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Original Article

The effect and mechanism of dl-3-n-butylphthalide on angiogenesis in a rat model of chronic myocardial ischemia

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Abstract: Objective: To assess the effect of dl-3-n-butylphthalide (NBP) on angiogenesis and its underlying mechanism in a rat model of chronic myocardial ischemia (CMI). Methods: Forty Sprague-Dawley rats were randomly divided into four groups: model, low-dose NBP (L-NBP), middle-dose NBP (M-NBP), or high-dose NBP (H-NBP) (n=10/group). All groups received intraperitoneal injections of isoprinosine hydrochloride daily for 14 days. Additionally, the L-NBP, M-NBP, and H-NBP groups received NBP at 3, 6, and 12 mg per kg body weight, respectively, by intraperitoneal injection. An additional 10 rats (control group) received 0.9% sodium chloride via intraperitoneal injection for 14 consecutive days. Echocardiography was used for the measurement of heart function. Immunohistochemical staining for factor VIII-related antigen and microvascular density determination were performed. The protein and mRNA expression of hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) in CMI areas were measured by western blot and RT-PCR, respectively. Results: Electrocardiograms showed that NBP improved cardiac function by regulating left ventricular end-diastolic and end-systolic diameters, ejection fraction, and fractional shortening. Compared with the control and model groups, the L-NBP, M-NBP, and H-NBP groups showed increased mRNA and protein expression of VEGFA and HIF-1α in myocardial tissue. The mRNA and protein expression of VEGFA and HIF-α in the H-NBP group were the highest. Conclusion: NBP treatment promotes VEGF and HIF-1α protein expression during myocardial ischemia, which may represent useful biomarkers for coronary collateral establishment and offer potential targets for therapeutic induction of angiogenesis in patients with CMI.

Keywords: Dl-3-n-butylphthalide, chronic myocardial ischemia, angiogenesis, hypoxia-inducible factor 1-α, vascular endothelial growth factor

Introduction

Coronary heart disease (CHD), which is the most common type of heart disease worldwide, refers to heart disease caused by abnormality or stenosis of the coronary arteries that supply blood and oxygen to the myocardium. In patients with CHD, the myocardium undergoes a process of chronic ischemia, resulting in heart failure [1]. Despite advances in pharmacological and interventional treatments, the mortality rate remains high in patients with heart failure caused by CHD. Improving blood perfusion in the ischemic myocardium is the key to treating ischemic heart failure. Angiogenesis is activated after myocardial ischemia and hypoxia and may improve the patient's clinical symptoms and cardiac function. Therefore, a current research focus is the therapeutic induction of angiogenesis in the ischemic myocardium.

Among the signaling pathways involved in angiogenesis processes, hypoxia-inducible factor-1α (HIF-1α)/vascular endothelial growth factor (VEGF) is one of the most important [2]. HIF-1α is a core angiogenesis activator and can activate the expression of VEGF, thereby initiating the angiogenesis process after ischemia [3]. VEGF is considered as an initiator of new capillary formation and promotes angiogenesis by regulating multiple processes [4]. Dl-3-N-butylphthalide (NBP), which was independently developed in China, is a new drug extracted from celery seeds and used to treat acute ischemic stroke and poststroke cognitive impairment. Its beneficial effects include increasing

serum VEGF levels and promoting cerebrovascular collateral circulation [5-8]. A previous study found that NBP reduced the infarct size in rats with acute myocardial infarction [9]. However, whether NBP has a role in promoting myocardial angiogenesis remains uncertain. The purpose of this study was to observe the angiogenesis effect of NBP, as well as to investigate its underlying mechanism, in a rat model of chronic myocardial ischemia (CMI).

Materials and methods

Animals

A total of 50 male Sprague-Dawley rats weighing 250±20 g (Wanleibio Co., Ltd.; Shenyang, China) were maintained under standard conditions and fed a regular diet. The experimental protocols were approved by the Ethics Committee of the Second Hospital of Hebei Medical University (No. 2018-P049).

Chemicals and reagents

NBP (butylphthalide sodium chloride injection, 25 mg:100 ml) was obtained from Shijiazhuang Pharmaceutical Group Co., Ltd. (Shijiazhuang, China). Primary antibodies against VEGFA and VIII-related antigen were obtained from Wanleibio Technology Inc. (Shenyang City, China) and Abcam (Massachusetts, USA), respectively. Antibodies against HIF-1α and VEGF receptor were purchased from Wanleibio Technology Inc. (Shenyang City, China). All reagents were analytically pure.

Preparation of the CMI model

The rats were randomly divided into control, model, low-dose NBP (L-NBP), middle-dose NBP (M-NBP), or high-dose NBP (H-NBP) groups (n=10 rats/group). According to a method reported by Yin et al. [10], all groups were administered isoprinosine hydrochloride (2 mg per kg body weight per day) by intraperitoneal injection for 14 consecutive days, except the control group, which was injected with an equivalent volume of 0.9% sodium chloride injection. NBP was given to the rats in the L-NBP, M-NBP, and H-NBP groups at doses of 3, 6, and 12 mg per kg body weight, respectively, by intraperitoneal injection once per day for 14 consecutive days. A 0.9% sodium chloride injection with an equivalent volume was injected into rats in the control and model groups.

Echocardiographic examination

Motion-mode echocardiography was performed on each study animal using a digital echograph (Philips, USA). The settings of the echocardiography device were held constant. The values of left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were conventionally measured and compared among groups. Echocardiographic variables, including left ventricular ejection fraction (LVEF) and fractional shortening (FS), were calculated for the evaluation of heart function.

Factor VIII-related antigen staining and microvascular density determination

The rats were sacrificed after model establishment and drug intervention. The myocardial tissue was fixed with formaldehyde. After the paraffin embedded section of myocardial tissue was completed, immunohistochemical staining of endothelial cells was performed with an anti-factor VIII-related antigen antibody using the streptavidin-peroxidase conjugate method. Sections were blocked with 20% normal goat serum for 30 min. After digestion in pronase for 10 min, polyclonal rabbit antibody against rat factor VIII-related antigen was applied to the sections at a dilution of 1:600 for 90 min at room temperature. After washing in phosphate-buffered saline (PBS; pH, 7.6), the sections were incubated with biotinylated goat anti-rabbit immunoglobulins (Thermo Fisher, USA) for 30 min at room temperature. Slides were washed in PBS buffer, and an avidin-biotin-peroxidase complex (Solarbio, China) was applied to the sections according to the instructions of the manufacturer. The slides were then developed using diaminobenzidine (Solarbio, China) with H_2O_2 to produce a brown-colored reaction product and were counterstained with methyl green.

Microvessels were counted according to the modified method of Fu et al. [11]. A single vessel was considered to be any dark brown–stained endothelial cell or cell cluster that was clearly separated from adjacent capillaries, cardiomyocytes, and other cardiac fibroblasts. The microvascular density of positive anti-factor VIII staining in five random high-power fields (×400) was counted by two blinded investigators. Additionally, the average microvascular

density of the five high-power fields was calculated for comparison [12].

Western blot detection for HIF-1α and VEGFA protein expression

Western blot was used to measure the levels of HIF-1α and VEGFA protein expression in the ischemic myocardium. Approximately 50 mg of left ventricular myocardial tissue was removed from the heart and placed on ice; afterward, the myocardial tissues were ground in a tissue homogenizer and lysates were collected. The lysates were then centrifuged at 4000× g for 10 min, and the supernatants were transferred to another tube for testing. Equivalent amounts of protein (40 μg/lane) were resolved electrophoretically and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with diluted (1:1000) primary antibodies against HIF-1α, VEGF, or β-actin, as the internal control, at 4°C overnight. Then, the membranes were incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG; both 1:5000, Wanleibio, Shenyang, China) for one hour at room temperature. Enhanced chemiluminescence was used for the visualization of proteins.

RT-PCR detection for Hif1a and Vegfa mRNA expression

TRIzol (BioTeke, Beijing, China) was used to isolate total RNA, and cDNA was synthesized from 2 μg total RNA using Superscript II reverse transcriptase (Invitrogen). The SYBR Green RT-PCR kit (Biyuntian, Shanghai, China) was used to perform quantitative real-time polymerase chain reaction (RT-PCR) in a total reaction volume of 20 μL, including 2 μL reverse-transcription product, 5 pmol of each primer, 10 μL 2× SYBR Green mix (Solarbio), and 0.4 μL diluted (1:1000) reference dye (Solarbio). The primers used to detect *Hif1a* expression were 5'-CTACTATGTCGCTTTCTTGG-3' (forward) and 5'-GTTTCTGCTGCCTTGTATGG-3' (reverse). The primers used to detect *Vegfa* expression were 5'-CGGACAGACAGACAGACACC-3' (forward) and 5'-AGCCCAGAAGTTGGACGAAA-3' (reverse). As an internal control, we detected the expression of β-actin mRNA using following primers: 5'-GGAGATTACTGCCCTGGCTCCTAGC-3' (forward) and 5'-GGCCGGACTCATCGTACTCCTGCTT-3' (reverse).

The Applied Biosystems Prism model 7900HT sequence detection system was used to perform RT-PCR with the following settings: initial denaturation at 95°C for 5 min to ensure complete denaturation of the DNA and activation of the Taq polymerase, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec. Fluorescence was detected after each cycle. All reactions were performed in triplicate. We normalized the relative expression levels to those of the β-actin internal control and calculated the *Hif1a* and *Vegfa* expression levels using the $2^{-\Delta\Delta CT}$ method.

Statistical analyses

All statistical analyses were performed using SPSS software (version 22.0, USA). Continuous variables are presented as means ± standard deviation. Multiple comparisons among groups were evaluated using one-way analysis of variance followed by two-sided Tukey's test. A *P*-value <0.05 was considered statistically significant.

Results

Comparisons of echocardiographic parameters

The changes in echocardiographic parameters among groups are shown in **Figure 1** and **Table 1**. LVEDD and LVESD were significantly higher in the model group than those in the control group after 14 days of NBP treatment, whereas the levels of LVEF and FS were significantly lower. Additionally, heart function improved significantly compared with rats in the model group after 14-day treatment with the different doses of NBP. The heart function parameters of the H-NBP group were similar to the control group.

Measurement of capillary density in ischemic myocardium

Immunohistochemical staining demonstrated that the expression of factor VIII-related antigen in capillary endothelial cells (**Figure 2**; **Table 2**) was scattered within the sections from all five groups. Significantly higher numbers of endothelial cells and higher capillary density could be detected in all groups, except the control group. Furthermore, the highest number of endothelial cells and the highest capillary density was found in the H-NBP group, suggesting that high doses of NBP might promote the generation of capillary endothelial cells and increase capillary density in chronic ischemic myocardium.

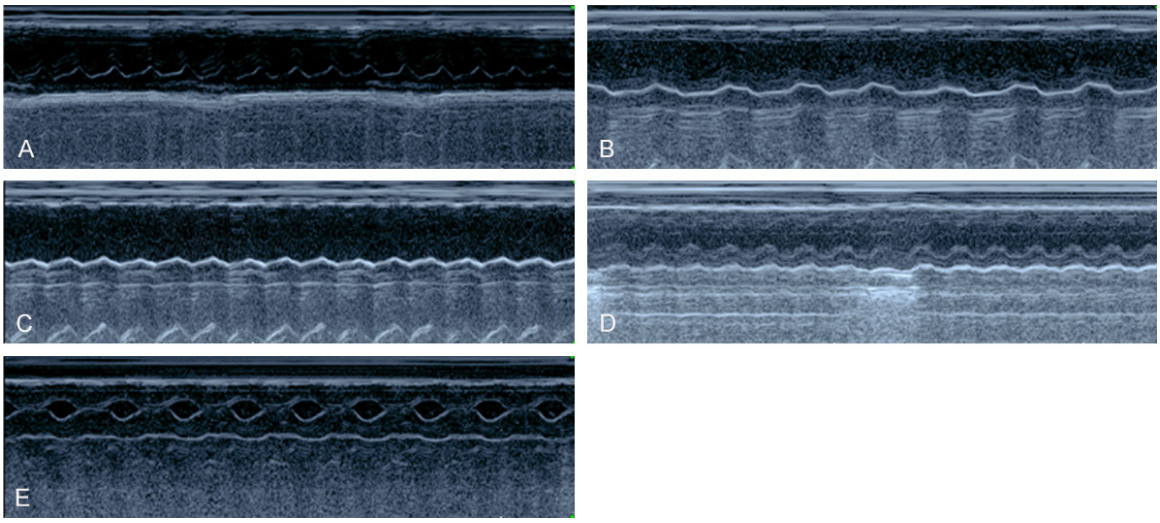

Figure 1. Motion-mode echocardiography images of rats among the control group (A), model group (B), L-NBP group (C), M-NBP group (D), and H-NBP group (E). Compared with the model group, the L-NBP, M-NBP, and H-NBP groups showed significantly improved heart function. The H-NBP group recovered heart function to the level of the control group.

Table 1. Comparisons of echocardiographic parameters

	Control group (n=10)	Model group (n=10)	NBP (n=10)	NBP (n=10)	NBP (n=10)	*P*-value
LVEDD (mm)	3.91±0.64$^{\&,*}$	6.15±1.04$^{\#}$	4.95±0.48$^{\#,\&}$	4.60±1.10$^{\&,*}$	4.43±0.26$^{\&,*}$	<0.001
LVESD (mm)	2.62±0.71$^{\&,*}$	5.51±1.06$^{\#}$	3.50±0.24$^{\#,\&}$	3.14±0.18$^{\&,*}$	2.88±0.74$^{\&,*}$	<0.001
LVEF (%)	73.81±4.23$^{\&,*}$	42.44±3.95$^{\#}$	57.56±8.95$^{\#,\&}$	60.29±10.78$^{\#,\&}$	71.49±3.25$^{\&,*}$	<0.001
FS (%)	34.49±2.44$^{\&,*}$	17.04±1.52$^{\#}$	26.24±5.16$^{\#,\&}$	26.58±6.56$^{\#,\&}$	32.41±5.52$^{\&,*}$	<0.001

Note: FS, left ventricular fractional shortening; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; NBP, dl-3-n-butylphthalide. $^{\#}$Compared with the control group, P<0.05. $^{\&}$Compared with the model group, P<0.05. *Compared with the L-NBP group, P<0.05.

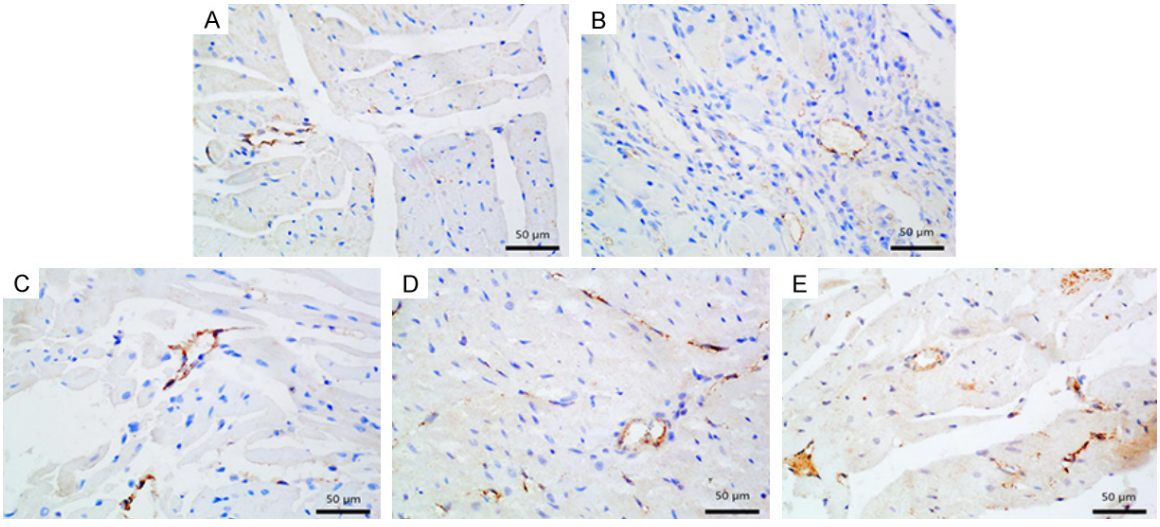

Figure 2. The effect of NBP on factor VIII-related antigen expression in capillary endothelial cells of rats with CMI. Immunohistochemical staining in the control group (A), model group (B), L-NBP group (C), M-NBP group (D), and H-NBP group (E). All images are 400× in magnification.

Table 2. Comparisons of the numbers of endothelial cells and capillary density among the groups

	Control group (n=10)	Model group (n=10)	L-NBP (n=10)	M-NBP (n=10)	H-NBP (n=10)	*P*-value
Endothelial cells	2.70±1.16	4.60±2.27#	6.10±1.79#	7.60±2.07#,&	10.10±2.03#,&,*	<0.05
Capillary density	1.10±0.99	4.60±2.37#	3.70±2.31#	6.70±2.75#,*	11.40±3.98#,&,*	<0.05

Note: NBP, dl-3-n-butylphthalide. #Compared with the control group, P<0.05. &Compared with the model group, P<0.05. *Compared with the L-NBP group, P<0.05.

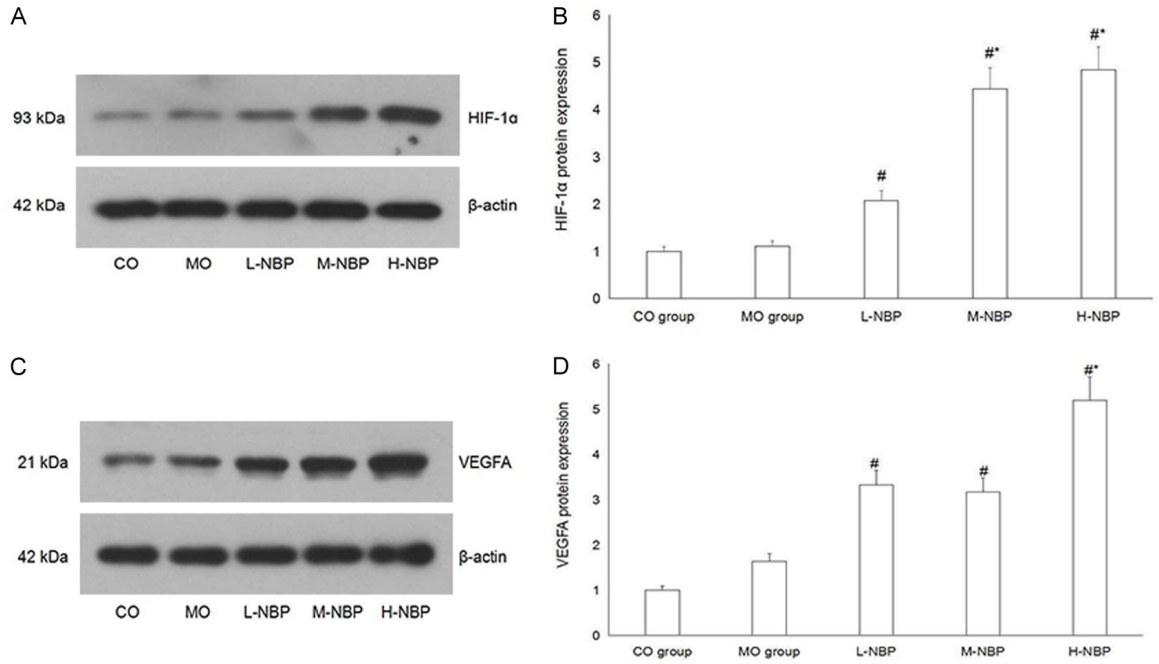

Figure 3. The effect of NBP on HIF-1α and VEGFA expression in rat ischemic myocardium. Photographs show representative western blots by group for HIF-1α and VEGFA. Graphs show the gray levels of bands. Quantitative data were normalized to β-actin. A. Protein bands for HIF-1α by western blot. B. Protein expression of HIF-1α. C. Protein bands for VEGFA by western blot. D. Protein expression of VEGFA. Values are the means ± SD (n=10 per group). #Compared with the control group, P<0.05; *Compared with the L-NBP group, P<0.05.

HIF-1α and VEGFA protein expression levels in the ischemic myocardium

HIF-1α and VEGFA protein expression levels in the chronic ischemic myocardium in each group were measured by western blot (**Figure 3**). Compared with the control and model groups, increased levels of HIF-1α protein expression in myocardial tissue were detected in all NBP groups, with the H-NBP group having the highest expression level (**Figure 3A**, **3B**). Additionally, a similar trend in the VEGFA protein expression level was also found in each group. There was no significant difference in the level of VEGFA protein expression between the control and model groups. Compared with the control and model groups, increased VEGFA protein expression was found after 14-day NBP treatment in chronic ischemic myocardium. As NBP concentration increased, VEGFA protein expression also increased, with the highest level in the H-NBP group (**Figure 3C**, **3D**).

Hif1a and Vegfa mRNA expression levels in the ischemic myocardium

Hif1α and *Vegfa* mRNA expression levels were higher in the model group than those in the control group. Furthermore, compared with the control and model groups, the administration of different doses of NBP increased *Hif1a* and *Vegfa* mRNA expression in the ischemic myocardium from the rat model of CMI. Similar to protein expression, the highest mRNA expression levels of *Hif1a* and *Vegfa* were detected in the H-NBP group after high dose of NBP administration (**Figure 4A**, **4B**).

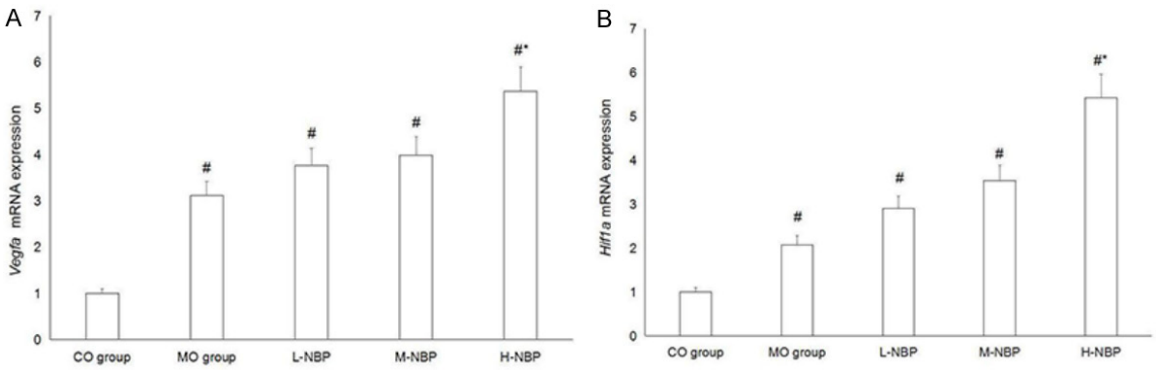

Figure 4. *Hif1a* and *Vegfa* mRNA expression levels in rat ischemic myocardium. Data are the means ± SD (n=10 per group). A. mRNA expression of *Hif1a*. B. mRNA expression of *Vegfa*. #Compared with the control (CO) group, P<0.05; *Compared with the L-NBP group, P<0.05.

Discussion

Despite continuous progress in medication and interventional therapies in recent years, CHD is still one of the most important health problems worldwide. Chronic myocardial ischemia in patients with CHD results from an imbalance of energy supply and demand and occurs when blood supply to the myocardium is reduced or the tissue demand for oxygen is increased [13]. Angiogenesis, which is the growth of new blood vessels, could help to establish collateral circulation in ischemic tissues [3]. Promoting angiogenesis through drug and non-drug treatments may be one of the most effective methods to restore the oxygen supply to ischemic tissue, thereby improving cardiac function. Mechanism for improving cardiac function by promoting angiogenesis is primarily due to the establishment of coronary collateral circulation to improve the blood supply to the ischemic myocardium [14, 15]. The aim of the present study was to observe the effect of NBP on angiogenesis and to investigate the mechanism in a rat model of CMI. We found that administration of different doses of NBP promoted angiogenesis in ischemic myocardial tissue by increasing expression of HIF-1α and VEGF. This result may help to promote further study of NBP in the field of angiogenesis in CHD and ischemic heart failure.

Myocardial ischemia induced by administration of isoproterenol is one of the classic models of myocardial ischemia. Isoproterenol causes a rapid increase in myocardial oxygen consumption by increasing heart rate and myocardial contractility, which leads to cardiac overload and myocardial microcirculation disorders [16]. In our study, we used this method in a rat model of CMI. Echocardiographic parameters were measured, and LVEF and FS values were calculated. Our results indicated that heart function decreased after the establishment of the CMI model, and 14-day NBP treatment improved heart function in these rats.

In the study of angiogenesis, the number of microvessels is often used to directly reflect establishment of collateral circulation [17]. Factor VIII-related antigen, also known as human von Willebrand factor, is a multimeric cytoplasmic glycoprotein with a molecular weight of 270 kD expressed by human endothelial cells and is also present in megakaryocytes and platelets. Staining for factor VIII-related antigens by immunohistochemistry can be used to determine the origin of endothelial cells [18]. Furthermore, anti-factor VIII immunostaining may be used to confirm the prognostic significance of microvessel density. In this study, the number of endothelial cells and capillary density significantly increased with increasing doses of NBP, indicating that administration of high-concentration NBP promotes angiogenesis in the area of CMI. This result may be similar to the previously uncovered role of NBP in the treatment of cerebral ischemia [8], suggesting its usefulness for limiting infarct size, as well as improving heart function.

Angiogenesis is critical for a positive response to ischemia, and vascular endothelial cells increase in hypoxemia through contact with blood [19]. One of the body's immediate responses to hypoxia is to activate the tran-

scription of several genes that lead to angiogenesis and metabolic adaptation. There are many molecular pathways involved in angiogenesis, and the HIF-1α/VEGF pathway plays an important role [20-22]. The hypoxia-inducible factor (HIF) protein family is a central mediator of the transcriptional activation process [23]. HIF-1 is a pivotal transcription factor that is very important for VEGFA expression during angiogenesis [24] and includes two subunits, HIF-1α and HIF-1β. Modulation HIF-1α generation constitutes the rate limiting step of the hypoxic response. The VEGF family is very important in regulating angiogenesis, which also contributes to the development of CHD [25, 26]. There are many members in the VEGF family, and VEGFA is essential for promoting angiogenesis. Therefore, we used HIF-1α and VEGFA as markers of angiogenesis and analyzed the effect of different doses of NBP on their protein and mRNA levels during CMI, in order to explore the potential mechanism by which NBP promotes angiogenesis.

In the last ten years, NBP has been widely used in clinical treatment of acute cerebral infarction. Previous studies have found that it could improve neurological function after cerebral ischemia by promoting angiogenesis and collateral blood circulation [5-7]. In studies of NBP used to treat acute ischemic stroke, NBP was found to increase VEGF expression through the regulation of multiple pathways, including upregulating sonic hedgehog expression and enhancing the expression of fibroblast growth factor (bFGF), thereby achieving its role in promoting angiogenesis [27, 28]. In the current study, the results indicated that the administration of different doses of NBP could promote angiogenesis in rats with CMI via the HIF-1α/VEGF pathway.

There are several novel observations in our study. We demonstrated that early NBP treatment exerts beneficial effects on postischemic heart functional changes. Most importantly, we provided evidence of the potential pathway that contributes to the angiogenesis effect of NBP. This is the first demonstration that cardioprotection by NBP occurs during an early stage of CMI and subsequent functional changes occur via promoting angiogenesis through the HIF-1α/VEGF pathway. Although the potential mechanism requires further investigation, we believe that our results may broaden the clinical application of NBP to include early myocardial ischemia.

In our study, VEGF and HIF-1α protein levels were not changed in the model group, but mRNA levels were increased. The reason for this result may be the difference in sensitivity between western blot and RT-PCR. RT-PCR is a very sensitive method to detect mRNA levels. However, protein detection methods are prone to interference, and the sensitivity of the detection methods may vary. The linear relationship between mRNA and protein expression levels detected by RT-PCR and western blot, respectively, is only approximately 0.4 to 0.5. This may be why mRNA expression levels of *Hif1α* and *Vegfa* were higher in the model group than those in the control group, whereas no significant differences were found in HIF-1α and VEGFA protein expression between the two groups. This result may also be one of the main limitations of this study, and it suggests that we need to analyze the changes in expression of relevant parameters in the HIF-1α/VEGF pathway using different or varied methods.

In conclusion, this study suggests that NBP treatment improves heart function and ventricular remodeling in rats with CMI. NBP treatment may represent an important protective mechanism that induces angiogenesis via changes in VEGF and HIF-1α expression. These observations require confirmation in future studies.

Acknowledgements

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Disclosure of conflict of interest

None.

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