MORRONISIDE PROMOTES ANGIOGENESIS IN INFARCTED RATS WITH ACUTE MYOCARDIAL INFARCTION THROUGH PRO-ANGIOGENIC GROWTH FACTOR AND ITS DOWNSTREAM ERK12 SIGNALING PATHWAY RELATED PROTEINS OF VEGF

Lei Li[1,2], Jingjing Wang[3], Wei Huang[2], Jun Zhang[4,*]
[1]Tianjin Medical University, Tianjin 300070, PR China - [2]Department of Geriatrics, Tangshan Workers' Hospital, Tangshan 063000, PR China - [3]Department of Laboratory, Tangshan Maternal and Child Health Hospital, Tangshan 063000, PR China - [4]Department of Cardiology, Cangzhou Central Hospital, Cangzhou 061001, PR China

ABSTRACT

Objective*: To investigate the effect and mechanism of morroniside on angiogenesis in rats with acute myocardial infarction (AMI) by regulating proteins related to downstream extracellular signal-regulating enzymes 1 and 2 (ERK1/2) signaling pathways through angiogenic growth factor and its vascular endothelial growth factor (VEGF).*

Methods: *Fifty-five SPF-grade healthy adult male SD rats were selected to establish an AMI model by ligating the left anterior descending coronary artery. After successful modeling, the rats were randomly divided into a sham operation group, a model group, a low-dose M (45 mg/kg morroniside) group, a medium-dose M (90 mg/kg morroniside) group, and a high-dose M (180 mg/kg morronoside) group. The low-dose M group, medium-dose M group, and high-dose M group were given 45 mg/kg, 90 mg/kg and 70 mg/kg by gavage once a day for 2 weeks, respectively. The sham operation group and the model rats were treated with the same amount of distilled water by gavage once a day for 2 weeks. On the seventh day, 5 rats in each group were sacrificed. Their myocardial tissues were taken to compare the density of new small blood vessels, pro-angiogenic growth factors [angiogenin-1 (Ang-1), fibroblast growth factor-2 (FGF-2), and VEGF], and protein expression levels of VEGF downstream ERK1/2 signaling pathway-related factors [p-Src, Src, p-PKC, protein kinase C (PKC), p-ERK1/2, and ERK1/2] in the myocardial tissue of rats in each group. At 14 d, 6 rats in each group were sacrificed, and the blood vessel density around the infarction of each group was compared.*

Results: *After 7 days, the density of α-SMA$^+$ arterioles in the model group was significantly higher than that in the sham operation group ($P<0.05$). The α-SMA$^+$ arteriole density of rats in the middle-dose M group and the high-dose M group was significantly higher than that of the model group ($P<0.05$). After 14 days, the lectin$^+$ vessel density around the infarction in the model group was significantly lower than that in the sham operation group ($P<0.05$). The density of lectin$^+$ blood vessels around infarction in the high-dose M group was significantly higher than that in the model group ($P<0.05$). After 7 days, the expression level of Ang-1 protein in the ischemic myocardium of the model group was significantly higher than that of the sham operation group ($P<0.05$). There was no significant difference in the level of FGF-2 protein in the ischemic myocardium of the model group compared with the sham operation group ($P>0.05$). The expression levels of Ang-1 and FGF-2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group ($P<0.05$). The expression level of VEGF protein in the ischemic myocardium of the model group was significantly higher than that of the sham operation group. The expression levels of p-Src, p-PKC and p-ERK1/2 in the ischemic myocardium of the model group were not significantly different from those in the sham operation group ($P>0.05$). The expression levels of VEGF, p-Src, p-PKC and p-ERK1/2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group ($P<0.05$).*

Conclusion: *Morroniside can effectively promote angiogenesis in rats with acute myocardial infarction. Its mechanism of action may be achieved by regulating pro-angiogenic growth factors and related proteins in the downstream ERK1/2 signaling pathway of VEGF.*

Keywords: *Morroniside, VEGF, ERK1/2, acute myocardial infarction.*

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Introduction

Cardiovascular disease is one of the major public health problems facing the world. According to relevant surveys in 2017, the incidence and mortality of cardiovascular diseases in China are still on the rise and seriously threaten the lives and health of the population[(1)]. Coronary atherosclerotic heart disease (CAD) is a pathological type of cardiovascular disease causing myocardial ischemia and hypoxia in patients and inducing myocardial infarction and angina pectoris[(2)]. Acute myocardial infarction (AMI) is a critical CAD illness mainly manifested as persistent and severe chest pain, usually

accompanied by acute circulatory disturbances and arrhythmias. In severe cases, it may even lead to heart failure and death[3].

Surgical bypass, drug thrombolysis, and percutaneous coronary intervention are the main clinical treatment methods of AMI. They have the effects of reducing the infarct size and protecting heart function. However, the above measures are relatively risky and expensive, and other shortcomings have led to their limitations in AMI treatment[4].

Therefore, we are eager to find a method that can effectively promote the microangiogenesis of the ischemic myocardium to realize myocardial revascularization. The vascular endothelial growth factor (VEGF) downstream extracellular signal-regulated enzymes 1 and 2 (ERK1/2) signaling pathway can regulate many neovascularization-related proteins. Under the mediation of VEGF, protein kinase C (PKC) can control the phosphorylation of downstream mitogen-activated protein kinase 1 (Mek1/2) and regulate the phosphorylation of ERK1/2 to promote endothelial cells proliferation[5, 6].

Clinical studies have shown that monoglycoside can protect the nerves of focal cerebral ischemia-reperfusion rats by up-regulating the expression levels of neurotrophic factors. In addition, monoglycoside can also maintain the integrity of neurovascular units and promote vascular homeostasis refactoring[7]. However, its mechanism of action in angiogenesis in rats with acute myocardial infarction has not yet been elucidated.

Therefore, the purpose of this study was to explore the effect and mechanism of morroniside on angiogenesis in rats with acute myocardial infarction through the pro-angiogenic growth factor and its downstream ERK1/2 signaling pathway-related proteins.

Materials and methods

Subject

Fifty-five SPF-grade healthy adult male SD rats were purchased from Guangzhou Fuerbo Biotechnology Co., Ltd., bodyweight 250±30 g, production batch number: SYXK (Guangdong) 2018-0809. All rats were kept in separate cages, with a 12 h day cycle. The temperature was set to 25±1 °C, the humidity was set to 50±10%, and all rats were pre-adapted to the environment for 7 days before the study.

Main reagents and instruments

Reagents

BCA protein quantitative kit was purchased from Wuhan Yunclone Diagnostic Reagent Research Institute Co., Ltd.; Rabbit anti-GAPDH antibody was purchased from Wuhan Chundu Biotechnology Co., Ltd.; Rabbit anti-VEGF purchased from; p-Src, Src, p-PKC, PKC, p-ERK1/2, ERK1/2 antibodies were purchased from PeproTech Biotechnology (Suzhou) Co., Ltd.; FITC-labeled α-SMA antibody was purchased from Shanghai Yubo Biotechnology Co., Ltd.; Rabbit anti-GAPDH antibody was purchased from Xiamen Huijia Biotechnology Co., Ltd.

Instruments

The ultra-clean workbench was purchased from Beijing QiWei YiCheng Tech Co., Ltd.; The electrophoresis instrument was purchased from Hangzhou Big Fish Biological Technology Co., Ltd.; The chemiluminescence gel imaging system was purchased from ProteinSimple, USA; The low-temperature high-speed centrifuge was purchased from Shimadzu (Shanghai) Laboratory Equipment Co., Ltd.; The Multiskan Sky microplate spectrophotometer was purchased from Hong Kong Boqi Technology Co., Ltd.

Method

• AMI models were established in all rats by ligating the left anterior descending coronary artery. After successful modeling, the rats were randomly divided into a sham operation group, a model group, a low-dose M (45 mg/kg morroniside) group, a medium-dose M (90 mg/kg morroniside) group, and a high-dose M (180 mg/kg morroniside) group. Rats in the low-dose M group, middle-dose M group, and high-dose M group were given 45 mg/kg, 90 mg/kg, and 70 mg/kg, respectively, once a day for 2 weeks. Both the sham operation group and the model rats were treated with the same amount of distilled water by gavage once a day for 2 weeks. At 7 d and 14 d, 5 and 6 rats in each group were sacrificed, and the myocardial tissue was taken and stored for testing.

• On the seventh day, the α-SMA immunofluorescence staining method was used to detect the density of new arterioles in the myocardial tissue of each group. On the fourteenth day, the lectin staining method was used to detect the peripheral blood vessel density of the rats in each group by immunofluorescence staining.

• On the seventh day, Western blot was used to

detect the expression levels of pro-angiogenic factors [angiogenin-1 (Ang-1), fibroblast growth factor-2 (FGF-2), and VEGF] and VEGF downstream ERK1/2 signaling pathway-related factors [p-Src, Src, p-PKC, PKC, p-ERK1/2, ERK1/2] in the ischemic myocardium of each group.

Statistical methods

The research data were processed and analyzed by SPSS17.0. All measurement data, such as the expression levels of pro-angiogenesis factors Ang-1 and FGF-2 in the ischemic myocardium of rats in each group, were expressed in ($\bar{x}\pm s$). The comparison between the two groups was performed by the t-test, and the comparison between multiple groups was analyzed by ANOVA. P<0.05 was considered statistically significant.

Results

Comparison of neonatal arterioles in each group

After 7 days, the density of α-SMA$^+$ arterioles in the model group was significantly higher than that in the sham operation group (P<0.05).

The α-SMA$^+$ arteriole density of rats in the middle-dose M group and the high-dose M group was significantly higher than that of the model group (P<0.05). See Table 1 and Figure 1.

Group	Cases	Density of α-SMA$^+$ arterioles (/mm^2)
Sham operation	11	10.02±1.02
Model	11	18.26±3.89*
Low-dose M	11	20.35±3.72
Middle-dose M	11	27.19±2.58$^\#$
High-dose M	11	33.19±5.26$^\#$

Table 1: Comparison of neonatal arterioles in each group ($\bar{x}\pm s$).

Note: *Means compared with sham operation group, P<0.05; $^\#$Means compared with model group, P<0.05.

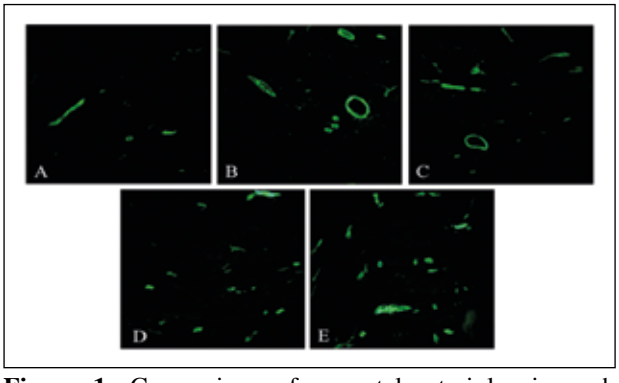

Figure 1: Comparison of neonatal arterioles in each group.

Note: A: Sham operation group; B: Model group; C: Low-dose M group; D: Medium-dose M group; E: High-dose M group.

Lectin$^+$ vessel density around infarct in each group

After 14 days, the lectin+ vessel density around the infarction in the model group was significantly lower than that in the sham operation group (P<0.05). The density of lectin$^+$ blood vessels around the infarction in the high-dose M group was significantly higher than that in the model group (P<0.05). See Table 2 and Figure 2.

Group	Cases	14 d (/mm^2)
Sham operation	11	523.06±15.48
Model	11	265.48±24.15*
Low-dose M	11	297.05±19.70
Middle-dose M	11	351.20±56.31
High-dose M	11	427.14±35.70$^\#$

Table 2: The density of lectin+ blood vessels around the infarct of each group ($\bar{x}\pm s$).

Note: *Means P<0.05 compared with the sham operation group; $^\#$Means P<0.05 compared with the model group.

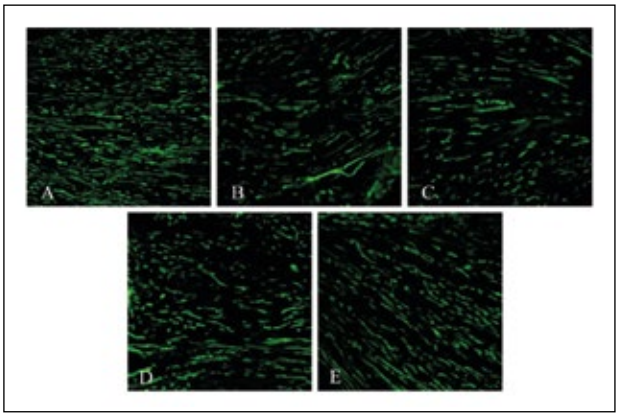

Figure 2: The density of lectin+ blood vessels around infarction in each group.

Note: A: Sham operation group; B: Model group; C: Low-dose M group; D: Medium-dose M group; E: High-dose M group.

Comparison of the expression levels of Ang-1 and FGF-2 proteins in the ischemic myocardium of rats in each group

After 7 days, the expression level of Ang-1 protein in the ischemic myocardium of the model group was significantly higher than that of the sham operation group (P<0.05).

There was no significant difference in the level of FGF-2 protein in the ischemic myocardium of the model group compared with the sham operation group (P>0.05).

The expression levels of Ang-1 and FGF-2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group (P<0.05). See Table 3 and Figure 3.

Group	Cases	FGF-2	Ang-1
Sham operation	11	0.36±0.05	0.30±0.04
Model	11	0.42±0.05	0.58±0.03*
Low-dose M	11	0.49±0.07	0.66±0.04
Middle-dose M	11	0.54±0.08	0.80±0.09
High-dose M	11	0.69±0.07#	0.92±0.06#

Table 3: Comparison of the expression levels of Ang-1 and FGF-2 proteins in the ischemic myocardium of rats in each group ($\bar{x}\pm s$).

*Note: *Means compared with the sham operation group, P<0.05; #Means compared with the model group, P<0.05.*

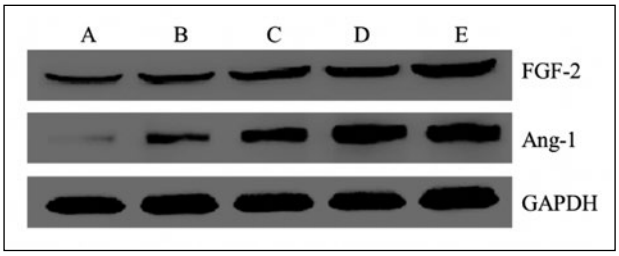

Figure 3: Comparison of the expression levels of Ang-1 and FGF-2 proteins in the ischemic myocardium of rats in each group.

Note: A: Sham operation group; B: Model group; C: Low-dose M group; D: Medium-dose M group; E: High-dose M group.

Comparison of the expression levels of VEGF and its downstream ERK1/2 signaling pathway-related proteins in ischemic myocardium of rats

The expression level of VEGF protein in the ischemic myocardium of the model group was significantly higher than that of the sham operation group. The expression levels of p-Src, p-PKC and p-ERK1/2 in the ischemic myocardium of the model group were not significantly different from those in the sham operation group (P>0.05). The expression levels of VEGF, p-Src, p-PKC and p-ERK1/2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group (P<0.05). See Table 4 and Figure 4.

Group	Cases	VEGF	p-Src/Src	p-PKC/PKC	p-ERK1/2/ ERK1/2
Sham operation	11	0.51±0.03	0.58±0.04	0.39±0.05	0.49±0.07
Model	11	0.74±0.08*	0.69±0.07	0.49±0.09	0.69±0.06
High-dose M	11	0.79±0.07	0.72±0.05	0.54±0.06	0.79±0.08
Middle-dose M	11	0.82±0.07	0.93±0.08	0.67±0.04	0.90±0.10
High-dose M	11	0.98±0.05#	0.98±0.08#	0.79±0.03#	1.04±0.06#

Table 4: Comparison of the expression levels of VEGF and downstream ERK1/2 signaling pathway-related proteins in the ischemic myocardium of rats in each group ($\bar{x}\pm s$).

*Note: *Means compared with the sham operation group, P<0.05; #Means compared with the model group, P<0.05.*

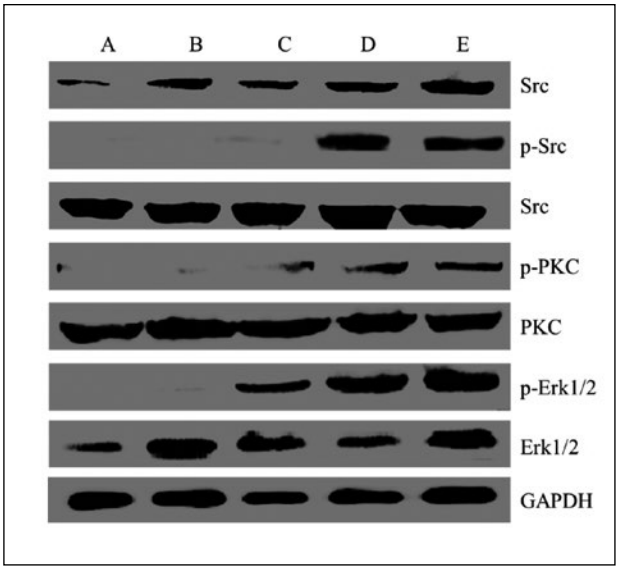

Figure 4: CComparison of the expression levels of VEGF and downstream ERK1/2 signaling pathway-related proteins in the ischemic myocardium of rats in each group.

Discussion

At present, the incidence and mortality of cardiovascular diseases in China are increasing year by year, seriously endangering human life and health. In 2000, the concept of drug bypass surgery (therapeutic angiogenesis) was proposed and provided a reference theory for future exploration of ischemic heart disease. Therefore, therapeutic angiogenesis has become an important topic in clinical research on ischemic heart disease[8]. Clinical studies have shown that morroniside has the function of promoting neurogenesis, and its mechanism of action is achieved by controlling the Wnt/β-catenin signaling pathway[9]. Other studies have shown that morroniside can effectively reduce the infarct volume, help angiogenesis and improve microcirculation[10].

Clinical studies have confirmed that arteriole production promotes the recovery of blood oxygen perfusion in ischemic myocardial tissue and reduces the risk of myocardial cell death. It plays an important role in restoring cell contraction. In this study, we first detected the density of neoplastic arterioles in each group of rats after 7 days of modeling and found that the number of arterioles in the infarcted area was significantly increased, and the lumens were larger. After the morroniside intervention, the number of neoplastic arterioles increased, indicating that morroniside can promote the blood supply recovery to myocardial tissue around the infarct area and improve myocardial function.

Myocardial infarction can cause cell death, degradation of the intercellular matrix, and dysfunction of microvascular function around the infarction, eventually leading to left ventricular remodeling and hypofunction of the heart. We measured the blood vessel density in the area around the infarction after 14 days of modeling in each group of rats and found that the microvessel density in the area around the infarction in the model group was significantly lower than that in the sham operation group. After the intervention of morroniside, the microvessel density was significantly higher than that in the model group, indicating that morroniside can increase the vascular density in the peripheral area of the infarction, which relieves the vascular perfusion of ischemic myocardium.

Clinical reports have shown that angiogenesis-promoting factors Ang-1, FGF-2, and VEGF are the keys to angiogenesis. However, when these factors act alone, they can trigger the penetration or immaturity of new blood vessels. Therefore, angiogenesis requires many factors to play a regulatory function together[11]. FGF-2 can stimulate proteolytic enzymes to accelerate the proliferation and migration of endothelial cells and ultimately plays an important role in the process of angiogenesis[12]. Other studies have shown that FGF-2 can cooperate with VEGF to act on the proliferation and differentiation of endothelial cells induced by VEGF, thereby increasing the expression level of integrin, enhancing the adhesion between endothelial cells, and ultimately promoting the production of neovascularization[13]. Ang-1 can eliminate the leakage or immaturity of new blood vessels caused by VEGF, which is beneficial to the interaction between endothelial cells and cells, and ultimately promotes angiogenesis[14]. After 7 days of modeling, we detected the expression levels of pro-angiogenic factor-related proteins in the myocardial tissue of each group of rats.

The results showed that after 7 days, the expression level of Ang-1 protein in the ischemic myocardium of the model group was significantly higher than that of the sham operation group ($P<0.05$). The expression levels of Ang-1 and FGF-2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group ($P<0.05$). It was suggested that morroniside could promote angiogenesis in rats with acute myocardial infarction by enhancing the expression of Ang-1. VEGF is a key regulatory factor that can effectively repair capillary circulation and endothelial tissue. In addition, it can also be an important regulatory factor for angiogenesis and angiogenesis. Clinical studies have shown that VEGF can activate its downstream Src tyrosine-protein kinase and regulate vascular permeability. In addition, VEGF can also activate downstream PCC, Mek1/2, ERK1/2 and other proteins to ultimately promote endothelial cell proliferation[15]. We detected the expression levels of VEGF and its downstream ERK1/2 signaling pathway-related proteins in the myocardial tissue of each group after modeling. The results showed that the expression level of VEGF protein in the ischemic myocardium of the model group was significantly higher than that in the sham operation group. The expression levels of VEGF, p-Src, p-PKC and p-ERK1/2 in the ischemic myocardium of rats in the high-dose M group were significantly higher than those in the model group ($P<0.05$). These results showed that morroniside could promote angiogenesis in rats with acute myocardial infarction, and its mechanism of action may be achieved by mediating VEGF and its downstream VEGF, p-Src, p-PKC, and p-ERK1/2.

In summary, morroniside can effectively promote angiogenesis in rats with acute myocardial infarction, and its mechanism of action may be achieved by regulating pro-angiogenic growth factors and related proteins in the downstream ERK1/2 signaling pathway of VEGF.

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Corresponding Author:
Jun Zhang
Email: ztn5rt@163.com
(China)