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Exploring the molecular mechanism of Panax notoginseng saponins on coronary atherosclerosis let-7b using miRNA

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ABSTRACT

Objective: The miRNA was used to explore the molecular mechanism and effects of Panax notoginseng saponins (PNS) on coronary atherosclerosis let-7b and understand the variations in let-7b molecular mechanism after the application of PNS, which verified the therapeutic effects of PNS on coronary atherosclerosis and provided guidance for the clinical treatment and diagnosis of coronary atherosclerosis. Methods: Patients who were clinically diagnosed as coronary atherosclerosis in the hospital were taken as research objects and divided into two groups, i.e. the experiment (treatment) group, and the control group. The peripheral venous blood sample of each patient was taken in the morning on an empty stomach to extract the total RNA. In addition, patients in the two groups received symptomatic treatments, such as anti-ischemic therapy, anti-platelet therapy, and hypo-lipidemic therapy. Simultaneously, patients in the treatment group took Xuesaitong Capsule, while patients in the control group took the simulant Xuesaitong Capsule. After four weeks, blood samples were drawn again to extract the total RNA. Then, the target gene and related pathways of let-7b were determined by using the micro RNA target gene prediction software miRBase. Finally, the real-time fluorescence quantitative polymerase chain reaction (QRT-PCR) was used to investigate the effect of PNS on the molecular mechanism of coronary atherosclerosis let-7b. Results: Both let-7b and ADRB2 were up-regulated after treatment in the treatment group, while the let-7b and ADRB2 were down-regulated after treatment in the control group. In addition, the levels of molecules in the treatment group were down-regulated, including GS, AC, cAMP, PKA, and PLIN3, which indicated that PNS might regulate the downstream signaling pathway through let-7b/ADRB2 to achieve the therapeutic effects. Conclusion: The study found that the application of PNS could up-regulate let-7b and ADRB2 in patients with unstable angina pectoris, indicating that PNS functioned through let-7b and ADRB2 channels, such as protecting ischemia reperfusion of myocardial cells, mobilizing the bone marrow stem cells for angiogenesis, improving the survival rate after transplantation of bone marrow mesenchymal stem cells, reducing the apoptosis of myocardial cells, and promoting the repair of myocardial-related protein expressions.

1. Introduction

Coronary atherosclerotic heart disease is referred to as coronary heart disease. It has been reported that the global mortality rate of coronary heart disease has exceeded 20%. According to the Report on Chinese Cardiovascular Diseases, the mortality rate of coronary heart disease in China continued to increase in 2002–2019, which has become one of the diseases that seriously endanger the health of Chinese people (Zhu et al., 2018; Jiang et al., 2017). Drug treatment for coronary heart disease is an important basic treatment for patients with coronary heart disease. It is an important task for traditional Chinese medicine to strengthen the treatment and rehabilitation of patients with coronary heart disease by using the advantages of traditional Chinese medicine (Yang et al., 2016). Modern medicine believes that coronary stenosis and thrombosis are the most important pathogenesis of coronary heart disease. Traditional Chinese

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medicine believes that the key pathogenesis of coronary heart disease includes the stagnation and the weak chest Yang. Thrombosis and lipid deposition in coronary arteries are considered as phlegm and blood turbidity. Blood stasis syndrome is the most important and most common syndrome in coronary heart disease. The rule of promoting blood circulation and removing blood stasis is the characteristic of traditional Chinese medicine for treating coronary heart disease (Sun et al., 2018). Clinically, angina pectoris of coronary heart disease is divided into unstable angina pectoris stable angina pectoris. Many studies have shown that Panax notoginseng saponins (PNS) have a curative effect on both unstable angina pectoris and stable angina pectoris. PNS can reduce the frequency of angina pectoris and improve the electrocardiogram conditions, as well as improving the blood rheology and other indicators, and is of high security (Table 1).

Panax notoginseng is one of the most commonly used drugs for promoting blood circulation and removing blood stasis in clinical practice. Panax notoginseng is the dry root and root lotus of Panax notoginseng plant. It is warm, slightly sweet, and bitter, which can relieve the blood stasis, swelling, and pains (Madonna et al., 2016). Panax notoginseng consists of multiple chemical compositions, in which PNS is one of the most important medicinal ingredients. PNS mainly contains ginsenoside Rbl, ginsenoside Rgl, and notoginsenoside Rl (Andrews et al., 2017). The drug "Xuesaitong Capsule" and "Xuesaitong Injection" with PNS as the major component are used to treat cardiovascular and cerebrovascular diseases with blood stasis as the major pathogenesis, which have obtained excellent curative effects (Hibi et al., 2018). In recent years, the research on the treatment of coronary heart disease by PNS has been deepened, mainly in the evaluation of clinical efficacy and mechanism of action. Previous studies have found that let-7b is an anti-oncogene, and the recovery of let-7b expression may be an effective way to treat tumors. Recent studies have found that the let-7b family plays an important role in cardiovascular physiological processes and pathological mechanisms. In major cardiovascular cells, let-7b is highly expressed, including vascular smooth muscle cells, endothelial cells, myocardial cells, and coronary artery smooth muscle cells. In particular, differences in endothelial cell let-7b expressions have been observed in different cells, such as cerebral microvascular endothelial cells, human coronary endothelial cells, and human pulmonary artery endothelial cells, suggesting that let-7b leads to a unique phenotype diversity of endothelial cells.

The effects of PNS on coronary atherosclerosis let-7b molecular mechanisms were explored by using miRNA. Patients with coronary atherosclerosis treated in the hospital clinically were enrolled in the experiment. The total RNA of each patient was obtained through blood sample extraction. Then, the target gene and the related pathways of let-7b were determined. Finally, the real-time fluorescence quantitative polymerase chain reaction (QRT-PCR) was used to investigate the effect of PNS on the molecular mechanism of coronary atherosclerosis let-7b. The research results provided guidance for the clinical treatment and diagnosis of coronary atherosclerosis and were of great significance in myocardium repairing.

Table 1
Primer design.

Gene	Primer sequence
β-Catenin-F	5′-GGAATGGCTACCCAAGCTGA-3′
β-Catenin-R	5′-AAGACTGTTGCTGCCAGTGA-3′
GAPDH-F	5′-ATTCCACGGCACAGTCAAGG-3′
GAPDH-R	5′-ACATACTCAGCACCAGCATC-3′

2. Materials and methods

2.1. Experimental objects and grouping

A total of 80 patients were enrolled in the study from our hospital. The collection time was from March 2018 to July 2019. There were 40 males and 40 females, aged between 42 and 79 years old. Informed consent was obtained from all patients and their families prior to the study, and the study was approved by the Ethics Committee of The First People's Hospital of Yunnan Province.

Inclusion criteria: coronary angiography confirmed coronary heart disease and syndrome differentiation as blood stasis syndrome; thrombolytic, anticoagulation, crown expansion, and blood circulation and phlegm drugs were not used in the past two weeks; age 30–75 years old; patients signed informed consent.

Exclusion criteria: patients who participated in other clinical trials; recent history of trauma; pregnant or lactating women; patients with severe liver, kidney, hematopoietic system, nervous system, and other primary diseases and mental and malignant tumors; insulin-dependent diabetes Severe valvular heart disease.

Grouping: The treatment group and the control group were randomly assigned 1:1, in which 40 patients were in the control group and 40 patients were in the treatment group. The treatment allocation corresponding to the drug serial number was listed, and the corresponding numbered medicine was issued according to the order of the subject's visit. The opaque file bag was used to hide the random distribution scheme, make a random card, and put it in a file bag to seal. The sequence number is attached to the bag and the distribution plan is sealed on the card in the bag.

2.2. Intervention methods

The treatment group and the control group were given conventional treatments such as anti-ischemic treatment, anti-platelet therapy, and hypolipidemic therapy, and symptomatic treatment was given according to the complication. The treatment group was given Xuesaitong Capsule based on routine treatment of Western medicine, 2 capsules per time, 2 times a day, and 0.5 h after the interval with western medicine, and then taken for 4 weeks. The control group was based on the routine treatment of Western medicine. Adding Xuesaitong Capsule Simulator (appearance and packaging is the same as Xuesaitong Capsule of the treatment group, provided by Kunming Shenghuo Pharmaceutical (Group) Co., Ltd.). In order to avoid interference of other Chinese medicines on the observation indicators, the subjects should not take other Chinese medicine decoctions or Chinese patent medicine preparations during the test.

2.3. Target gene and signaling pathway

According to the preliminary research results of the research project, the gene miRNA let-7b related to coronary heart disease blood fatigue syndrome was found. The target gene ADRB2 of let-7b was predicted using the microRNA target gene prediction software miRBase, DIANA-microT, PicTar, TargetScanS, miRanda, and the like. The function and signaling pathways of the genes in the above miRNA list were analyzed by mas3.0, and the cardiac-related signal pathway and signal molecule ADRB2/GS/AC/cAMP/PKA/PLIN3 were found.

2.4. The QRT-PCR detection of let-7b and its target gene

The peripheral blood mononuclear cells were separated and purified by miRcute miRNA extraction and separation reagent kit (Certificate No. N2424). First, the peripheral blood samples were

diluted according to the ratio of physiological saline: blood = 1:1, and then the lymphocyte separation solution was added to the bottom of the glass tube, and the diluted blood: lymph solution = 2:1. Warm up to room temperature. Then dilute the diluted blood sample with a plastic pipette and slowly spread it over the tube wall to the lymphocyte separation solution, so as not to disturb the liquid layer interface. Centrifuge for 20 min at 2000 rpm/min. Next, insert directly into the mononuclear cell layer with a pipette and aspirate the layer. Add another 10 ml of physiological saline to dilute the isolated monocytes at 2000 rpm/min. Centrifuge for 10 min on a high-speed refrigerated centrifuge (HITACHI Corporation, Japan) and discard the supernatant. Repeat washing 1 or 2 times. Finally, 1 ml of Trizol was added and the total RNA was extracted in a freezer at −80 °C.

After RNA preparation and quality inspection, first take out the sample and add Trizol, put it in normal room temperature for 10 min, then centrifuged at 12,000 rpm/min for 10 min, discard the precipitate, and the supernatant was pipetted into a new tube and placed at 4 °C. Under the environment, add 200ul of chloroform (pre-cooling) to mix, put it at normal room temperature for 5 min, then apply 2000 rpm/min, centrifuge for 15 min, move the supernatant to a new tube and place it at 4 °C, then add 500ul of isopropanol (Pre-cooling), placed in an environment of −20 °C for 30 min or overnight, continuously discard the supernatant and mix the steps and finally transcribe to remove genomic DNA (gDNA) interference: prepare the total mixture in the RNase-free centrifuge tube, after the configuration is completed Put into the PCR instrument (Applied Biosystems, USA), the temperature was 42 °C, and the time was 2 min. After the configuration was completed, the sample was put into the PCR instrument, the temperature was 37 °C, and the time was 15 min. After real-time PCR, the primers were designed first, as shown in Table 2:

Preparation of the reaction system: The head used in the q RT-PCR reaction should be immersed in DEPC water. PE gloves should be worn throughout the process to prevent enzyme contamination. According to the required reagent amount and the number of reaction tubes, prepare the mixture in the RNase-free centrifuge tube. Add the dosage of the required reagent to the tube when preparing. The specific configuration is as follows:

After shaking for 10 s on the oscillator, centrifuge at low speed. Detection using the StepOnePlus Real-Time PCR System: Stage 1 Pre-denaturation: reaction at 95 °C for 30 sec, repeated 1 time; Stage 2 PCR reaction: 95 °C for 5 s, 60 °C for 30 s, 40 cycles; Stage3 Dissolution curve: reaction at 95 °C for 15 s, reaction at 60 °C for 1 min, reaction at 95 °C for 15 s. The results were calculated using 2-ΔΔCT. The relatively lowest group of m RNAs that silence the β-catenin gene was used in subsequent experiments.

2.5. Statistics analysis

All data in this experiment were statistically analyzed using SPSS 23.0. For statistical method selection, the paired *t* test was used for comparison between the two groups, and all results were expressed as mean ± standard deviation (MEAN ± SD). Statistically significant when P < 0.05, suggesting differences between the different groups.

Table 2
Usage of configuration solution.

Reagent	Usage
SYBR Premix Ex Taq II	10 μL
PCR Forward Primer (10 μM)	0.8 μL
PCR Reverse Primer (10 μM) 0.8 μL	0.8 μL
ROX Reference Dye (50 ×)	0.4 μL
DNA Template	2.0 μL
Sterile distilled water	6.0 μL
Total	20 μL

3. Results

3.1. General materials

The personal situation and clinical data of 80 patients were statistically analyzed, including gender, age, and past medical history. According to Table 3, there was no significant difference in the pretreatment status between the treatment group and the control group in personal information and diabetes history, high-pressure history, blood lipids, medication, and various information (P > 0.05).

3.2. PCR results of let-7b

The study found that the trend of let-7b in the two groups after treatment is shown in Fig. 1. The treatment group's let-7b was up-regulated after PNS intervention and let-7b was up-regulated almost twice as much as before treatment. The control group had a downward trend after treatment, and the downregulation was half of the time before treatment.

3.3. The ADRB2 results of target gene of let-7b

The study found that the trend of ADRB2 in the two groups after treatment is shown in Fig. 2, the treatment group ADRB2 was up-regulated after PNS intervention, while the control group of let-7b was down-regulated after treatment.

Table 3
Analysis results of general information.

General materials	The treatment group	The control group
Age (years old)	66.25 ± 8.68	64.40 ± 7.24
Gender (male %)	3 (60%)	3 (60%)
Current or Ex-smoker (%)	3 (60%)	4 (80%)
Hypertension history (%)	2 (40%)	2 (40%)
Diabetes mellitus history (%)	2 (40%)	1 (20%)
TC (mmol/L)	3.62 ± 0.87	3.47 ± 0.75
TG (mmol/L)	1.90 ± 1.25	1.98 ± 1.36
LDL-C (mmol/L)	2.58 ± 0.82	2.37 ± 0.73
HDL-C (mmol/L)	0.98 ± 0.15	1.06 ± 0.41
Aspirin medication (%)	5 (100%)	4 (80%)
Statin medication (%)	4 (80%)	4 (80%)

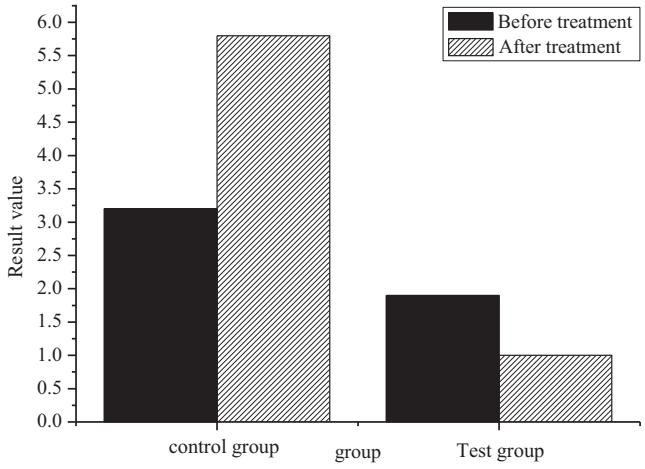

Fig. 1. PCR results of let-7b.

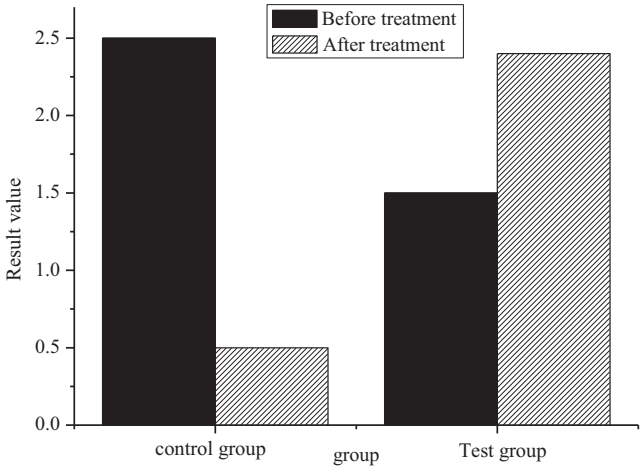

Fig. 2. The ADRB2 results of target gene of let-7b.

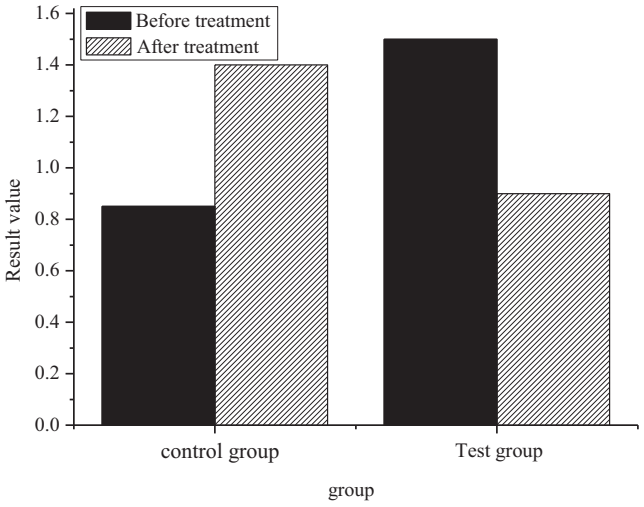

Fig. 4. The PCR results of cAMP.

3.4. Signaling pathway

After research, it was found that the molecules downstream of ADRB2 were GSI, AC, cAMP, PKA, PLIN3, GS, AC, PLIN3 of patients with coronary heart disease and blood stasis syndrome compared with normal people, coronary heart disease non-blood stasis syndrome, non-coronary heart disease, and blood stasis syndrome. Shows a difference of more than 2 times. Therefore, ADRB2/GSI/AC/cAMP/PKA/PLIN3 was selected as the observation object.

The study found that GSI changed in the opposite trend after treatment in both groups. As shown in Fig. 3, the GSI of the treatment group was up-regulated after PNS intervention; while the GSI of the control group was down-regulated after treatment.

The study found that cAMP changes in the opposite trend after treatment in both groups, as shown in Fig. 4. The cAMP in the treatment group was down-regulated after PNS intervention; the cAMP in the control group was up-regulated after treatment.

The study found that PRKACA showed consistent trends after treatment in both groups, as shown in Fig. 5. PRKACA in the treatment group was down-regulated after PNS intervention; PRKACA in the control group was also down-regulated after treatment.

The study found that the trend of PLIN3 changes after treatment in both groups of patients, as shown in Fig. 6. PLIN3 in the treatment group was down-regulated after PNS, and PLIN3 in the control group was also down-regulated after treatment.

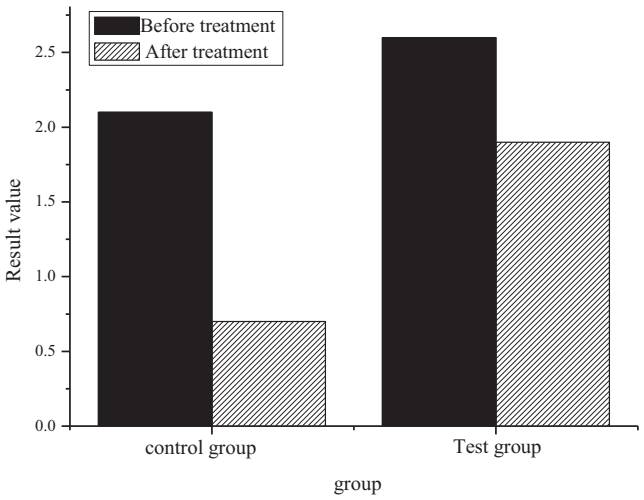

Fig. 5. The PCR results of PRKACA.

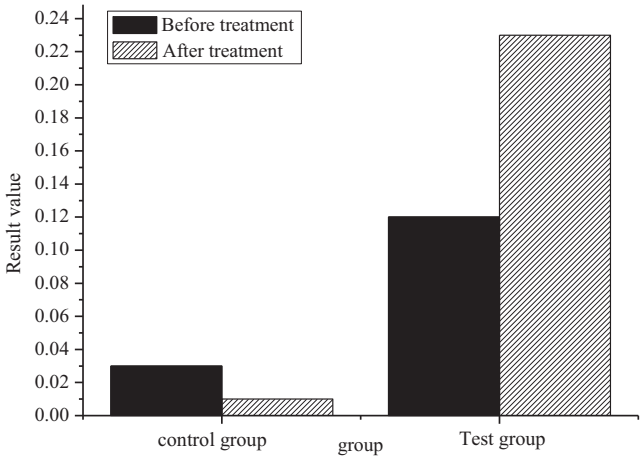

Fig. 3. The PCR results of GSI.

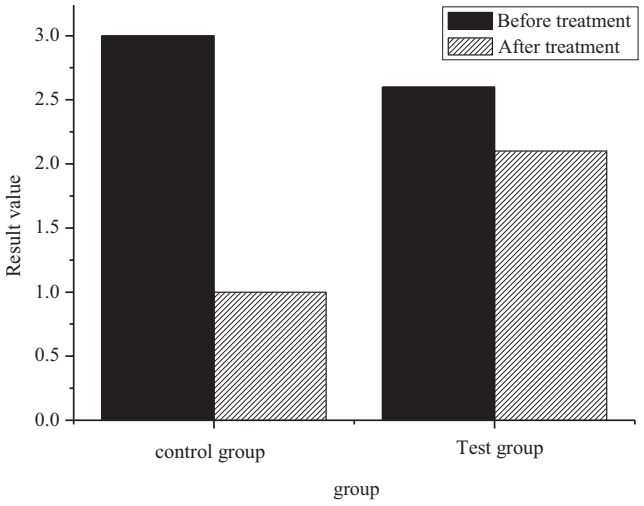

Fig. 6. The PCR results of PLIN3.

4. Discussion

Let-7 is a gene of the miRNA let-7 family, and the miRNA is an endogenous non-coding small RNA containing 21–25 nucleotides (Budoff et al., 2018). These small single-stranded RNAs target one or more mRNAs and activate regulatory target genes through degradation, translational inhibition, or targeting translational activations. It has been reported that miRNAs can directly regulate approximately one-third of the genes in cells. Early studies suggested that let-7 is a tumor suppressor gene, and the role of let-7 in cardiovascular biology and disease has received attention in recent years (Rong et al., 2016). Therefore, based on domestic and foreign research reports, further research and exploration are needed on let-7b and coronary heart disease.

PNS are currently the most common therapeutic drugs. It can effectively promote blood stasis and blood circulation, effectively inhibit platelet aggregation, and increase the cerebral blood flow of patients for the treatment of cerebral vascular sequelae. The therapeutic effects on diseases such as central venous obstruction of the retina are satisfactory after treatment. In recent years, diseases such as heart disease are particularly common and have brought serious damages, which has plagued many patients. Therefore, PNS can be used as a treatment for these diseases. Most patients have achieved extremely excellent results after taking PNS. A number of studies have shown that the addition of PNS before PCI in patients with acute myocardial infarction can improve the myocardial blood supply, relieve the myocardial damage, reduce the cardiovascular adverse events, and have a positive effect on patient prognosis. For patients with acute ST-segment elevation myocardial infarction, PNS intravenous infusion was performed based on western medicine before and after surgery. TIMI blood flow classification, MPG classification, cTnT, and CKMB were observed. It was found that pre-operative addition of PNS was significant in improving the myocardial blood supply in patients with acute ST-segment elevation myocardial infarction, reducing the inflammatory factor levels, relieving the myocardial damages, and improving the cardiac functions. In patients with acute myocardial infarction, it was found that PNS combined with Tirofiban intravenous infusion can effectively improve the myocardial reperfusion in patients with acute myocardial infarction after emergency PCI. Also, 12 months after PCI operation, it can reduce the incidence of cardiovascular adverse events.

The PCR and ADRB2 results of let-7b showed that both let-7b and ADRB2 were up-regulated after treatment in the treatment group, while both let-7b and ADRB2 were down-regulated after treatment in the control group. First, the consistency of changes in let-7b and ADRB2 indicated that ADRB2 was very likely to be a target gene of let-7b. In the process of predicting target genes, ADRB2 was also a target gene of kt-7b that had not been verified yet. The experimental results further provided a basis for the inference. The in vivo and in vitro studies have shown that most vascular diseases (such as coronary artery disease) are manifested as down-regulation of let-7b AMI at 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h. This study found that PNS could up-regulate the let-7b and ADRB2 in patients with unstable angina pectoris, suggesting that PNS might regulate the downstream signaling pathway through let-7b/ADRB2, thus achieving therapeutic effects. According to the review of previous researches, let-7b is of great significance in the treatment of coronary heart disease. The myocardial cells were transfected with let-7b, which was found to have protective effects on myocardial cells after ischemia-reperfusion. Besides, let-7b could promote angiogenesis and revascularization by mobilizing bone marrow stem cells after myocardial infarction. By transfecting the let-7b human bone marrow mesenchymal stem cells into the myocardial infarction areas, it was found that the bone marrow mesenchymal stem cells transfected with let-7b significantly increased the expression of related proteins in repairing myocardium and decreased the apoptosis and the autophagia positive cells.

This study found that PNS up-regulated the expressions of let-7b and ADRB2 in patients with unstable angina pectoris, suggesting that PNS could achieve the following potential therapeutic effects through let-7b and ADRB2: the protection of myocardial cells from ischemia-reperfusion; the mobilization of bone marrow stem cells thereby angiogenesis; the improvement of the survival rate of bone marrow mesenchymal stem cells after transplantation; the reduction of myocardial cell apoptosis; and the promotion of the repair of myocardial related protein expression. The research results would provide new ideas for the clinical application of PNS in the treatment of coronary heart disease, which might play an important role in the repairing treatment of myocardial cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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