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Original Article

Qiliqiangxin ameliorates cardiac dysfunction in heart failure rats post myocardial infarction via activation of HIF/VEGF pathway

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Abstract: Objective: To evaluate the effects of superfine Qiliqiangxin powder (QL) on cardiac function and microangiogenesis in heart failure (HF) rats post myocardial infarction, and to explore the related mechanisms. Methods: Myocardial infarction was induced by ligation of the left anterior descending coronary artery in male Sprague-Dawley (SD) rats. Two weeks after MI, rats with a left ventricular ejection fraction < 50% were divided into HF+NS (saline, orally) group, HF+QL (0.6 g/kg/day, via intragastric administration) group and HF+QL (0.6 g/kg/day, via intragastric administration)+HIF (2-MeOE2, HIF-1α inhibitor, 1 mmol/kg/week, via intraperitoneal injection) group, respectively, rats in sham group underwent similar surgical procedure without coronary ligation (n=6 for each group). Six weeks later, cardiac structure and function were examined by echocardiography, microangiogenesis was assessed by immunostaining CD31, and myocardial protein and mRNA express ions of p53, HIF-1α and VEGF were determined by Western blotting and RT-PCR, respectively. Results: Compared with sham group rats, heart failure rats exhibited enlarged cardiac chamber, reduced left ventricular ejection fraction and rare microvascular density (all $p < 0.05$). QL treatment ameliorated cardiac remodeling, improved cardiac function and promoted microangiogenesis around infarct border zone. Moreover, myocardial p53 expression downregulated and HIF-1α and VEGF expressions upregulated post QL treatment, above beneficial effects were partly counteracted by cotreatment with HIF-1α inhibitor 2-MeOE2. Conclusions: Our results showed that QL treatment could significantly improve cardiac function and enhance microangiogenesis in HF rats post myocardial infarction via modulating HIF-1α/VEGF pathway.

Keywords: Qiliqiangxin, myocardial infarction, heart failure, microangiogenesis

Introduction

Heart failure (HF) is the final stage of a variety of cardiovascular diseases, and myocardial infarction (MI) is one of the most prevalent causes of heart failure. Despite advances in reperfusion strategies and optimized pharmacological therapies, myocardial remodeling is still observed in a substantial proportion of patients and progression to HF can still occur in 30% of patients suffering MI [1]. These data suggest current therapies are inadequate to prevent myocardial remodeling and transition to heart failure in patients undergoing myocardial infarction. Hence, there is a need for comprehensive understanding of the mechanisms of cardiac remodeling after MI.

When myocardial infarction occurs, a spontaneous angiogenic response was initiated to reestablish myocardial blood flow, however, molecular studies showed that the blood supply of myocardium was mainly from a large amount of blood capillary, and the pathophysiological process of blood capillary was significantly different from that of the large blood vessel. Thus the protective response is usually insufficient to restore the physiological level of perfusion [2]. Under the stimulation of ischemia and hypoxia, blood capillary would secrete a large number of cytokines, such as hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF), inducing the regeneration of microvessels around infarct area, thus reducing the degree of myocardial necrosis [3]. Plenty of studies demonstrate that angiogenesis abnormality plays important roles in the pathogenesis of heart failure and targeting angiogenesis is an effective option for the treatment of heart failure [4-7], hence, therapeutic

induction of angiogenesis is recognized to be a valid approach to restore the supply of oxygen to the ischemic regions and improve compromised cardiac function.

In recent years, Chinese traditional compound, Qiliqiangxin (QL), has been proven to exert multiple physiological functions for the prevention and treatment of heart failure [8-10]. Animal studies demonstrated QL capsule could reduce the excessive neuroendocrine activation in heart failure through diuretic and vasodilator effects [11]. To our knowledge, there is no literature concerning the therapeutic effects and mechanisms through angiogenesis of QL in chronic heart failure post MI. The present study aims to establish the role of QL in ameliorating myocardial remodeling, improving cardiac function and enhancing HIF-1α and VEGF production after myocardial infarction.

Materials and methods

Animals and drugs

Male Sprague-Dawley rats, weighing 200-250 g, were purchased from Experimental Animal Center of Shanghai Medical College, Fudan University. All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996) and approved by Animal Care Committee of Zhongshan Hospital, Fudan University (DF-384). Superfine QL powder was provided by Yiling Pharmaceutical Corporation (Shijiazhuang, China); 2-MeOE2 (2-Methoxyestradiol, which is a special inhibitor suppressing HIF-1α protein levels and its transcriptional activity) was purchased from Sigma-Aldrich; Ketamine hydrochloride purchased from Jiangsu Hengrui Medicine Co., Ltd. (Lot H32025255).

MI model and experimental groups

All rats were anaesthetized with intraperitoneally injection of ketamine hydrochloride (50 mg/kg) and the chest was opened by left thoracotomy at a fourth or fifth intercostal space. A single 5-0 nylon suture was passed under the left anterior descending coronary artery (LAD) which was 2.0~3.0 mm below the left coronary artery starting point, LAD was permanently ligated in MI rats and rats in sham group received the same procedure without LAD ligation. Two weeks after operation, the survived rats were subjected to echocardiographic examination and MI rats with left ventricular ejection fraction < 50% were chosen for subsequent studies. After echocardiography, MI rats were randomly divided into 3 groups (n=6 for each group): HF+NS group (EF=41.69%); HF+QL group (EF=40.08%) and HF+QL+HIF group (EF=39.46%). QL (0.6 g/kg/d) was administered orally for HF+QL group, 2-MeOE2 (1 mmol/kg) was injected intraperitoneally once a week and 0.6 g/kg/d QL was orally fed for 6 weeks for HF+QL+HIF group. Equal volume of saline was orally administered for sham group and HF+NS group.

Echocardiographic examinations

Cardiac structure and function were evaluated by echocardiography with a 17.5-MHz high-frequency scan head (Vevo770; VisualSonics Inc., Toronto, ON, Canada) 2 weeks after surgical procedure and at the end of the study. LV interior dimension of end diastole and systole (LVIDd and LVIDs), LV posterior wall thickness at diastole and systole (LVPWd and LVPWs), LV ejection fraction (LVEF) and fractional shortening (FS) were determined. All parameters were obtained from 5 consecutive cardiac cycles and the average values were calculated.

Immunohistochemical and serological analysis

After 6 weeks of intervention and final echocardiography examinations, deep anesthesia was achieved by additional intraperitoneal administration of ketamine hydrochloride. An incision was made in the abdomen, blood was sampled from the inferior vena cava, commercially available enzyme linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp, USA) were used to determine the serum NT-proBNP levels among groups according to the manufacturer's instructions. The whole heart was then harvested and weighed immediately to calculate heart weight/body weight ratio. Middle sections of left ventricular short axis around papillary muscle level were then fixed in 10% formalin, embedded in paraffin, and cut into 5-μm thick sections and stained with either Sirius Red or CD31 antibody (Boster Biotech Company, Wuhan, China). Digital photographs were thereafter taken and analyzed using a high-resolution digital image analysis system (Qwin V3,

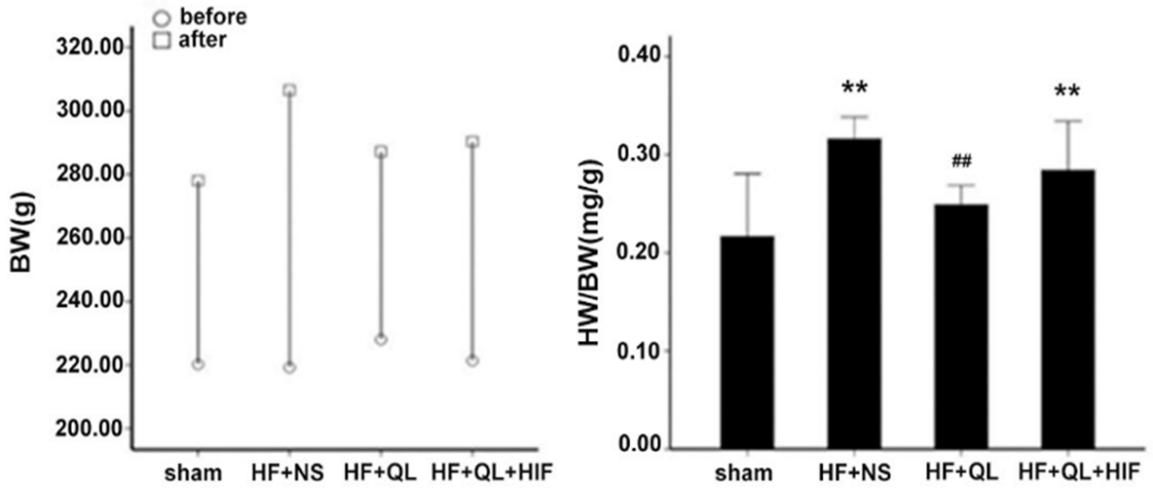


Figure 1. Comparisons of body weight (BW) and heart weight to body weight (HW/BW) ratio among groups. *p < 0.05 versus sham group, **p < 0.01 versus sham group; #p < 0.05 versus HF+NS group, ##p < 0.01 versus HF+NS group.

Leica, Germany). The percentage of fibrosis was determined on Sirius Red-stained sections and presented as percent of the fibrosis area to that of the whole LV. CD31 positive endothelial cells including single endothelial cells, cluster and tubular structures were observed and newborn capillary density was calculated. The remaining tissue samples were flash-frozen in liquid nitrogen and stored at -80°C for further studies.

Real-time quantitative reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from around the infarct border tissues using Trizol Reagent (Invitrogen) and cDNA was synthesized according to the manufacturer's instructions. According to the gene sequence provided by Genbank, the primers were designed using design primer software as follows: GAPDH forward primer 5'-GGAAAGCTGTGGCGTGATGG-3' and reverse primer 5'-GTAGGCCATGAGGTCCACCA-3'; p53 forward primer 5'-TGGCTCCTCCCCAACATCTTATC-3' and reverse primer 5'-CTTCCTCTGTCCGACGGTCTCTC-3'; HIF-1α forward primer 5'-TGACTGTGCACCTACTATGTCACTT-3' and reverse primer 5'-GGTCAGCTGTGGTAATCCACTC-3'. The PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 34 s. Relative changes in expression were calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cardiac tissues were lysed using RIPA buffer containing a protease inhibitor cocktail. The protein concentration was determined by BCA method. Then equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA for 1 hour, and then probed with antibodies for HIF-1α (KangChen Bio-tech, Shanghai, China) and VEGF (Santa Cruz, Dallas, TX, USA) overnight at 4°C. On the next day, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours. Finally the immunocomplexes were visualized using ECL Plus (Thermo Scientific). Bands were scanned and processed by densitometry on a gel documentation system with Bio-Rad Image Lab Software 3.0.

Statistical analysis

Values were expressed as means ± SD. Differences among groups were assessed by one-way analysis of variance with a Student-Newman-Keuls test for post hoc analysis. Differences before versus after treatment were assessed by paired t-test or differential analysis. P < 0.05 was considered statistically significant. All analyses were carried out with SPSS16.0 statistical package for Windows (SPSS Inc, Chicago, IL).

Results

QL treatment reduced body weight increment and heart weight to body weight ratio

A total of 32 rats were used in this study, of which 6 rats were randomly assigned to sham

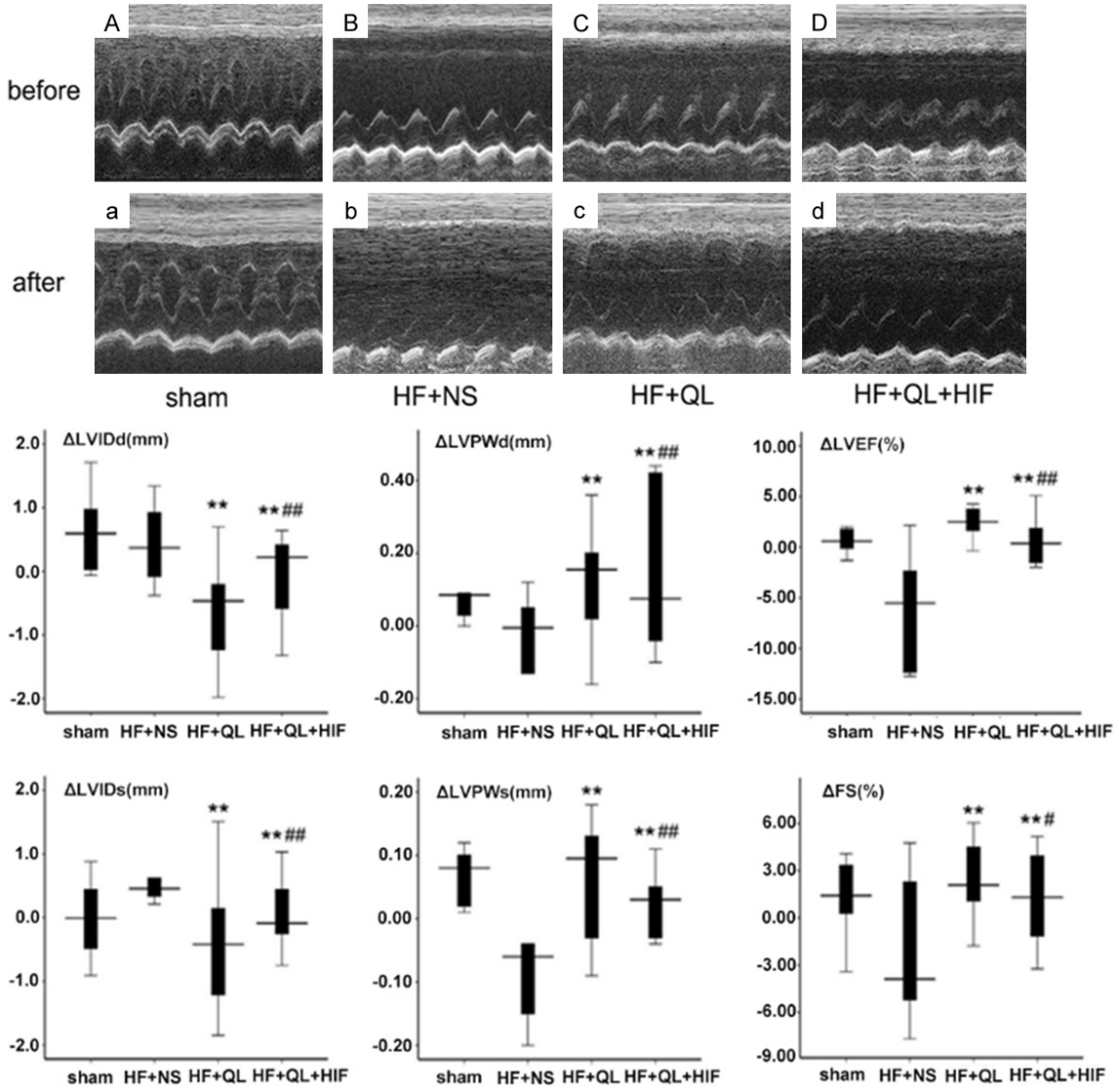


Figure 2. Comparisons of cardiac structure and function by echocardiography with differential analyses before and after treatment among different groups. A-D. Stand for sham group, HF+NS group, HF+QL group and HF+QL+HIF group before intervention, respectively; a-d. Stand for sham group, HF+NS group, HF+QL group and HF+QL+HIF group after intervention, respectively. *$p < 0.05$ versus HF+NS group, **$p < 0.01$ versus HF+NS group; #$p < 0.05$ versus HF+QL group, ##$p < 0.01$ versus HF+QL group.

group, the rest rats received LAD ligation. Two weeks later, two rats died and one rat excluded from the subsequent study with LVEF higher above 50%. Five rats were sacrificed for histopathological studies and the remaining rats were divided into HF+NS group, HF+QL group and HF+QL+HIF group and survived across the study.

Body weight was significantly higher in HF+NS group rats compared with sham group rats (306.50±14.18 g vs 278.00±14.45 g, $p < 0.01$). However, body weight increment was less higher in HF+QL group rats (287.17±8.80 g for HF+QL group vs 306.50±14.18 g for HF+NS group, $p < 0.01$) as well as in HF+QL+HIF group rats (290.33±14.77 g for HF+QL+HIF group vs 306.50±14.18 g for HF+NS group, $p < 0.01$) at the end of the study, similar results were found for heart weight to body weight ratio (**Figure 1**).

QL treatment alleviated left ventricular enlargement and delayed cardiac dysfunction

All heart failure rats exhibited enlargement of LV cavity compared to sham group rats.

Table 1. Cardiac structure and function before and after treatment among groups (means ± SD)

	sham		HF+NS		HF+QL		HF+QL+HIF	
	Before	After	Before	After	Before	After	Before	After
LVIDd (mm)	5.41±0.67	6.05±0.31**	7.88±0.48	8.31±0.92**	7.91±0.22	7.31±0.93**	7.90±0.48	7.83±0.72**
LVIDs (mm)	1.89±0.33	1.88±0.52	5.98±0.99	6.56±0.94**	6.13±0.21	5.75±1.18**	5.90±0.39	5.95±0.54*
LVPWd (mm)	1.43±0.23	1.49±0.22*	1.51±0.16	1.49±0.09*	1.49±0.09	1.61±0.16**	1.51±0.11	1.65±0.25**
LVPWs (mm)	1.57±0.14	1.64±0.17**	1.63±0.19	1.58±0.16**	1.63±0.08	1.69±0.14**	1.61±0.11	1.64±0.12**
LVEF (%)	83.31±2.41	83.89±2.67	41.69±6.67	35.63±5.02**	40.08±1.66	43.46±0.57**	39.46±4.37	41.16±6.04**
FS (%)	62.98±4.92	64.17±4.24	25.49±2.56	23.22±3.84*	24.61±2.23	26.94±0.85**	26.81±4.32	28.02±6.18**
NT-proBNP (pg/ml)	NA	141.65±7.03	NA	455.45±15.61	NA	299.87±10.36	NA	309.82±13.47

LVIDd, LV interior dimension of end diastole; LVIDs, LV interior dimension of end systole; LVPWd, LV posterior wall thickness at diastole; LVPWs, LV posterior wall thickness at systole; LVEF, LV ejection fraction; FS, fractional shortening; NA, not available. *$p < 0.05$ versus comparative group before intervention, **$p < 0.01$ versus comparative group before intervention.

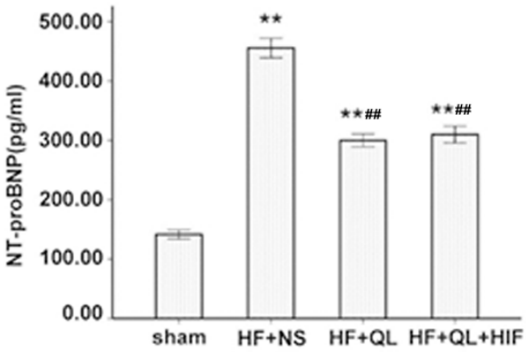


Figure 3. Plasma NT-proBNP levels among different groups. *$p < 0.05$ versus sham group, **$p < 0.01$ versus sham group; #$p < 0.05$ versus HF+NS group, ##$p < 0.01$ versus HF+NS group.

Treatment with QL significantly delayed cardiac expansion as evidenced by less increment of LVIDd and LVIDs with increased LVEF and FS (**Figure 2**). Meanwhile, NT-proBNP level significantly decreased in HF+QL group than that in HF+NS group, all these beneficial effects were partially abrogated by cotreatment with HIF-1α inhibitor (**Table 1**, **Figure 3**).

HF rats exhibited remarkable interstitial fibrosis around infarction area as shown in **Figure 4**, QL treatment reduced fibrosis which could be counteracted by HIF-1α inhibitor.

QL promoted microangiogenesis and inhibition of angiogenesis partly abrogated the beneficial effects of QL on heart

Microvascular angiogenesis assessed by CD31 staining showed that papillary density significantly decreased around infarct border zone in heart failure rats compared to that of sham group rats, QL significantly increased microvessel density, which was partly blocked after treatment of HIF-1α inhibitor (**Figure 5**).

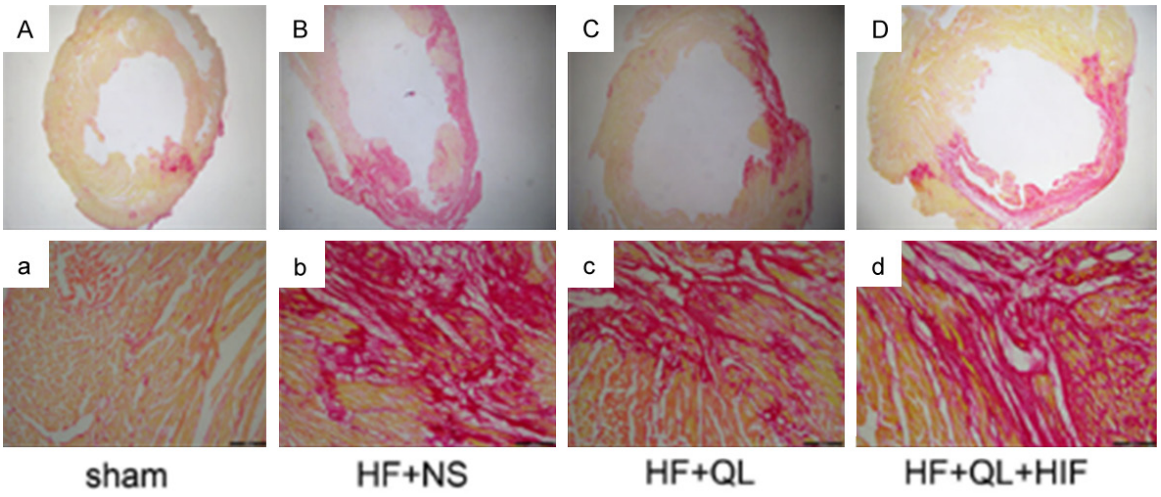
QL improved cardiac function by activating proangiogenic signalings

To explore whether angiogenesis was mainly involved in QL mediated cardiac function improvement, the principal proagniogenic molecules, such as p53, HIF-1α and VEGF, were determined. The results demonstrated that, compared to sham group or HF+NS group rats, QL treatment could downregulate p53 mRNA expression, upregulate HIF-1α mRNA expression, and increase HIF-1α and VEGF protein expressions; while inhibition of HIF-1α significantly downregulated HIF-1α and VEGF protein expressions (**Figure 6**).

Discussion

The present study demonstrated that QL treatment could significantly improve cardiac function, attenuate cardiac remodeling and promote microangiogenesis at infarct border zone in heart failure rats, these beneficial effects were largely related to activated myocardial HIF-1α/VEGF pathway.

Myocardial remodeling is a critical process underlying the progression to heart failure, and coronary microcirculation disturbance is an important pathological element contributing to myocardial remodeling, thus attenuation of myocardial remodeling is regarded as one of the most important aspects for improving compromised cardiac function [12, 13]. Myocardial microcirculation disturbance can lead to a series of cardiovascular events including abnormalities of blood hemodynamics, regulation disorders of neuroendocrines and cytokines, disturbances of endothelial system and energy metabolism [14]. It has been demonstrated in experimental models that stimulation of blood vessel growth leads to growth of



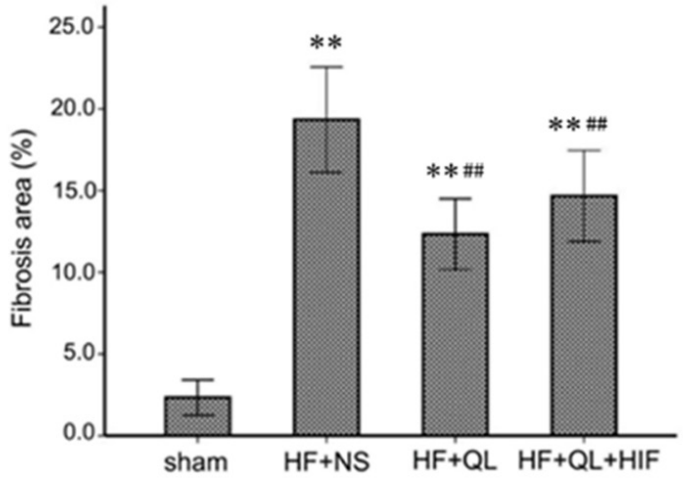


Figure 4. Histological observations of fibrosis by Sirius Red (upper) and percentages of fibrosis area (lower) among groups. A-D. Stands for sham group, HF+NS group, HF+QL group and HF+QL+HIF group with a magnification of 50 times, respectively. a-d. Stands for sham group, HF+NS group, HF+QL group and HF+QL+HIF group with a magnification of 200 times, respectively. *p < 0.05 versus sham group, **p < 0.01 versus sham group; #p < 0.05 versus HF+NS group, ##p < 0.01 versus HF+NS group.

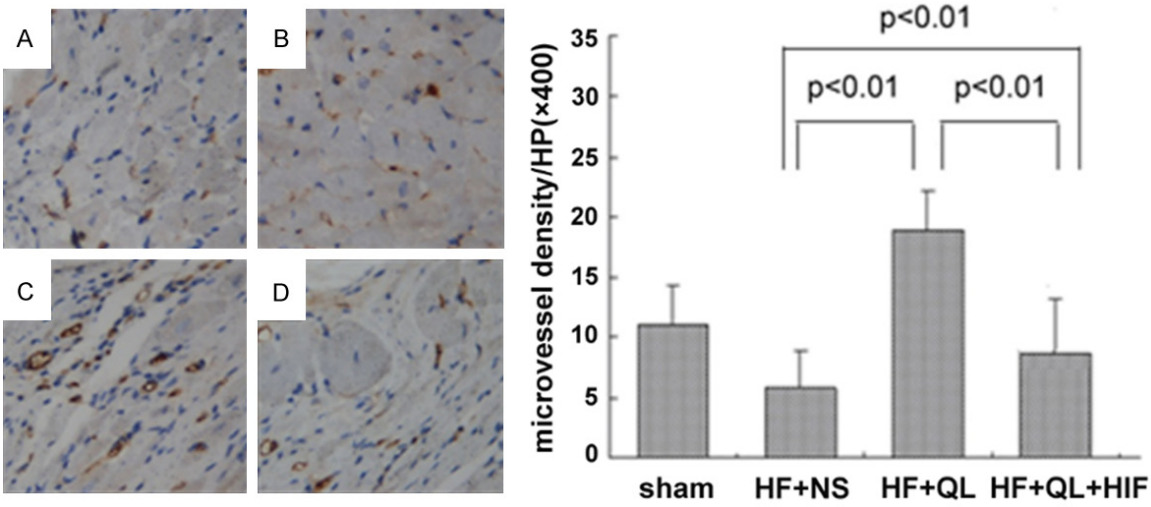


Figure 5. Infarct marginal zone microangiogenesis assessed by CD31 staining (left) and microvessel density (right). A-D. Stands for sham group, HF+NS group, HF+QL group and HF+QL+HIF group, respectively.

the whole vascular tree as well as improvement of ischemic tissue perfusion and muscle aerobic energy metabolism [15]. In the progression of ischemic heart disease, MI-induced angio-

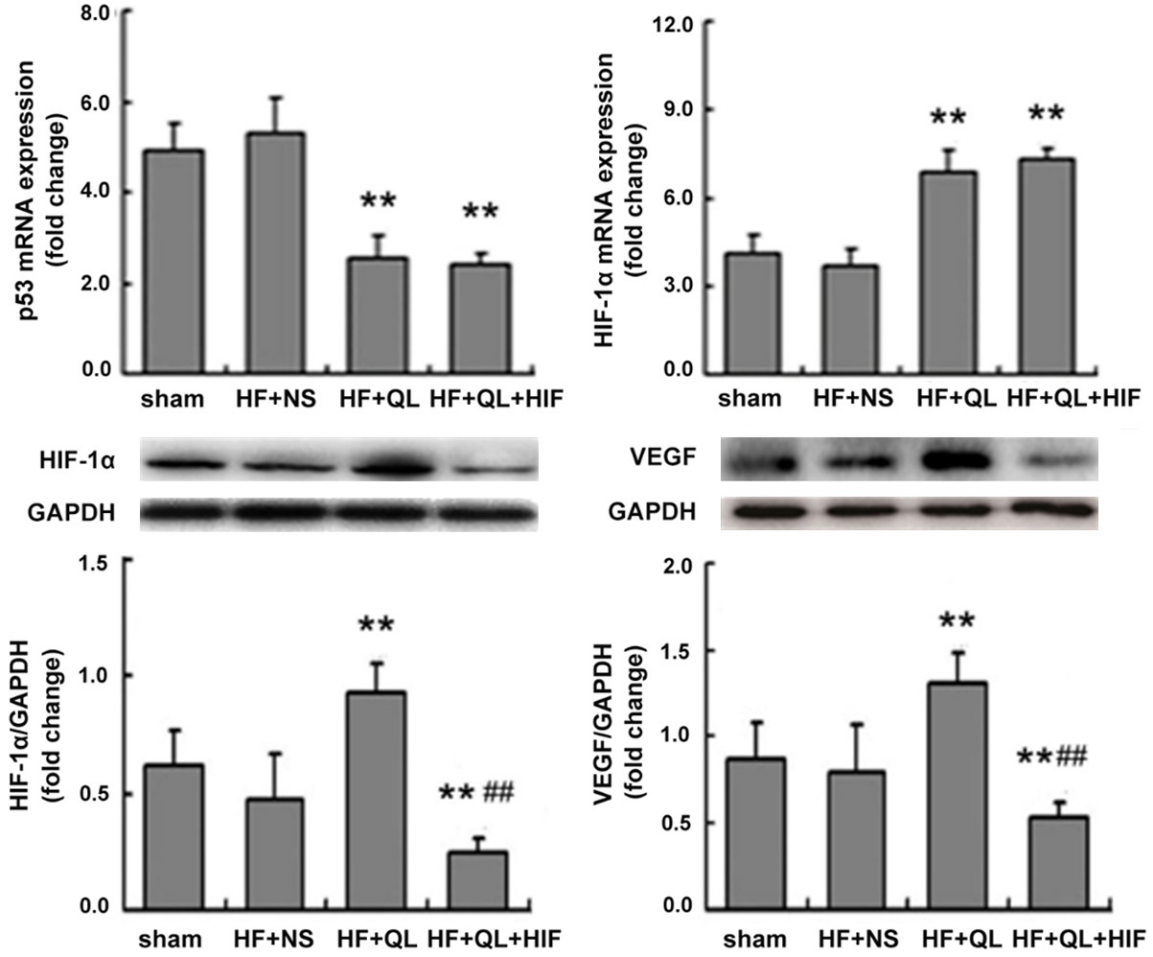


Figure 6. Changes of angiogenic signaling molecules among groups. *$p < 0.05$ versus HF+NS group, **$p < 0.01$ versus HF+NS group; #$p < 0.05$ versus HF+QL group, ##$p < 0.01$ versus HF+QL group.

genesis is a slow process and may only partially compensate for the blood supply to the heart [16]. Recent advancement in the field of angiogenesis in myocardial ischemia indicates that therapeutic angiogenesis may improve cardiac perfusion which in turn mitigates ischemic tissue necrosis, leading to reduced infarction size and improved left ventricular function [17].

Of proangiogenic factors, HIF-1α is a crucial nuclear transcription factor which has highly specificity and activity under hypoxic/ischemic conditions to play a core role in promoting angiogenesis as well as reducing reperfusion injury [18]. VEGF is a main downstream effector of HIF-1α which is important for cardiac remodeling and repair within the diseased myocardium. VEGF could promote the blood vessel regeneration and stromal deposition so as to guarantee the microcirculation establishment during the recovery process [19, 20]. In normal ventricular tissues, there is no HIF-lα and VEGF mRNA expression, but in the specimen under myocardial infarction advanced phase, HIF-1α and VEGF mRNA expression could be detected [21, 22]. The expression of HIF-1α and VEGF after myocardial infarction could promote the formation of new vessels to develop compensatory adaptation which stimulates collateral circulation in the ischemic heart [23]. In recent years, there has been an increased focus on the influence of traditional Chinese medicine (TCM) on angiogenesis such as QL [24].

QL is prepared from 11 Chinese herbs and the main active constituents are Astragali Radix and Aconite Root [25]. A recently published clinical trial proved the efficacy and safety of QL in chronic heart failure patients [26]. In line with previous studies [11, 27, 28], we demonstrated that QL could improve cardiac function in heart failure rats post myocardial infarction,

specifically QL enhanced microangiogenesis via upregulating myocardial HIF-1α and VEGF expression, while inhibiting HIF-1α partially abrogated the protective effects of QL. Plenty of evidence suggests that Astragali Radix and Aconite Root extract (such as Rg1 and calycosin) exhibit proangiogenic effects in vivo and in vitro in a HIF-1α and/or VEGF-dependent manner [29-33]. Although the present study could not provide which compound(s) in QL exerted the cardioprotective effects, our results did show that the beneficial effects of QL is largely related to HIF-1α and VEGF upregulation. Evidently, the results of our study have not been verified by HIF-1α gene transfection, nor involved in any specific downstream mechanism of HIF-1α and VEGF in promoting blood vessel regeneration. Thus, these problems still require further verification.

Acknowledgements

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