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Amelioration of cardiac dysfunction and ventricular remodeling after myocardial infarction by danhong injection are critically contributed by anti-TGF-β-mediated fibrosis and angiogenesis mechanisms

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ABSTRACT

Ethnopharmacological relevance: Danhong injection (DHI) is a standardized product extracted from Radix et Rhizoma Salviae Miltiorrhizae and Flos Carthami , which has been long applied mainly used to treat ischemic encephalopathy and cardiac diseases including myocardial infarction and angina in clinical practice.

Aim of the study: Aim of this study was to investigate the salutary effects of DHI by slowing ventricular remodeling and improving cardiac function after myocardial infarction (MI) in rats.

Materials and methods: In this study, Male Sprague-Dawley rats were subjected to ligation on left anterior descending coronary artery to establish MI models and valsartan was selected as positive control. Cardiac function examination was conducted at the 1st, 3rd, 7th, 14th and the 28th days after LAD. Haematoxylin and Eosin (HE) staining and Masson staining were conducted to observe cardiac pathology and morphological changes levels of VEGF, TGF-β, MMP-2, and MMP-9 in the myocardial tissue were determined in gene and protein expressions.

Results: After 3 days post-treatment and thereafter, EF and FS in DHI group were greater than that of model group ($p < 0.05$). Compared with the MI group, ratio of infarct was markedly decreased in treated-DHI group($p < 0.05$). TGF-β1 protein and fibrosis-related proteins MMP-2 and MMP-9 were up-regulated after MI, and they were significantly suppressed by the administration of DHI($p < 0.05$ and $p < 0.01$, respectively). Moreover, DHI improved the mRNA expression of VEGF and increased the blood vessel density of myocardial infarct border zone. DHI decreased the expression of cell apoptosis protein of caspase-3 and increased the anti-apoptotic protein, bcl-2.

Conclusions: We provided direct evidences that DHI improves cardiac remodeling and preserves ventricular function post-MI in rats. DHI conferred cardio-protection in rats with MI via anti-myocardial apoptosis, angiogenesis, reduction of myocardial fibrosis and many other aspects of joint actions.

1. Introduction

Myocardial infarction (MI) is a common clinical acute critical illness which is crucial in cardiovascular disease mortalities (Yellon and Hausenloy, 2007). MI induces global changes of ventricular architecture, called post-MI ventricular remodeling (Pfeffer et al., 1995). Left ventricular (LV) remodeling is the process by which ventricular size, shape, and function are regulated by mechanical, neuro-hormonal, and genetic factors (Pfeffer and Braunwald, 1990; Rouleau et al., 1993). Early changes in LV architecture after MI are adaptive responses of the heart during acute loss of functional myocardium to initially preserve cardiac performance. In actual fact, cardiac remodeling was deemed as a determinant of clinical course of heart failure. It comprises structural and functional changes at

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different levels, such as molecular, cellular, tissue and whole-organ levels (Cohn et al., 2000; Pfeffer and Braunwald, 1990; Sutton and Sharpe, 2000). It is well established that hemodynamic load and neurohormonal activation are major factors that have influences on cardiac remodeling (Vantrimpont et al., 1998). However, once these processes develop after large MI, the infarcted heart progressively dilates and accelerates the deterioration of ventricular dysfunction that eventually results in heart failure (Pfeffer et al., 1992, 1995). LV remodeling after MI is a complex process that is sustainable for weeks or months until the distending forces are counterbalanced by the tensile strength of the collagen scar. This process consists of acute phase LV dilatation due to lengthening of infarct and non-infarcted myocardium and subsequent development of myocyte hypertrophy and interstitial fibrosis in the residual myocardium (Pfeffer and Braunwald, 1990; Warren et al., 1988; White et al., 1987). Consequently, LV function deteriorates and is usually associated with high rate of mortality. Thus, effective therapeutic strategy for the prevention of cardiac dysfunction in patients with myocardium ischemia is imperative.

DHI is a standardized product extracted from Rhizoma Salviae Miltiorrhizae (Salvia miltiorrhiza Bge., Labiatae, Danshen in Chinese) and Flos Carthami (Carthamus tinctorius L, Compositae, Honghua in Chinese). with the raw material dose ratio of 3:1 (Tai et al., 2005). DHI was awarded the first Chinese medicine patent gold medal in 2010 (Guan et al., 2013). Rhizoma Salviae Miltiorrhizae and Flos Carthami are two well-known traditional medicines in China. Both of them have potent therapeutic effects on “activating blood circulation and dissipating blood stasis, dredging meridians and collaterals” according to the traditional Chinese Medical theory (Wan et al., 2013). Since the former is ‘cold’, while the latter is ‘warm’ in nature, these two herbs are widely applied together to achieve synergistic therapeutic efficacy on cardiovascular diseases, while reducing the side effects in clinical decoctions (Liu and Zhang, 2011; Zhan et al., 2008). The main pharmacological active components of DHI include; phenolic acids, diterpenes and flavonoids, for example, salvianolic acid B, danshensu, tanshinoneIIA and safflor yellow (Huang et al., 2005; Wang, 2006; Wang et al., 2010). These components have been hypothesized to be the main bioactive ingredients of DHI and used as index components for quality control according to the standard of State Food and Drug Administration of China (2002). High-performance liquid chromatography (HPLC) fingerprinting of DHI also has been widely performed(Dissection of Mechanisms of a Chinese Medicinal Formula: Danhong Injection Therapy for Myocardial Ischemia/Reperfusion Injury In Vivo and In Vitro). Pharmacological studies demonstrate that DHI has been long applied mainly used to treat ischemic encephalopathy and cardiac diseases including myocardial infarction and angina in clinical practice (He et al., 2012; Sun et al., 2009). DHI is one of the best-selling Chinese medicine injections to treat myocardial infarction, atherosclerosis and coronary heart disease. Although DHI has been extensively used to treat cardiovascular disease, it remains unknown whether DHI has an effect of delaying the ventricular remodeling after myocardial infarction. In this study, we investigated the pharmacological effects of DHI on LV remodeling during the 2-week and 4-week periods after coronary ligation.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (SD) rats [220–250 g body weight] were purchased from Beijing HFK Bioscience. Co., Ltd (Certificate no.: SCXK Jing 2009-0004), kept in a 12-h dark/light cycle in a temperature 22 ± 2 °C and humidity 40 ± 5%, and fed on standard laboratory diet and water ad libitum. This study was carried out in strict accordance with the recommendations in the Guidance Suggestions for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of China. The experimental procedures followed the European Union (EU) adopted Directive 2010/63/EU, and all procedures were performed to reduce the number of animals and suffering during the experiments. All animals were handled according to the guidelines of Tianjin University of Traditional Chinese Medicine (TCM) Animal Research Committee (TCM-LAEC2015026).

2.2. Drugs and reagents

DHI (Country Medicine Accurate Character Number: Z20026866) was obtained from Heze Buchang Pharmaceutical Co., Ltd. of China. According to the statement of National Drugs Surveillance administrative bureau standard, The quality control standard of DHI is that the total amount of protocatechuic aldehyde (molecular formula is C7H6O3) and danshensu (molecular formula is C9H10O5) should not be lower than 0.5 mg analyzed by high performance liquid chromatography (HPLC) as a reference in 1 mL injection. Simultaneously, the total flavonoids determined by visible spectrophotometry should not be lower than 5.0 mg/mL against rutin (molecular formula: C27H30O16)(He et al., 2012). The quality control of DHI was performing by an established method in our lab (Mao et al., 2016). We converted a commonly used dosage of DHI in clinical practice as the chosen dose 0.76 mL/kg in the experiment. Valsartan (Batch number: X1651) was purchased from Beijing Novartis Pharma Co., Ltd. (Beijing, China). Valsartan was dissolved in saline to make a solution at concentrations of 1 mg/mL and the dosage is 10 mL/kg for experiments.

Chloral hydrate (Batch number: Q/12HB 4218-2009) was purchased from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China), freshly prepared to 5% solution with saline before experiment. Rabbit-anti-rat monoclonal antibodies for Bcl-2 and Caspase-3 and goat-anti-rabbit polyclonal antibodies were purchased from Uscn Life Science Inc. (Wuhan, China). The mouse-derived antibody for α-sarcomeric actin (α-SARC) was purchased from Sigma Chemical Company (USA). Vascular endothelial growth factor (VEGF) rabbit anti-goat antibody was purchased from R & D. Antibodies for transforming growth factor (TGF)-β1, Matrix metalloproteinases-2 (MMP2) and MMP-9 were from Abcam Inc. (USA).

2.3. Animal model of myocardial infarction and drug administration

For MI model preparation, rats were subjected to ligation on left anterior descending coronary artery (LAD) according to the previous studies (Pfeffer et al., 1979). Anesthesia was achieved by an intraperitoneal injection of 5% chloral hydrate (300 mg/kg). Skin preparation was completed, and rats were fixed on an appropriate board for surgery. The rat was intubated with a catheter through the mouth while the trachea was exposed and was connected to the ventilator (Ventilator parameters: Respiratory rate 60; Respiration ratio 1:1; Tidal volume 1.5 mL). After completion of the preparatory work, the left 3th and 4th lateral intercostal was opened to accomplish ligation at the left anterior-descending coronary artery (LAD) with a 6-0 polypropylene suture. The chest was then closed and rats were placed on a hot plate. The procedure for sham group was totally the same except without ligating the suture.

The overall mortality of rats that underwent induction of coronary ligation during the entire experimental period was 20–25%. The majority of death occurred during or after coronary ligation surgery, probably because of acute pump failure or lethal arrhythmias.

After an echocardiographic evaluation following the surgery at one day, rats were randomized into four groups according to the echocardiographic data (38% < left ventricular ejection fraction < 50%) and were administered immediately. Among them, sham and coronary ligature group were given normal saline (intramuscular); and drug treatment groups were given valsartan (intragastric) or DHI (intramuscular), and continuous administration lasted 28 days. After 28 days of administration, the mortality rates of the sham, coronary

ligature, valsartan and DHI group were 0%, 31%, 20% and 24%, respectively. Number of animals in each group for determination of each parameter (See Table 1 for detail).

2.4. Echocardiographic and hemodynamic assessment of left ventricular function

Echocardiography was operated by an observer blinded to the experiment, 6 times of measurements were taken referring to the surgery date : at 1 day before and 1 day, 3 day, 7 day, 14 day, 28 day after the date. Short-axis view was obtained using a Vevo 2100 Ultra-high resolution small animal ultrasound imaging system in real time (Visual Sonics Vevo 2100, Canada) with a 16/20-Hz echocardiographic transducer (MS250, model C5). Left ventricular end-systolic, end-diastolic diameters and some other indicators were calculated as indicators of function and remodeling: left ventricular internal diameter (diastole, LVIDd), left ventricular posterior wall (diastole, LVPWd), and left ventricular internal diameter (systole, LVIDs). These measurements were used to determine left ventricular ejection fraction (EF), which is considered as an important indicator of left ventricular systolic function. All data were analyzed off-line at the end of the study with software resident on the ultrasound system (Chen et al., 2015).

After 4 weeks of administration, rats were anesthetized with chloral hydrate (5%, peritoneal injection, 300 mg/kg for maintenance during surgery), and the cannulation was done to the left ventricle. Briefly, the right common carotid artery was dissected and separated from the connective tissue. A catheter was inserted into the left ventricle through the carotid artery. Hemodynamic parameters were recorded by bio-function experiment system MP100-CE (BIOPAC Systems, Inc., Santa Barbara, CA, USA). Heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), left ventricular maximum upstroke velocity (+dp/dtmax), and left ventricular maximum descent velocity (−dp/dt) were measured at baseline.

Adequacy of anesthesia was controlled by monitoring corneal reflex and the lack of response to toe-pinching. Hearts were excised from rats for further investigation after terminal anesthesia by i.p. injection of 5% chloral hydrate at dose 300 mg/kg and heparin (100U). Euthanasia was performed by excessive inhalation of isoflurane. Death was monitored by the cardiac activity and respiration.

2.5. Histopathological examination

Myocyte size and interstitial fibrosis were shown by pathological section. After the end of the cardiac ultrasound, rats were sacrificed under anesthesia. The heart was removed, fixed in 4% paraformaldehyde (PFA) solution for more than 48 h, and further prepared for paraffin sectioning. For infarct size calculation, 5-μm thick sections were cut from each segment and stained with hematoxylin eosin (H & E) and Masson trichrome. In order to observe cardiac cell morphology, the pathological sections were observed under microscope at 400× amplified. Moreover, we got the whole outline of left ventricular. Infarct size measurements were performed by midline length measurement (Takagawa et al., 2007). In this way, the proportion of infarction was measured with the auxiliary of Image J.

2.6. Relationship network analysis by IPA

The relationship between apoptosis, fibrosis and angiogenesis related to MI was searched in the Ingenuity Pathways Analysis system (IPA, http://www.ingenuity.com) that enabled the discovery visualization and exploration of molecular interaction in order to identify the biological mechanisms, pathways and functions most relevant to the experimental datasets, genes, or proteins of interest (Niu et al., 2015). We search for molecules correlation with heart from published literature and other databases in IPA software with the key word of apoptosis, fibrosis and angiogenesis. ‘Diseases and functions’ module was utilized to analyze the shared and key molecules which can regulate the apoptosis, fibrosis and angiogenesis function, and establish a relationship network. 58 useful molecules related the three functions were obtained from the network, all of them we selected have a relationship with two or three functions. “Path designer” module was launched to modify the network.

2.7. Quantitative real-time PCR

Total RNA was isolated from LV samples (non-infarcted LV myocardium around infarcted part) with TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA samples (1 μg) were reverse transcribed with oligo (dT) primers using Superscript II (Roche). Quantification of cardiac gene expression was determined by real-time polymerase chain reaction with Bio-Rad CFX system. Published primer and probe sequences (Table 2) were used to amplify and detect VEGF, MMP-2, MMP-9, TGF-β and GAPDH.

2.8. Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed with standard protocols on the basis of H & E staining. The presence of endothelial cell was determined using VEGF (AF564, R & D) staining. Cardiomyocytes were marked with sarcomeric actin (α-SARC) (A2172, Sigma) was detected using anti-smooth muscle actin antibody (A5228, Sigma) while DAPI (70317525, Roche) marked nucleus. Apoptosis was determined by expression of cleaved caspase-3 (PAA626Ra81, USCN) and bcl-2 (PAA778Ra81, USCN). Fluorescence images were captured by use of the OLYMPUS DP71 inverted fluorescence microscopy.

Table 1
Number of animals for different experimental groups and various parameters at 28 day after administration.

	Sham group	Coronary ligature group	Valsartan group	DHI group	Total
Echocardiographic and hemodynamic	8	8	8	8	32
HE staining	3	3	3	3	12
Masson and myocardial infarct size	4	4	4	4	16
Quantitative real-time PCR	3	3	3	3	12
Western blot analysis	4	4	4	4	16
Immunohistochemistry and immunofluorescence	3	3	3	3	12
Total	25	25	25	25	100

Table 2
Primers sequences used for real-time PCR.

mRNA (rat)		Sequence
VEGF	Forward	5′GTCCTCACTTGGATCCCGACA3′
	Reverse	5′CCTGGCAGGCAAACAGACTTC3′
MMP-2	Forward	5′TTTGCTCGGGCCTTAAAAGTAT3′
	Reverse	5′CACGCTCTTCAGACTTTGGTTCT3′
MMP-9	Forward	5′GTGCCCTGGAACTCACACAAC3′
	Reverse	5′CCAGAAGTATTTGTCATGGCAGAA3′
TGF-β	Forward	5′AGGACCTGGGTTGGAAGTGG3′
	Reverse	5′AGTTGGCATGGTAGCCCTTG3′
GAPDH	Forward	5′ATGACTCTACCCACGGCAAG3′
	Reverse	5′CTGGAAGATGGTGATGGGTT3′

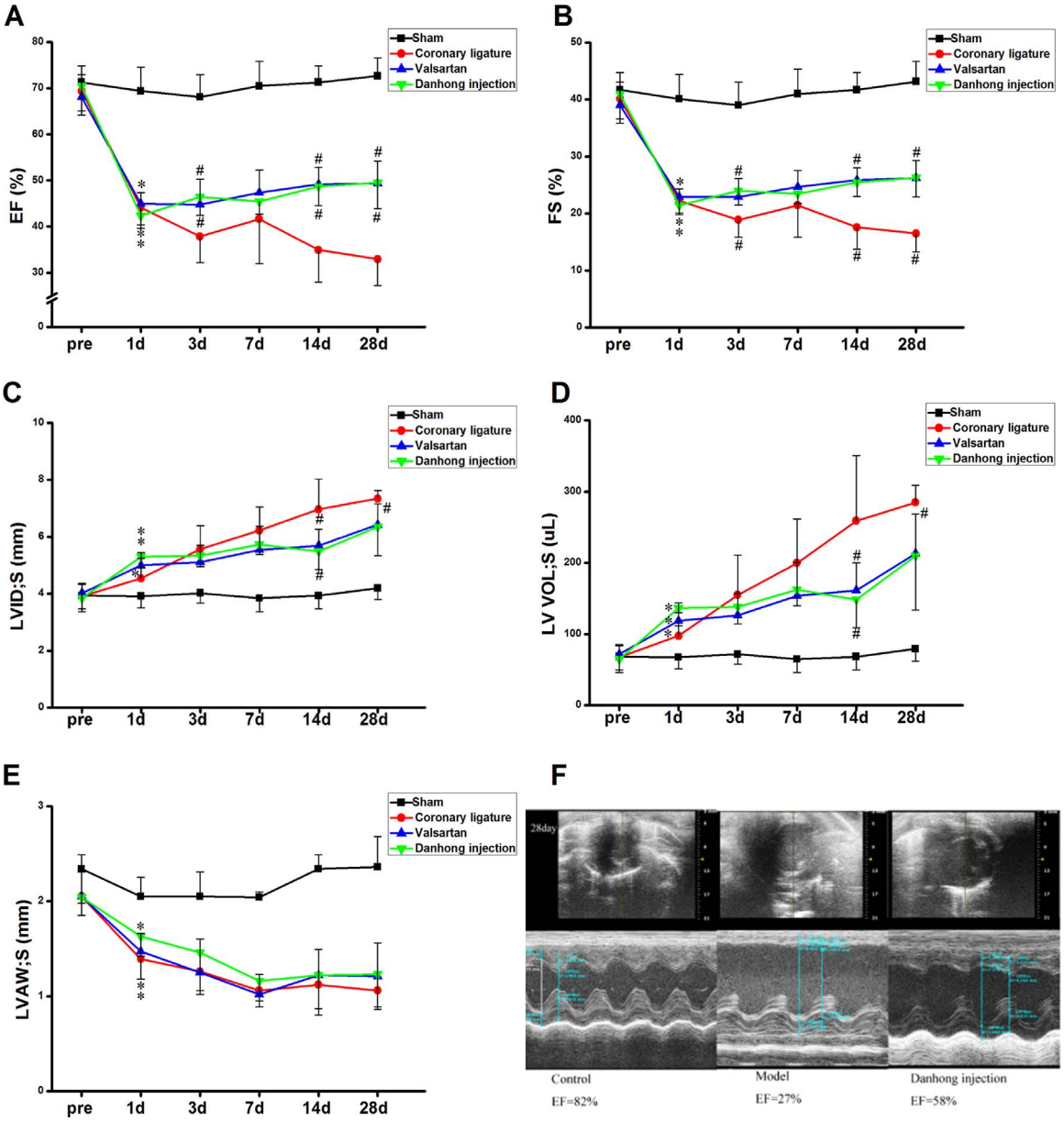

Fig. 1. Effect of DHI on rat cardiac function. Presented are the time courses of LVEF: LV ejection fraction (A), LVFS: LV fractional shortening (B), LVIDs: LV end-systolic dimension (C), LVVOL;s: LV systolic volume (D), LVAWs: LV end-systolic anterior wall (E); (n=8, each group). (F) Representative echocardiographic images (*m*-mode) in different groups. All values are means ± SD; $^{*}p < 0.05$ versus sham group; $^{\#}p < 0.05$ versus coronary ligature group.

2.9. Western blot analysis

Myocardial tissues were cut from the infarct border zone. The whole protein was extracted and the concentration was determined with a Bicinchoninic acid (BCA) protein assay kit. Equal amount of proteins (30 μg) underwent SDS-PAGE (8%) and were transferred to polyvinylidene difluoride membranes. The membranes with target proteins were cut and incubated with the primary antibodies and rinsed three times, 5 min each, and then incubated with secondary antibody for 2 h at room temperature. Blots were visualized with use of chemiluminescence HRP substrate (Millipore Corporation, Billerica, MA 01821 USA). The densities of bands were quantified by scanning densitometry in the X-film using a multifunctional imaging analysis system (VersaDoc MP 5000; Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.10. Statistical methods

All data were expressed as mean ± SD. Multiple comparisons were evaluated by one-way ANOVA. Statistical analysis was performed using the SPSS17.0 statistic program. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Progressive LV dilatation and impaired cardiac function in the 28 days after MI

All animals had similar baseline measurements of cardiac function before surgery. Echocardiographic analysis revealed that EF, FS, LVAW, LVID, and LVVOL were similar in the four groups 1 day after coronary ligature. And compared with sham group, there was a significant difference in the other three groups ($p < 0.05$). After 3 days post-treatment and thereafter, EF and FS were greater in DHI and valsartan group compared to coronary ligature group (Fig. 1A and B), but only in 3th, 14th and 28th day the difference was statistically significant ($p < 0.05$). Compared with Sham group, the coronary

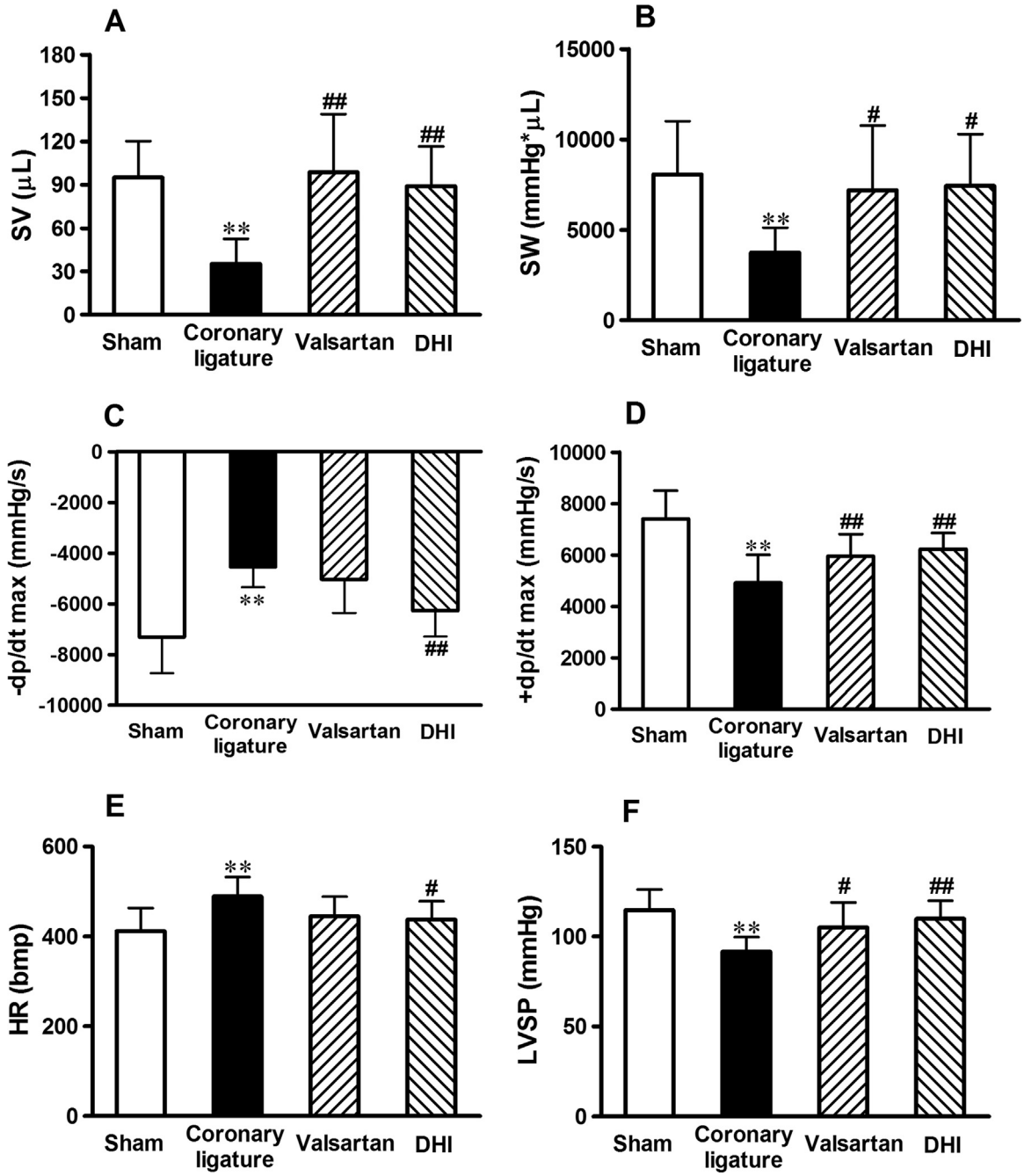

Fig. 2. Hemodynamic assessment was carried out on the left ventricle through right carotid artery to evaluate the role of DHI for MI. (A–E) Quantitative assessment of hemodynamic on cardiac function based on stroke volume (SV) (A) and stroke work (SW) (B), left ventricular maximum descent velocity (−dp/dtmax) (C), left ventricular maximum upstroke velocity (+dp/dtmax) (D), heart rate (HR) (E), and left ventricular systolic pressure(LVSP)(F) (n=8, each group). Data are expressed as means ± S.D. $^{**}p < 0.01$ compared with sham group; $^{\#}p < 0.05$ and $^{\#}p < 0.01$ compared with coronary ligature group.

ligature group had a significant increase in both LVID and LVVOL , which was attenuated apparently by treatment with DHI and Valsartan in 14 and 28 days after surgery ($p < 0.05$) (Fig. 1C and D). Likewise, the extent of regional wall motion abnormalities was improved in the treatment groups, but no significant difference between the groups for LVAW (Fig. 1E). All the results showed that Dan Hong injection had similar effect with valsartan. The representative echocardiograms in different groups are presented in Fig. 1F.

3.2. Hemodynamics

Improvement of the heart functionality by DHI after coronary ligature was further confirmed by hemodynamic measurement in each group on the 28 day after operation. As shown in Fig. 2, DHI and valsartan group markedly increased stroke volume (SV) ($p < 0.01$)and stroke work (SW) ($p < 0.05$)compared to coronary ligature group. Evidently, the maximum rate of rise and decline of ventricular pressure (+dp/dt max and −dp/dt max, respectively) was significantly enhanced because of the injection of DHI compared to coronary ligature group($p < 0.01$). Valsartan showed the same curative effect on +dp/dt max($p < 0.01$), but had no significant difference in −dp/dt max. . As noticed in Fig. 2E and F, in comparison with sham group, coronary ligature caused a significant increase in heart rate (HR) and decrease left ventricular systolic pressure(LVSP), injection with DHI significantly restrained the increase of HR and decrease of LVSP($p < 0.05$, $p < 0.01$ respectively). But valsartan only increased LVSP($p < 0.05$), and has no

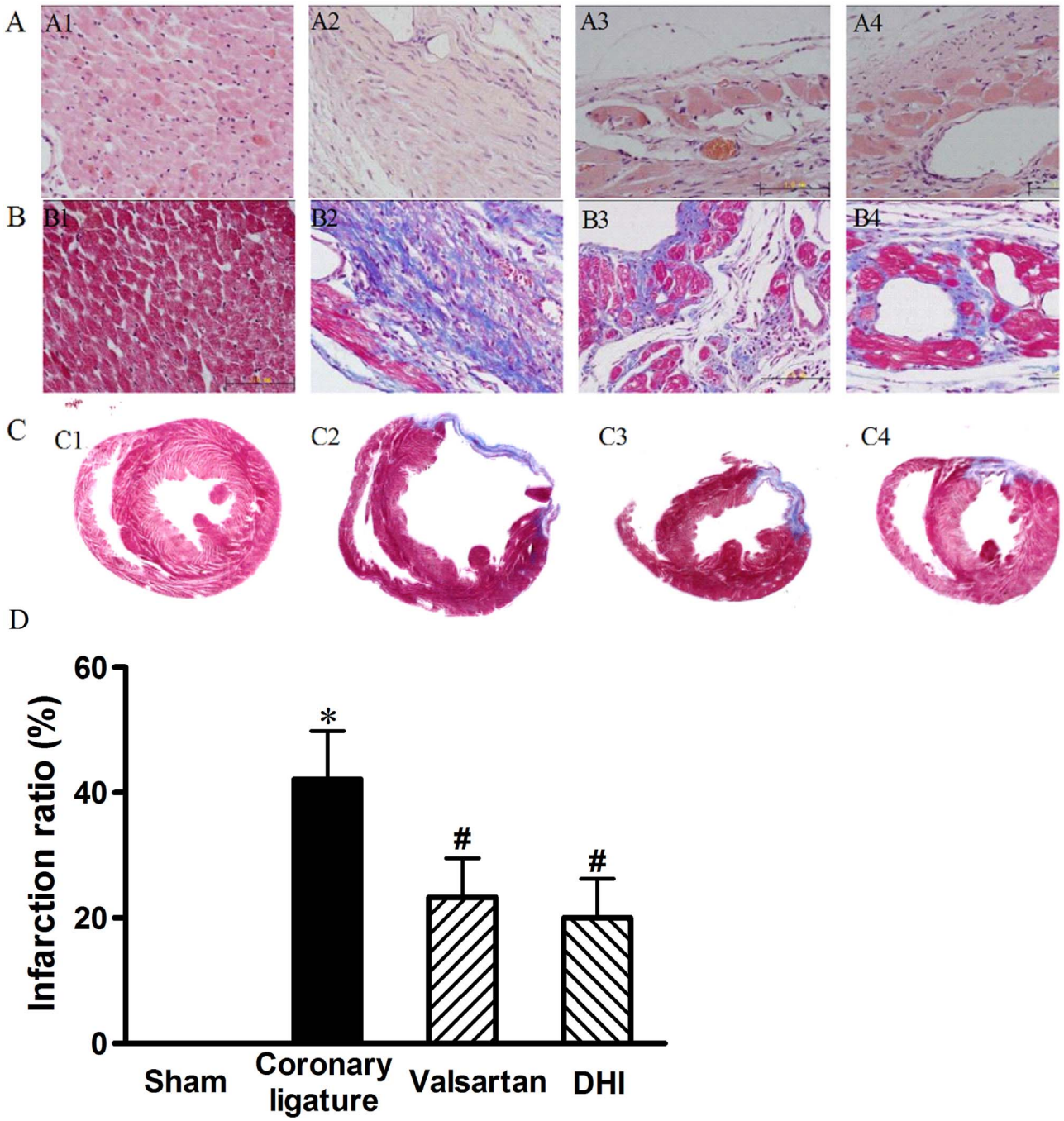

Fig. 3. H & E and Masson stain of left ventricle at 28th day after the MI. A: Representative photographs of myocardium by H & E. 400×; B: Representative photographs of myocardium by Masson stain. 400×; C: Representative panoramic of heart. They were sham(A1, B1, C1), model(A2, B2, C2), valsartan(A3, B3, C3) and DHI(A4, B4, C4) group from left to the right; the cavity on the right is left ventricular; red is cardiomyocyte, and blue is collagen fiber. D: Proportion of infarct ratio (n=4). All values are means ± SD; $^{*}p < 0.05$ versus sham group; $^{\#}p < 0.05$ versus coronary ligature group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

effect on HR.

3.3. Myocyte size and myocardial fibrosis in infarcted hearts

Hearts were examined for gross morphological changes at the 28th day using H & E and Masson stains (Fig. 3). The myocyte cross-sectional area increased in MI rats and significantly reduced by DHI and valsartan treatment. Loose arrangement of cells was found in coronary ligature rats, while a relative slighter condition in drug-treated groups (Fig. 3A). We examined myocardial fibrosis at 4 weeks after coronary ligature, because reactive fibrosis at the non-infarcted area adversely alters myocardial stiffness, leading to cardiac dysfunction (Weber and Brilla, 1991). Significant left ventricular fibrosis, wall thinning, and less residual cardiomyocytes were observed on the panoramic image of LV. Residual myocardial cells were island-like distribution. Interstitial collagen density in the LV myocardium was increased in MI rats but was significantly reversed by DHI and valsartan therapy (Fig. 3B and C). The statistical collagen deposition was conducted by the application of image J. Compared with the coronary ligature group, ratio of infarct was markedly decreased in the treated-DHI and valsartan group($p < 0.05$) (Fig. 3D).

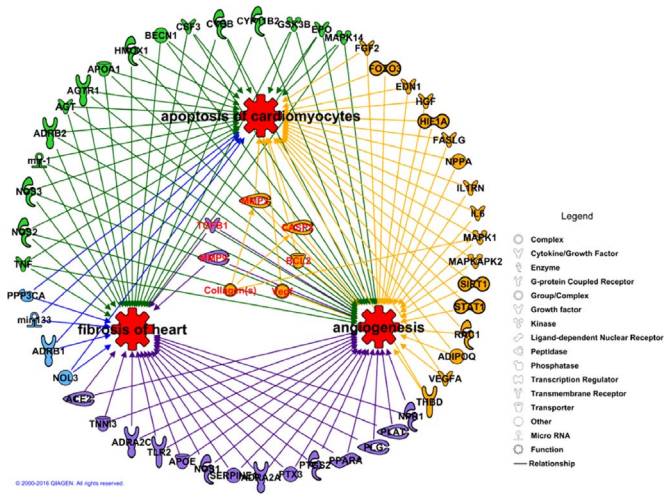

Fig. 4. The network relationship between apoptosis, fibrosis and angiogenesis with key molecules related to MI by IPA software. Green, apoptosis, fibrosis and angiogenesis related molecules ; Blue, apoptosis, fibrosis related molecules; Violet, fibrosis, angiogenesis related molecules; Yellow, apoptosis, angiogenesis related molecules. Red fond, key molecules focused: apoptosis (Caspase-3, BCL-2), angiogenesis (VEGF); fibrosis (TGF-β1, MMP-2, MMP-9). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.4. The MI-associated signaling pathways and molecular networks prediction by IPA

Based on the IPA analysis platform, Fifty-eight apoptosis, fibrosis and angiogenesis shared and seven key molecules play a very important role in MI and the network relationship between them was established. In order to elaborate the pharmacological mechanism, we focus on seven key molecules (MMP-2, MMP-9, CASP3, BCL-2, vegf, TGF-β1, collagen) involve apoptosis, fibrosis and angiogenesis biological process related to MI (Fig. 4).

3.5. Cardiac gene expression in infarcted hearts

The mRNA levels of TGF-β, MMP-2 and MMP-9 in non-infarcted LV myocardium around infarcted hearts were assessed by competitive RT-PCR analysis. TGF-β, a critical ventricular remodeling-related gene, was highly expressed in model group at 2 and 4 weeks after coronary ligature, but was lowered in both valsartan and DHI-treated groups($p < 0.05$). Similarly, MMP-2 and MMP-9 gene expressions were both elevated in coronary ligature group but significantly reduced with either valsartan or DHI-treatment group($p < 0.05$), suggesting that two proteins were important indicators of myocardial fibrosis (Fig. 5).

3.6. Regulation of fibrosis-related proteins MMP2, MMP9, and TGF-β1 expression by DHI after MI

To validate the cardiac protective effects of DHI after LAD ligation surgery , hearts were explanted and Western blotting was performed at 4 weeks after administration. We determined the expression of TGF-β1, MMP-2 and MMP-9, and the role of DHI in regulation of their expression. As shown in Fig. 6, TGF-β1 protein was up-regulated after coronary ligature, but they were significantly suppressed by the administration of DHI and valsartan($p < 0.05$, $p < 0.01$). MMP-2 and MMP-9, LAD ligation-induced up-regulation of fibrosis-related proteins, were also suppressed by DHI, consistent with valsartan results($p < 0.01$). The results suggest that DHI may attenuate myocardial fibrosis after coronary ligature,.

3.7. Angiogenesis in infarcted hearts

Immunohistochemical staining results showed the edge of myocardial infarction area has newly formed small blood vessels (Vascular endothelial cells performance round nucleus) in which blood vessels were stained brown. The capillaries content of treatment group with DHI and valsartan was significantly more than coronary ligaturegroup in danger zone (Fig. 7). Likewise, relatively high expression of VEGF was reflected on the level of gene expression in DHI and valsartan group especially in DHI group which was nearly normal($p < 0.05$).

3.8. Immunofluorescence staining

We detected myocardial cell survival with α-SARC (Sarcomeric actin). Myocardial cells displayed red fluorescence marked with α-SARC monoclonal antibody. After 28 days, MI was stained, results showed that α-SARC protein was rarely detected in the border infarct zones of the model group but increased in both valsartan and treated-DHI group (Fig. 8A). As shown in Fig. 8B, anti-apoptotic protein Bcl-2 and apoptotic protein Caspase-3 appeared with LAD ligation surgery. The results of Caspase-3 and Bcl-2 immunostaining showed that DHI and valsartan group compared with coronary ligature group, anti-apoptotic protein Bcl-2 (green stain) expression was significantly increased and a significant reduction in apoptotic protein Caspase-3 (yellow stain).

4. Discussion

LV remodeling after MI is a complex process consisting of three stages: acute phase infarct expansion, subsequent myocyte hypertrophy and interstitial fibrosis in the residual myocardium, all of which lead to LV dysfunction (Oishi et al., 2006). In the current study, effects of DHI on LV remodeling during the 4 weeks after coronary ligature were examined in rats. DHI effectively inhibited regional distortion and dilatation caused by thinning and lengthening of the infarct zone and LV dilatation. Additionally, DHI administration improved EF and FS values thereby, maintaining myocardial contractility and guaranteeing cardiac blood supply. On the other hand, LV Mass was estimated by wall thickness and LVID, LV Vol herein referred to as LV volumes, both of which reflect the expansion of the heart, are some of the manifestations of ventricular remodeling that were observed. Under the premise of little change in left ventricular mass, left ventricular volume changes directly reflect the left ventricular expansion. Left ventricular wall thickness and range of motion reflect the degree of myocardial fibrosis and left ventricular remodeling in the development process. Our results suggest that DHI can improve heart function, and delay the development of ventricular remodeling. In addition, DHI can significantly inhibit myocardial infarct size in rats after 4 weeks of coronary ligature. In the present study, the infarct ratio of model rats during a period of 4 weeks was also higher than that of DHI-treated rats.

It is noteworthy that the normal adult heart is composed of approximately 2 to 4% collagen, the presence of which confers high tensile strength whereas slight changes in the heart's composition may adversely affect cardiac contractility. Therefore, the higher the collagen content, the worse the contractile force exerted by the myocardium (Alp et al., 1976). In another study, it was demonstrated that an increase in collagen deposition contributes to ventricular chamber strain enhancement and compliance reduction (Brower et al., 2006). Hence, collagen reduction plays a key role in reducing adverse remodeling after MI (Sun, 2009), and participates in the normal distribution of contraction force during the cardiac cycle. As demonstrated in our study, the increase in collagen deposition in coronary ligation rats was accompanied by the reduction of both contraction force (+dP/dt) as well as a decrease in LVSP, as described by others (Vantrimpont et al., 1998; Distefano and Sciacca, 2012; Zornoff et al., 2009).

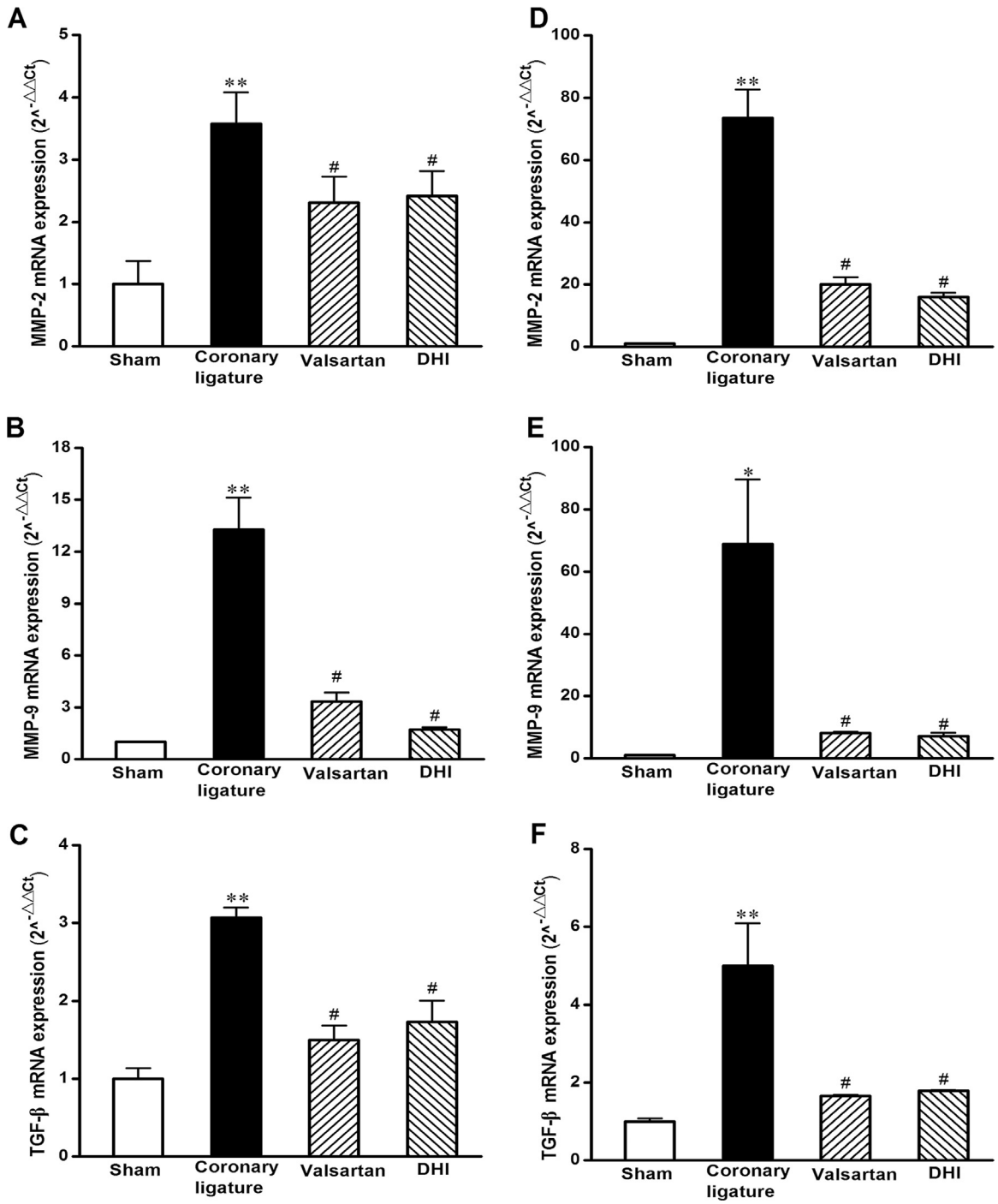

Fig. 5. Cardiac VEGF, TGF-β, MMP-2 and MMP-9 mRNA expression in rats 2(A–C) and 4(D–F) weeks after MI. The relative levels of mRNA were assessed by RT-PCR(n=3, each group). Results were normalized to GAPDH. All values are means ± SD; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus sham group; $^{\#}p < 0.05$ versus coronary ligature group.

The enhancement of collagen deposition plays an important role in adverse remodeling after MI (Brower et al., 2006). In our study, the rats subjected to 4 weeks of DHI showed a reduction in collagen deposition compared to the model group. HE and Masson results showed that cardiomyocytes in the coronary ligature rats were significantly larger and loosely arranged. Moreover, the cell gap widened and the residual myocardial cells reduced, showing overall fibrosis in myocardial infarct border zone. Compared with the drug treatment group, myocardial fibrosis and collagen content in coronary ligature group was significantly higher. Immunofluorescence results also showed that Sarcomeric actin dissolution was inhibited in DHI treatment group. Additionally, Cardiomyocytes survival rate was higher in DHI treated group than coronary ligature group, suggesting that DHI confers protective effect in myocardial cell.

The process of ventricular remodeling could be affected by many distinct factors, such as inflammation, fibroblast growth, myocardial apoptosis, collagen deposition, etc. TGF-β, MMP-2 and MMP-9 have been extensively involved in the modulation of fibrosis. It is important to note that TGF-β is a profibrotic cytokine that is extensively expressed after ischemia and is also important in the phenotypic transformation of interstitial fibroblasts to myofibroblasts (Desmouliere et al., 1993; Khan and Sheppard, 2006; Rosenkranz et al., 2002).

Early release of TGF-β1 from necrotic myocytes and macrophages not only promotes transformation of cardiac fibroblasts into myofibro-

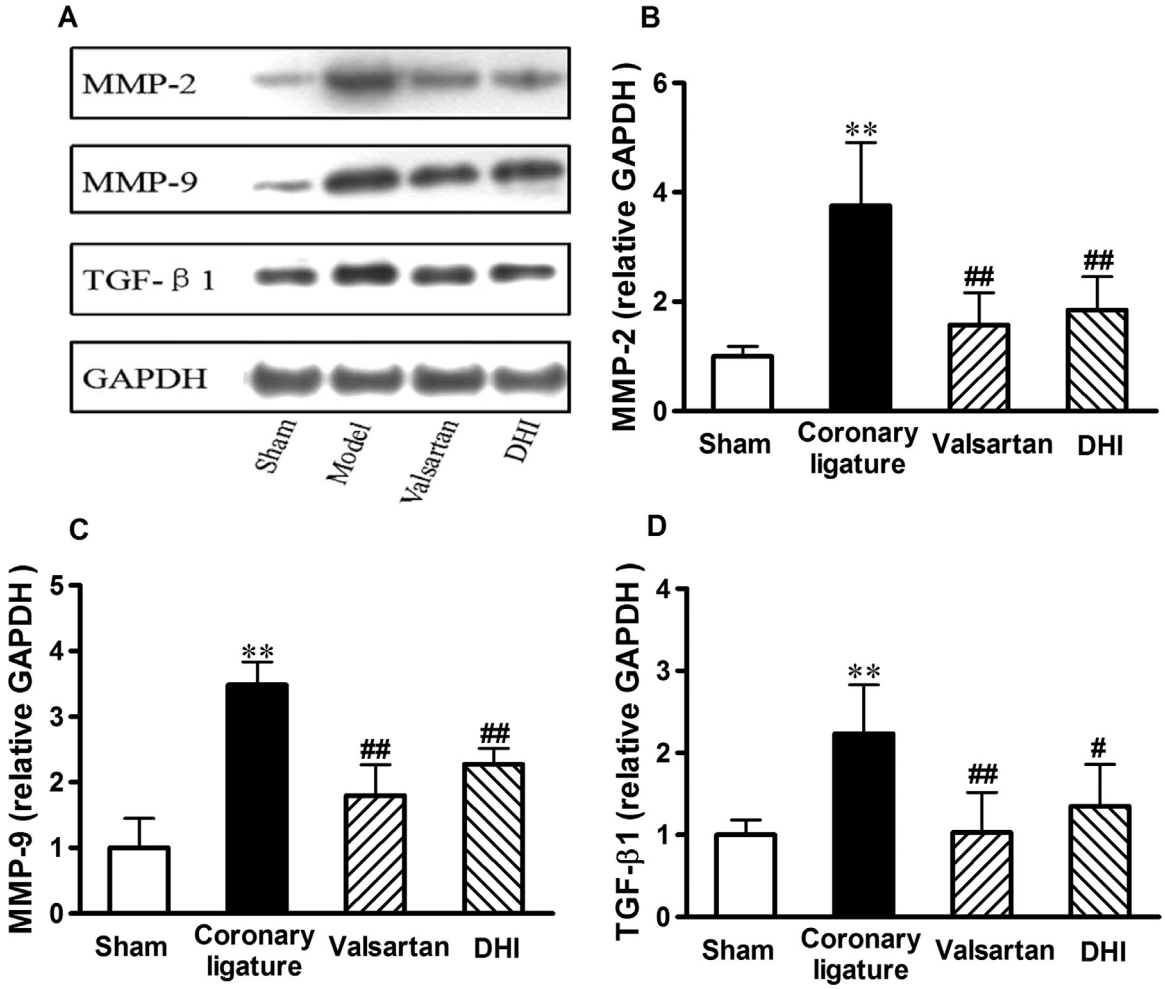

Fig. 6. Western blot of proteins related to myocardial fibrosis pathway in different groups. A: The representative western blotting bands of each protein in different groups. B–D: semi-quantitative analysis of MMP-2, MMP-9, and TGF-β1 in various groups. In A, each row represents images cropped from different gels probed with different antibodies as shown. Data are expressed as mean ± S.D. $^{**}p < 0.01$ compared with sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with coronary ligature group (n=4, each group).

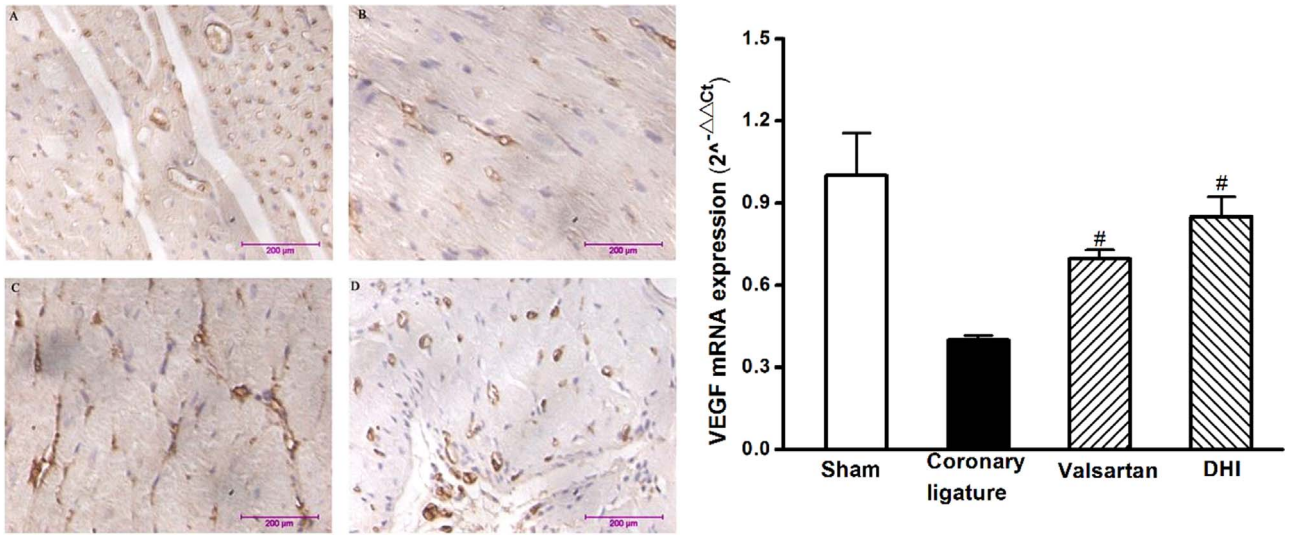

Fig. 7. Effect of DHI on angiogenesis in MI rats after 28 days of administration. Blood vessels were marked with VEGF (stained brown); bar=200 μm; A/B/C/D represent 4 groups sequentially (sham/model/valsartan/DHI). The relative levels of cardiac VEGF mRNA were assessed by real-time PCR(n=3, each group). Results were normalized to GAPDH. Data are expressed as mean ± S.D. $^{\#}p < 0.05$ compared with coronary ligature group.

blasts, but also directly mediates the formation of fibroblasts, thereby accelerating myocardial fibrosis. Previous studies have confirmed that increased expression of TGF-β worsens ventricular remodeling after acute myocardial infarction, leading to a poor prognosis for cardiac function (Bujak and Frangogiannis, 2007; Lijnen et al., 2000). Furthermore, it has been revealed that TGF-β-related pathways in

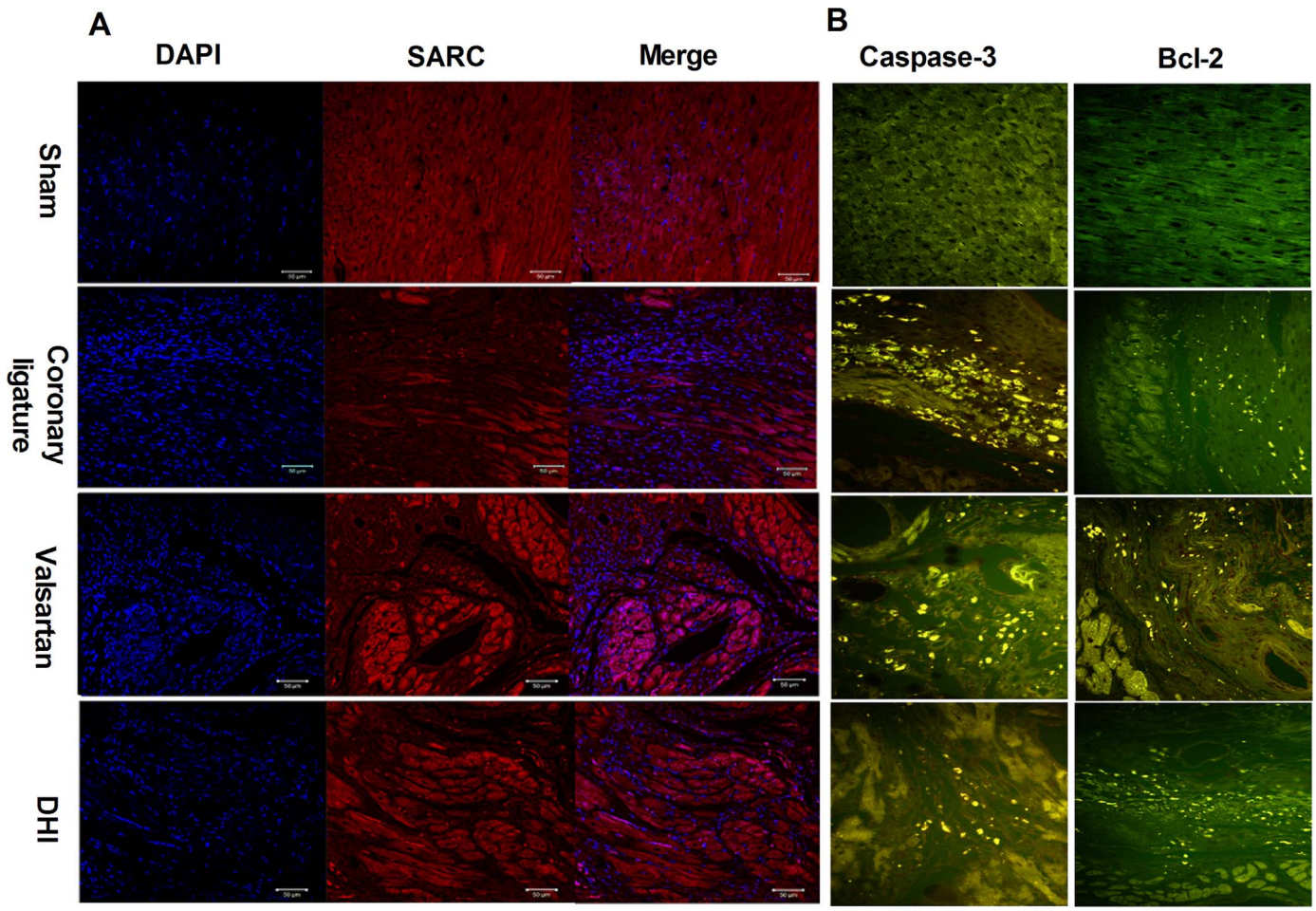

Fig. 8. The effect of DHI on myocardial apoptosis and survival. A: SARC expression in rat myocardial infarct border zone. (Nucleus were marked with DAPI displayed as blue; the last column was the merge graph); bar=50 μm. B: The expression of Bcl-2 and Caspase-3 in rat myocardial infarct border zone (Bcl-2 and Caspase-3 were stained with green fluorescent); bar=50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

fibrosis were related to the regulation of differentiation, recruitment, and activation of myofibroblasts that produced and remodeled extracellular matrix (Gabbiani, 2003; Kalluri and Zeisberg, 2006; Wynn and Ramalingam, 2012).

Based on the results of IPA, there are seven key molecules(MMP-2, MMP-9, Caspase-3, BCL-2, VEGF, TGF-β1, and collagen) play a very important role in ventricular Remodeling after MI. In this study, we confirmed that DHI can reduce collagen deposition through its downstream molecules MMP-2, MMP-9. Also, DHI attenuate MI through regulating the dynamic equilibrium of angiogenesis (VEGF), apoptosis (Caspase-3, BCL-2) and fibrosis (TGF-β1). Our RT-PCR analysis also revealed that mRNA levels of TGF-β, MMP-2 and MMP-9 were markedly increased 2- and 4-weeks after coronary ligature in the non-infarcted area of rat hearts. Interestingly, these effects were significantly ameliorated by DHI.

Since Sui and coworkers recently showed that valsartan prevents the deposition of collagen and inhibits fibrosis through regulation of TGF-β production (Sui et al., 2015), our observation that both DHI and valsartan could reduce the expression of TGF-β and its downstream fibrosis-related proteins, MMP-2 and MMP-9 in the border of myocardial infarction at 4 weeks after coronary ligature established a similar mechanism for DHI to that of valsartan as reported previously.

In this study, we also observed some other protective effects of DHI on cardiac function in addition to inhibiting fibrosis, such as increased vascularization and inhibition of apoptosis. VEGF expression induces and enhances the proliferation of vascular endothelial cells and migration to stimulate the growth of collateral blood vessels. Moreover, expression of VEGF inhibits apoptosis, increases vascular permeability, and promotes angiogenesis (Kajdaniuk et al., 2011). RT-PCR and immunohistochemistry results showed that DHI improved the mRNA expression of VEGF and increased blood vessel density of myocardial infarct border zone. In addition, DHI decreased the protein expression of one of the main pathways activating cell apoptosis, Caspase-3 and increased the anti-apoptotic protein, Bcl-2. These effects contributed to improvement of cardiac function and inhibited remodeling process.

Although we demonstrated the cardio-protective effect of DHI in myocardial fibrosis and remodeling after coronary ligation, we have yet to reveal the underlying mechanisms of DHI on TGF-β1 signaling pathways, a limitation of the study. However, this study has set the stage for an in-depth investigation to unravel whether TGF-β1/Smad signaling may have pro-fibrotic or anti-fibrotic effects and whether Smads are up-regulated or down-regulated during fibrosis. Furthermore, it is worthwhile to decipher in future studies, how apoptosis program and angiogenesis are regulated by DHI. Thus, the potential underlying mechanisms for these processes need further investigation.

In summary, we confirmed that DHI improved cardiac function by preventing adverse myocardial remodeling and reducing fibrosis after coronary ligation. Injection of DHI attenuated myocardial fibrosis via inhibiting TGF-β, MMP2 and MMP9 expression as well as collagen accumulation in the heart. We also found that the beneficial effects of DHI after coronary ligation is the increased expressions of VEGF the anti-apoptotic protein, Bcl-2 and the reduction of apoptotic protein,

Caspase-3. These findings may contribute to our understanding of the molecular mechanisms involved in cardioprotective effect of DHI and provide novel insights into future therapeutic strategy for post ischemic cardiac remodeling and heart failure.

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Disclosures

none declared.

Contributions of the authors to this study and their email address

Jingrui Chen performed the animal model, echocardiographic assessment of left ventricular function, western blot, and wrote the manuscript.

Wenjie Cao performed the real-time PCR, histopathological examination, immunohistochemistry and immunofluorescence assays.

Patrick Fordjour Asare reviewed the manuscript.

Ming Lv performed the Relationship network analysis.

Zhu Yan provided drugs and discussed interpretation.

Lan Li drew pictures and analyzed data.

Jing Wei performed the western blot.

Hui Gao performed the hemodynamic assessment of left ventricular function.

Han Zhang discussed interpretation.

Haoping Mao discussed interpretation.

Xiumei Gao conceived and designed the animal experiments.

Guanwei Fan conceived and designed the animal experiments.

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Glossary

α-SARC: α-sarcomeric actin
DHI: Danhong injection
HR: Heart rate
IPA: Ingenuity Pathways Analysis
LVAWs: left ventricle end-systolic anterior wall
LVEDP: left ventricular end-diastolic pressure
LVEF: left ventricle ejection fraction
LVFS: left ventricle fractional shortening
LVIDs: left ventricle end-systolic dimension
LVSP: left ventricular systolic pressure
LVVOLs: left ventricle systolic volume
MI: myocardial infarction
MMP-2/9: matrix metalloproteinases-2/9
SV: stroke volume
SW: stroke work
TGF-β: transforming growth factor - β
Val: valsartan
VEGF: vascular endothelial growth factor