (lot#20120712), *Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao* (lot#20121209), *Codonopisis pilosula(Franch.) Nannf.* (lot#20110503), and *Lepidium apetalum Willd* (lot#20111004). All herbs were accredited by a pharmacologist, Professor Zhentao Wang, according to the Pharmacopoeia of the People's Republic of China (2010). Their voucher specimens were deposited at the Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

The extraction process for the LHF (lot#20130925) was as follows (Li et al.,2015): (1) 9 g of *Cervus nippon Temminck*, 9 g of *Carthamus tinctorius* L., 9 g of *Cinnamomum cassia Presl*, 30 g of *Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao*, 30 g of *Codonopisis pilosula(Franch.) Nannf.*, and 20 g of *Lepidium apetalum Willd* were added to 8 times amount of water, soaked for 30 min, decocted two times(each time with 30 min); (2) the decoctions were combined and dried by decompression at 60 ± 2 °C (1 g of the LHF granule contained 3.28 g of herbal mixtures); and (3) the dry extract was dissolved in water to make LHF (1.3 g extract/ml) for intragastric administration. The dry extract was dissolved in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) in vitro experimental. The final concentrations of LHF used in the cellular assays were: 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, 1000 μg/ml,5000 μg/ml and 10000 μg/ml.

For quality control, the fingerprint spectrum for LHF was performed by UHPLC-Q Exactive system (Thermo, San Jose, CA, USA) equipped with a quaternary gradient pump, an autosampler and high-resolution mass spectrometry detector. The components were eluted with a gradient system consisting of acetonitrile (A) and water (B) in gradient (time, min/B%: 0/95, 25/5). The spectra data were recorded in the *m/z* range of 80–1000. Mass spectra were acquired in both negative and positive modes with ion spray voltageat 3.5 kV, capillary temperature at 320 °C, auxiliary gas heater temperature at 300 °C, sheath gas (nitrogen) flow at 35 AU, auxiliary gas (nitrogen) flow at 10 AU. The chromatographic column was ACQUITY UPLC HSS T3 (2.1 mm×100 mm, 1.8 μm). The mobile phase flow rate was 0.3 ml/min and column temperature was maintained at 40 °C. Otherwise, the contents of cinnamic acid, codonolactone, formononetin, calycosin, hydroxysafflor yellow A, quercetin-3-O-*b*-D-glucose-7-O-*b*-D-gentiobioside and astragaloside were detected by UPLC-MS method, and were 32.61 mg/g, 0.47 mg/g, 4.20 mg/g, 19.60 mg/g, 52.76 mg/g, 16.81 mg/g and 18.80 mg/g in the extracts respectively (Fig. 1).