Original Article

Astragalus membranaceus extract promotes angiogenesis by inducing VEGF, CD34 and eNOS expression in rats subjected to myocardial infarction

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Abstract: The aim of the study is to determine the angiogenesis effect of Astragalus membranaceus extract (AME) on myocardium of rats with MI in vivo and to study its possible mechanisms involved in vascular endothelial growth factors (VEGF), cluster of differentiation 34 (CD34) and endothelial nitric oxide synthase (eNOS). A Sprague-Dawley rat model of MI was established by ligation of the left anterior descending coronary artery. Forty rats were randomized into 5 groups: MI control group, 3 different doses of AME (10, 20, 40 mg·kg⁻¹·d⁻¹) groups, and the Sham-operated group, each group consisted of 8 rats. The MI control group and sham-operated group were fed 0.9% sodium chloride 20 ml·kg⁻¹·d⁻¹. The rats were sacrificed after treated 8 weeks, hematoxylin-eosin staining, Masson staining and electron microscopy scanning were used to observe the pathomorphological changes of the myocardial tissues and vessels structure in the ventriculus sinister of rats. Immunohistochemical staining and western blot were used to evaluate the expression of VEGF, CD34 and eNOS. Compared with the MI control group, the morphology and arrangement of cardiomyocytes and the integrity of endothelial cells were improved, the contents of collagen fibers in myocardial tissues were decreased and the number of the new formed microvessels were increased in the myocardial tissues of rats in the AME treated groups. The VEGF, CD34 and eNOS protein expression in the myocardial tissue of the rats treated with different doses of AME increased significantly (P < 0.01). AME can obviously improve the disorganized myocardial tissues and promote angiogenesis in the rats after MI, which was accompanied by significantly increased expression of VEGF, CD34 and eNOS protein.

Keywords: Astragalus membranaceus extract, myocardial infarction, angiogenesis, VEGF, CD34, eNOS

Introduction

Myocardial infarction (MI), commonly known as a heart attack, remains the leading cause of morbidity and mortality for patients with cardiovascular disease worldwide [1]. Despite the large number of novel therapies under basic scientific investigation, the current therapy strategies to prevent and reverse the progress of MI to heart failure have been disappointing [2]. Percutaneous coronary intervention (PCI) is a relative efficiency strategy for some patients with MI. However, it is not optimistic about the long-term clinical outcomes in those patients after PCI [3, 4]. Therefore, a new therapy approach inducing angiogenesis in the heart after MI thus restoring myocardial perfusion has been proposed in recent years, and achieved positive outcomes in some reports [5-7].

Numerous factors are expressed in the ischemic heart where they promote angiogenesis. Hypoxia inducible factor 1-α (HIF1-α) is activated by hypoxia after MI and targets a wide range of genes including proangiogenic genes, such as vascular endothelial growth factor (VEGF). VEGF has a prominent role in post-MI angiogenesis through direct effect on endothelial cell survival and proliferation, control of new vessel formation and permeability as well as recruitment of inflammatory and regenerative cells [8]. The endothelial nitric oxide synthase (eNOS) generate NO and are intimately involved in the angiogenic response to ischemia and hypoxia. Treatment with the eNOS enhancer AVE9488 improved left ventricular remodeling and contractile dysfunction after MI via enhancing circulating endothelial progenitor cell (EPC) levels, and endothelial vasomotor dysfunction [9]. CD34 is a member of a family of single-pass
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transmembrane sialomucin proteins that show expression in hematopoietic progenitor cells during the embryonic phase and then in the vascular endothelial cells [10]. CD34 is used as a marker to select whole bone marrow-derived mononuclear cells or medullar cells, which are often used as source of EPCs for preclinical studies of therapeutic cell therapy for angiogenesis [11].

As research continues, evidences show that proangiogenic treatments with a single growth factor, VEGF for example, may present multiple potential risk factors, such as hypotension, dyslipidemia [12], and some larger, more-rigorously designed clinical trials, such as the EURONJECT ONE [13] and NORTHERN [14] trials, did not demonstrate significant clinical benefit of VEGF therapy in patients with MI. For this reason, novel approaches and drugs to treat MI are currently being pursued.

Recently, there has been growing interest toward establishing the therapeutic potential of natural products or Traditional Chinese Medicine (TCM) against MI [15-17]. Natural products or TCM have advantages to treat MI due to the complexity, chemical diversity, and biological properties of them. The root of Astragalus membranaceus (Fisch) Bge, commonly known as “Huang Qi” in China, is one of the most widely used TCM with variety of biological activities, such as improved sensitivity to insulin, immune modulation, antiviral activity, antitumor activity, and enhancement of cardiovascular functions [18]. The protection of cardiovascular function might be explained in terms of protection of tissue structure and as a decrease in serum marker of ischemic injury, malondialdehyde (MDA) [19]. Zhang et al. reported that the root of Astragalus membranaceus extract and one of its constituents, astragaloside IV, exert proangiogenic effect via enhancing VEGF mRNA expression in human umbilical vein endothelial cells in vitro [20, 21].

Our previous study founded that the root of Astragalus membranaceus extract can inhibit myocardial fibrosis and ventricular remodeling by regulation of protein kinase D1 in a rat model of myocardial infarction [22]. Although previous studies have suggested that the cardioprotective effect of the root of Astragalus membranaceus is related to multi-mechanisms and multi-targets, the angiogenesis effect related to VEGF, eNOS and CD34 has not been performed in vivo. The aim of the present study was to investigate the biological effects of Astragalus membranaceus on angiogenesis and its underlying mechanisms in a rat model of myocardial ischemia in vivo.

Materials and methods

Animals

A total of 40 male Sprague Dawley (SD) rats, weighing 200-250 g, were obtained from Experimental Animal Center of Henan Province (Zhengzhou, Henan, China). They were kept in standard conditions with controlled a temperature of 22±2°C, a humidity level of 45% to 65%, and a 12/12 hours light/dark cycle, and were provided with standard pellet food and purified drinking water. All animals were handled according to the institutional guidelines complying with Chinese legislation.

Preparation of the Astragalus membranaceus extract

The Astragalus membranaceus extract (AME) was prepared using the following method after Professor Xianzhang Huang, from Nanyang Institute of Technology, authenticated the origin of the roots of Astragalus membranaceus Bge. The dried roots of Astragalus membranaceus were prepared as crude slices and extracted two times (120 min, 90 min) by reflux extraction method with 70% ethanol (EtOH). The extract was combined, filtered and evaporated under reduced pressure. The supernatant was adsorbed on D101 macroporous resin and successively eluted with deionized water first, and then eluted with 70% EtOH. The fraction of 70% EtOH elution was collected, concentrated under vacuum and sprayed to dryness to obtain the AME. The yield ratio of AME was 1.5% containing 66.9% astragaloside. AME was stored in a refrigerator (4°C) until use; the time limit for AME storage was 1 year. The extract was freshly dissolved in distilled water before giving each dose to animals.

Establishment of MI rat model

The rat model of MI was established as previously described [22]. Briefly, the male SD rats were intraperitoneally anesthetized with pentobarbital (70 mg/kg), intubated, and connected
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to a respirator. The heart was exposed using an angular incision on the left side of the thoracic cavity. The pericardium was opened and ligation was performed on the left anterior descending branch of the coronary artery, and then the thoracic cavity was closed. At 48 h after establishment of the MI rat model, the survival rats were randomly divided into 4 groups comprised 8 rats each group, and administered orally once a day for 8 weeks, with the control vehicle (distilled water; MI control group) or one of the doses of AME at 10, 20 and 40 mg/kg (namely AME low, middle and high dose group, respectively). The sham-operated rats were threaded in the corresponding parts of the left anterior descending coronary artery instead of ligation and treated with the same dosage regimen as that in the control group (sham-operated group, comprised 8 rats). The rats were euthanized at the end of the experiment. These heart tissues were snap-frozen in liquid nitrogen until they were processed for further biochemical and pathological analysis.

Hematoxylin-eosin staining

Hematoxylin-eosin staining was performed in the apex tissues of the heart. Briefly, apex tissues of the heart after surgery were rapidly frozen by liquid nitrogen. After being fixed in 10% formaldehyde and embedded in paraffin, the embedded tissues were cut into sections with a thickness of 4 μm at the midpoint of left ventricle long axis. After washing with running water and distilled water, sections were stained with hematoxylin for 3-5 min, and then washed with running water and differentiated with 1% HCl in 70% alcohol. After that, the sections were stained with eosin for 1-4 min after washing with running water. After dehydration and differentiation in alcohol, sections were mounted and observed under Nikon Ti-soptical microscope (Nikon Corporation, Tokyo, Japan).

Masson staining

Myocardial collagen could be stained by Masson’s trichrome staining. Apex tissues of the heart after surgery were rapidly frozen by liquid nitrogen. After being fixed in 10% formaldehyde and embedded in paraffin, the embedded tissues were cut into sections with a thickness of 4 μm at the midpoint of left ventricle long axis. The sections were dewaxed, dehydrated in graded alcohols and stained by hematoxylin for 3 min. After washing with running water, the sections were differentiated in a 1% hydrochloric acid alcohol solution. The sections were then stained in warm Ponceau-acid fuchsin solution for 3 min, washed with distilled water, and differentiated in a 1% phosphomolybdic acid solution for 1 min. After wiping the phosphomolybdic acid residue from the slides, the sections were stained in 2% aniline blue solution for 1 min. The sections were dehydrated in graded alcohols, dried with cold air, and mounted in neutral resin. Changes in the myocardial interstitial collagen were observed by HMIAS-2000 Imaging System (Champion Medical Imaging Co., Wuhan, China).

Ultrastructural evaluation

The myocardial specimens were harvested from the septal and external portions of the left ventricular wall and fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1-mol/L cacodylate buffer. Histological processing was performed according to standard methods. Transmission electron microscopy (JEM-1200 EX; JEOL Ltd, Akishima, Japan) at a magnification of × 5,000 (3 microscopic fields for each heart) was used to determine ultrastructural damage. This evaluation was conducted independently by two histologists who were blinded to sample origins.

Immunohistochemical staining

Apex tissues of the heart after surgery were rapidly frozen by liquid nitrogen. After being fixed in 10% formaldehyde and embedded in paraffin, the embedded tissues were cut into sections with a thickness of 4 μm at the midpoint of left ventricle long axis. To inactivate endogenous peroxidase, 3% fresh prepared hydrogen peroxide was added at room temperature for 15 min. After washing with distilled water, the antigen was repaired by citrate buffer (pH 6.0) with microwave heating. The sections were washed with PBS and blocked with serum blocking solution for 20 min. Then a polyclonal rabbit anti-eNOS (sc-654), a monoclonal rabbit anti-VEGF (sc-152) or a polyclonal rabbit anti-CD34 antibody (sc-9095; from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added and incubated at 4°C in dark overnight. The secondary biotined goat anti rabbit IgG (Santa Cruz Biotechnology Inc.) was added for a 20 min incubation at room temper-
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nature. After incubation with streptavidin-biotin complex, the sections were developed with 3, 3'-Diaminobenzidine chromogenic reagent. The sections were counterstained with haematoxylin. After hydrochloric acid differentiation and dimethylbenzene transparency, the sections were mounted with neutral gum. Ten fields at a magnification of × 400 or × 100 were taken from every section. Mean optical density from every field was analyzed by HMIAS-2000 Imaging System.

Western blot analysis

The cardiac muscles were homogenized in the Potter/Elvehjem device in a proportion of 1 g of tissue to 5 mL of buffer solution Tris-HCl 20 mM (pH 7.4) and sonicated for 30 s. After centrifugation at 14,000 g for 10 min, the supernatant of the homogenized tissue was utilized for identification of VEGF, CD34 or eNOS proteins. The total protein concentrations of supernatant were quantified using a bicinchoninic acid assay (BCA) kit, and equal amounts of protein were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat dried milk diluted with TrisBuffered Saline Tween-20 (TBST; in mmol/l: Tris-HCl 20, NaCl 150, pH 7.5 nd 0.1% Tween20) at room temperature for 1 h and probed overnight at 4°C with a polyclonal rabbit anti-eNOS (sc-654), a monoclonal rabbit anti-VEGF (sc-152) or a polyclonal rabbit anti-CD34 antibody (sc-9095), and then incubated for 2 h with anti-rabbit IgG (Santa Cruz Biotechnology Inc.). Incubation with polyclonal mouse β-actin antibody (#3700; Cell Signaling Technology) was used as the internal standard control. Finally, the membranes were washed with PBS three times and the immunoreactive bands were visualized using an ECL-PLUS/Kit according to the manufacturer’s instructions. The grey values of each band were measured with a computer-assisted imaging analysis system (Quantity One, Bio-Rad, Hemel Hempstead, UK).

Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analysis was performed using one way ANOVA with Tukey’s correction. Analysis of variance was used to analyze comparisons between groups. P value less than 0.05 was considered to be significantly different. All statistical analyses were performed with SPSS 20.0 (IBM software, Somers USA, www.ibm.com).

Results

Improved the morphology and arrangement of cardiomyocytes by AME

To investigate the effect of AME on the myocardial cells of MI rats, hematoxylin-eosin staining was performed to observe the morphology and arrangement of cardiomyocytes. As shown in Figure 1A, in the sham-operated group, the cardiomyocytes have clear structure and the myocardial tissues were neatly arranged and well-organized, the cardiovascular morphology is regular and integrity, the microvessels count showed a normal distribution. However, in the MI control group, the myocardial tissues were disordered arrangement, nucleolysis and blurred boundaries occurred in the cardiomyocytes with disorganized and aged granulation tissues and neutrophil infiltration, and the myocardial tissues were lighter in color, as compared with those in the sham-operated group (Figure 1B). After treated with the different

![Figure 1. Morphology and arrangement of cardiomyocytes. Hematoxylin-eosin staining was performed to observe the morphology and arrangement of cardiomyocytes. Representative micrographs were shown. A. Sham-operated group. B. MI control group. C. AME low dose group. D. AME middle dose group. E. AME high dose group.](image-url)
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doses of AME, the myocardial tissues arranged more regular and deeper stained with relative clear boundaries, the number of inflammatory cells obviously decreased, and the inflammatory infiltration improved, as compared with those in the MI control group. After treated with low dose of AME, new formed microvessels were shown in the myocardial tissues with aged granulation tissues among them. New granulation tissues were widespread in the myocardial tissues with rare aged granulation tissues and more neovascularization in the middle dose of AME group. Moreover, there have numerous new formed microvessels and few granulation tissues in the high dose of AME group (Figure 1C-E). These results suggest that the morphology and arrangement of cardiomyocytes were improved after treated with AME.

Decreased the contents of collagen fibers in myocardial tissues by AME

To investigate the distribution of myocardial interstitial collagen, Masson’s trichrome staining was performed to observe the sections of myocardial tissues using an ordinary optics microscope. In the sham-operated group, the myocardial tissues were predominant in the field with blue collagen intermingled, clear structure and regular shape in the myocardial tissues were observed; the vascular morphology was clear, integrity and well arranged; the red mucosal layer can be seen clearly with a few blue collagenous fibers around the microvessels (Figure 2A). However, in the MI control group, the disorganized myocardial tissues were found with some tissues ruptured, a large area of myocardial tissues were substituted by blue collagen with the hypertrophy of red myocardial tissues intermingled, and the disorganized arrangement of aged granulation tissues were especially obvious (Figure 2B). For the AME treated groups, the red myocardial tissues were more regular arranged and their proportion increased along with the dose increased, as compared with those in the MI control group. In the low dose of AME group, new microvessels formed obviously in the myocardial tissues and showed in fresh granulation tissue morphology, however, the red myocardium was not regular enough. In the middle dose of AME group, the new formed integrity microvessels and the granulation tissues were interwoven obviously. While the new formed regular microvessels were spread over the myocardial tissues with clear and integrated walls in the

Figure 2. Distribution of myocardial interstitial collagen and microvessels. Masson’s trichrome staining was performed to observe the distribution of collagen and new formed microvessels in myocardial tissues. Representative micrographs were shown. Cardiomyocytes were stained to be red and collagen was stained to be blue. A. Sham-operated group. B. MI control group. C. AME low dose group. D. AME middle dose group. E. AME high dose group, visualized by an optical microscope at high magnification (× 400).

Figure 3. Ultrastructural changes analysis of the myocardial tissues after treated with AME by Electron microscopy scanning (original magnification × 5,000). Representative micrographs were shown. A. Sham-operated group. B. MI control group. C. AME low dose group. D. AME middle dose group. E. AME high dose group.
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Figure 4. Expression of VEGF, CD34 and eNOS in the myocardial tissues of rats by immunohistochemical staining. Representative micrographs were shown. A. sham-operated group. B. MI control group. C. AME low dose group. D. AME middle dose group. E. AME high dose group. Visualized by an optical microscope (× 400 for VEGF and eNOS, × 100 for CD34).

high dose of AME group (Figure 2C-E). These results indicate that contents of collagen fibers in myocardial tissues were decreased and new formed microvessels were increased after treated with AME.

Improved integrity of endothelial cells by AME

Electron microscopy was performed to observe the ultrastructural changes of the myocardial tissues of rats after treated with AME. In the sham-operated group, the myocardial tissues were organized regularly, the intercalated disc and the vessel morphology were clear with full walls, the perfusion of red blood cells were showed and the endothelial cells inside the walls were integral, the intact nuclei and the smooth basilar membranes were showed clearly and surrounded with pericytes (Figure 3A). In the MI control group, the myocardial tissues arranged in disorder, some intercalated discs disappeared, the residual walls damaged, the endothelial cells inside the walls grossly damaged and almost disappeared. The dissolved endothelial cell nuclei, the crenated basilar membranes and fewer pericytes showed in Figure 3B. For the AME groups, the morphology of myocardial tissues was more orderly, the intercalated disc and the vessel morphology were clear relatively, as compared with those in the MI control group. In the low dose of AME group, the vessel walls in the myocardial tissues have defects, the endothelial cells partly damaged, the extent of shrinkage of the basilar membranes somewhat improved, but still serious relatively, as compared with those in the MI control group. In the middle dose of AME group, the more clearly microvessels with full walls were found in the myocardial tissues and the pericytes appeared, the endothelial cell nucleus formed clearly, the basilar membranes were smooth relatively. While in the high dose of AME group, a large number of the microvessels with full walls were found in the myocardial tissues, the endothelial cells proliferated obviously, the nucleus were larger and plump, the pericytes were clearly visible (Figure 3C-E).

Increased VEGF, CD34 and eNOS expression by AME

To determine the expression of VEGF, CD34 and eNOS in the myocardial tissues of rats with MI, immunohistochemical staining was performed. The results were shown in Figure 4 and Table 1. Positive granules were rarely detect-
able in cytoplasm in the sham-operated group (Figure 4A). However, in the MI control group, the positive granules in the cytoplasm of myocardial cells increased, but no statistical significance ($P > 0.05$) compared with those in the sham-operated group (Figure 4B and Table 1). For the AME groups, the VEGF, CD34 and eNOS expression increased significantly in a dose-dependent manner when compared with those in the MI control group ($P < 0.01$) (Figure 4C-E and Table 1).

The significant difference can be further confirmed by western blot analysis. The grey values of VEGF, CD34 and eNOS expression from the bands of AME treated groups were significantly higher than those in the MI control group ($P < 0.05$, Figure 5). Although there has an increased trend for the VEGF, CD34 and eNOS expression in the myocardial tissues of rats in the MI control group compared with those in the sham-operated group from the bands, the mean of duplicate points has no statistical significance between them ($P > 0.05$, Figure 5).

**Discussion**

In recent years, proangiogenic therapy appeared a promising strategy for the treatment of patients with MI, as angiogenesis has the potential to salvage ischemic myocardium at early stages after MI [8]. Proangiogenic-based therapies, including growth factors delivery and administration of stem/progenitor cells, have obtained several positive clinical efficacies [6, 23, 24]. However, these therapies showed negative results blocking the way for the optimization of proangiogenic therapies, such as can’t completely improve the myocardial perfusion abnormalities [13], insufficient evidence of efficacy [25]. More and more researchers realized that single therapy approach may result in a new dilemma at the time to solve the existing problem, so they are pursuing synthetical therapy or holistic healing plan for one disease. Preclinical studies have suggested, for example, that VEGF and fibroblast growth factor (FGF) or T-box transcription factor TBX5 can act synergistically to give a more robust angiogenic response than any of them alone [23, 26].

In this study, we provide evidences that AME exerts an angiogenic effect on blood vessel formation after MI in vivo via inducing eNOS, VEGF and CD34 expression synergistically. During ischaemia the constitutive eNOS is activated leading to a burst of NO production which causes subsequent HIF-1α mediated expression of numerous proangiogenic growth factors, such as VEGF, as well as the recruitment of endothelial progenitor cells to sites of vascularization [8, 27, 28]. To further elucidate the mechanism of action of AME, immunohistochemical staining and western blot were used to analyze the expression of eNOS. It was found that AME can induce eNOS expression directly in the myocardial tissues of rat after MI in a dose-dependent manner, suggesting the AME-induced angiogenesis was related to the increased expression of eNOS.

New vessel formation takes the form of vessel sprouting (angiogenesis) which creates a network that remodels into arteries and veins [5]. At the initial stage, the proangiogenic factors is active by the increase of degradation of extracellular matrix and vascular permeability, leading to the migration and proliferation of endothelial cells (EC), and then the new sprouts develop lumens with an increasing number of ECs. VEGF is known to be an important growth factor for vascular development and angiogenesis and the high-expression predicts an increased angiogenesis in the vascular endothelial cells [8, 28]. In this study, the expression of VEGF increased in the myocardial tissues of rats in the MI control group demonstrate it’s a normal pathological reaction to MI, because the electron microscopy results showed the ECs were not in their integrity in the same group. In the meanwhile, there has less
CD34 expression, which is also a characteristic of new vessel formation, in the MI control group; suggesting new sprouts may not always develop lumens finally. After treated with AME, the vascular morphology restored its integrity relatively and the increased VEGF expression further induced and promoted the new vessels formation and maturity, and the increased CD34 expression demonstrated AME can increase the number of new formed vessels sharply.

As showed in the hematoxylin-eosin staining and Masson’s trichrome staining results, the newly formed granulation tissues can be seen in the myocardial tissues of rats in the MI control group due to the stimulation of injury factors like ischemia after MI, and this phenomenon was further confirmed by the VEGF and CD34 expression in the same group by immunohistochemical staining and western blot, which increased seemingly when compared with those in the sham-operated group. However, although the evidence of angiogenesis was found, new vessels can’t form effectively because the appearance of granulation tissues in the injury areas is just a self-defense reaction occurred when the body is stimulated by the injury factors. In the electron microscopy ultrastructure analysis results in the MI control group, ECs were few in number and the cell nucleus were lack of integrity, degraded and even disappeared, the basilar membranes were shrank and damaged grossly, suggesting the emergence of ECs is essential for angiogenesis. In the myocardial tissues treated with AME, the new formed vessels increased obviously in a dose-dependent manner, and the electron microscopy results showed that the basilar membranes tended to be integrity and the ECs with intact nucleus increased. Immunohistochemical and western blot results showed that CD34 and VEGF expression were also increased obviously with the dose of RAM increased, as compared with those in the MI control group, suggesting the histopathological results were kept highly consistent with the immunohistochemical and western blot results.

In summary, our results show that treatment with AME promotes several features of angiogenesis, including improvement of the disorganized myocardial tissues and the integrity of endothelial cells, increased newly formed microvessels and decreased the contents of collagen fibers in the myocardial tissues of rats after MI through induction of eNOS, VEGF and CD34 expression. Additional studies are required to characterize the molecular mechanism of AME on regulating the angiogenic process.

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Disclosure of conflict of interest

None.

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