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Original Research Article

Neuropeptide Y3-36-loaded PVAX Nanoparticles Enhance Angiogenesis in Myocardial Ischemia

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Abstract

Background: Neuropeptide Y (NPY) is an abundant sympathetic co-transmitter widely found in the heart, as it affects cardiac function.
Objectives: This study aimed to explore the effect of PVAX nanoparticles carrying NPY3-36 (PVAX-NPY3-36) on acute myocardial ischemia.
Materials and Methods: A double emulsification assay was conducted to load NPY3-36 onto the PVAX. With 11 untreated mice taken as a sham group, 38 mice were induced with left anterior descending artery ischemia, and some received PVAX-NPY3-36 or normal saline (Vehicle group). Upon evaluating heart function and echocardiographic data, all mouse tissues (n = 49) were collected 2 weeks later for Western blot and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analyses.
Results: The presence of PVAX-NPY3-36 induced reduced infarct size and mortality (p = .01 and p = .05), accompanied by increased systolic and diastolic parameters and elevation of stroke volume and cardiac function. Furthermore, upon treatment with PVAX-NPY3-36, ischemic tissues exhibited increased expression levels of pro-angiogenic factors.
Conclusion: Collectively, administration with NPY3-36 using PVAX nanoparticles promotes angiogenesis and alleviates the damage caused by myocardial ischemia.

Keywords

NPY3-36, PVAX, myocardial ischemia, angiogenesis, nanoparticles

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Introduction

Myocardial metabolism is almost aerobic due to the special structure of the myocardium (Al-Lamee et al., 2018; Bae et al., 2016; Bates, 2010). In the case of myocardial infarction, blockage of the coronary arteries involved in the supply of nutrients to the myocardium results in a deficiency of oxygen necessary for cell survival. Restoring blood flow to the ischemic myocardium can trigger injury. This process is called ischemia/reperfusion (I/R) and induces various reactions to affect gene expression and influence cell survival and the long-term recovery of heart tissue (Bengel et al., 2006; Johnson et al., 2004). When suffering from an I/R injury, the heart exhibits decreased contractility and undergoes reconstruction. Cardiac remodeling is initially characterized by ventricular dilatation, elevated cytokine levels, and rhythm of cardiomyocytes (Camici & Crea, 2007; Carmeliet, 2000). Since myocardial cell death secondary to ischemia triggers myocardial remodeling, and microvascular dysfunction is also the leading cause of heart failure, developing treatments for microvascular dysfunction after acute myocardial I/R is of significance. The established therapies for acute I/R injury in animal models are still far from clinical practice (Dvorakova et al., 2014; Erlinge et al., 1993; Eshun et al., 2017). Microvascular disorder followed by myocardial I/R remains challenging. Neuropeptide Y (NPY) is a highly conserved

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peptide present in the animal central and peripheral nervous system, as it enhances angiogenesis and relieves myocardial ischemic injury (Freccero et al., 2004; Gacche & Meshram, 2014). NPY is expressed in the dorsal horn of the spinal cord and functions through Y1 and Y2 receptors. NPY3-36 can promote angiogenesis in various animal models (Grundemar & Hakanson, 1993; Kothawade & Bairey Merz, 2011; Löffler & Bourque, 2016). Additionally, the impact of NPY3-36 in selectively stimulating angiogenesis by activating receptors (Matyal et al., 2011). NPY is considered a drug to improve myocardial ischemic injury effectively, but its defect of a short half-life limits its application to practice. Therefore, the further modification of NPY is essential.

Given the therapeutic potential of NPY3-36, this study established an acute myocardial ischemia model and used novel nanoparticles carrying NPY3-36 to treat the modeled mice. Our results finally demonstrated that continuous administration of NPY3-36 stimulates angiogenesis and reverses cardiovascular ischemia, which may help determine the therapeutic target. The prepared NPY3-36 nanoparticles can be used to prevent and treat myocardial ischemia and improve microvascular dysfunction.

Materials and Methods

Synthesis of Polyvinylpyrrolidone (PVAX)

1,4-Cyclohexanedimethanol (21.96 mM) and 4-hydroxy-3-methoxybenzyl alcohol (5.49 mM) were dissolved in tetrahydrofuran (THF) under nitrogen at room temperature for 6 h. The solid polymer of PVAX was obtained by extraction with dichloromethane and separation, and its structure was detected by hydrogen nuclear magnetic resonance (HNMR) spectroscopy.

Nanoparticle Preparation

PVAX (50 g) and NPY3-36 peptides (250 μg, Sigma) were dissolved in 500 mL of trichloromethane neuropeptide-Y, followed by the addition of polyvinyl alcohol (PVA) solution. The mixture was sonicated for 30 s and stirred for 1 min. Following centrifugation at 11,000 *g* for 5 min, we finally obtained PVAX nanoparticles.

Enzyme-linked Immunosorbent Assay (ELISA)

The condition of NPY3-36 release was detected using an ELISA kit, as previously described (Eshun et al., 2017), to evaluate biodistribution and metabolism.

Animal Model

Forty-nine adult C57BL/J6 mice (Jiangsu Jicui Yaokang Biotechnology Co., Ltd.) aged 10–12 weeks were classified into PVAX-NPY3-36 ($n = 22$), empty vehicle group (Vehicle) (dissolved in saline) ($n = 16$), and sham operation group (sham) ($n = 11$). Thirty-eight mice were induced with artery ischemia through coronary artery ligation using a 7-0 silk thread with their hearts exposed. After 30 min, PVAX-NPY3-36 (3 mg/kg) or Vehicle was injected intraperitoneally, and 10 min later, the suture was cut (Figure 1).

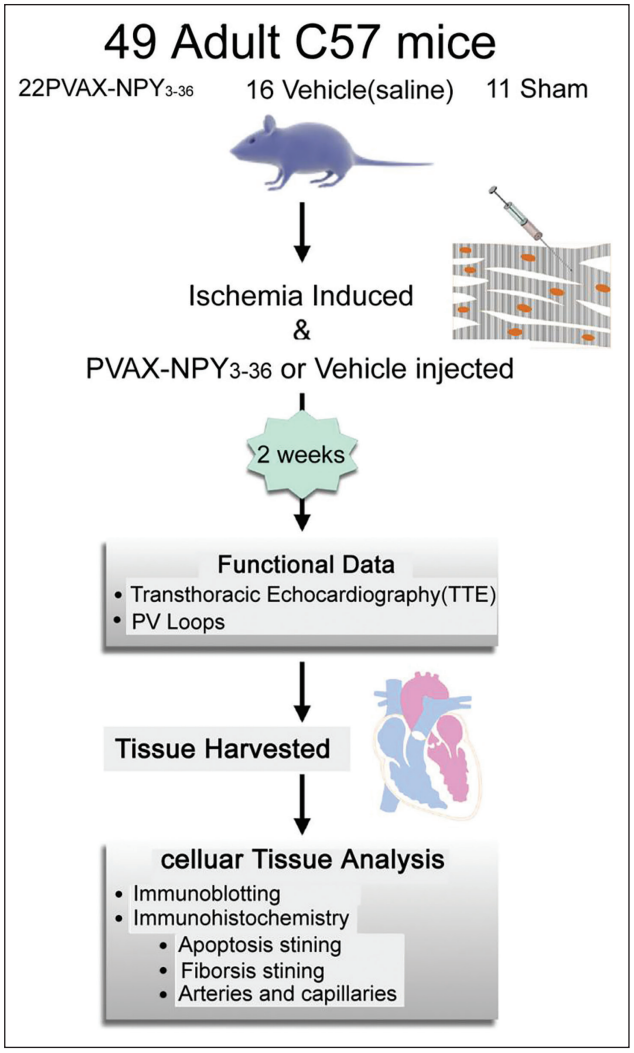


Figure 1. Schematic Graph of the Experimental Design. Fifty Mice were Divided into Three Groups: PVAX-NPY3-36 ($n = 22$), Empty Vehicle Group (Vehicle) ($n = 16$), and Sham Operation ($n = 12$).

Heart Function Test

The mouse's cardiac function was evaluated by echocardiography and the pulmonary venous circuit.

Quantification of Infarct Volume

The mouse hearts were extracted from the animals, fixed in formalin for 24 h, and embedded in paraffin. The paraffin sections were stained, and the infarction area was measured following the previous description.

Immunofluorescence

After dewaxing, tissue sections were incubated with 5% bovine serum albumin (BSA) (Sigma) for 1 h and probed with antibodies against CD-31 (1:100, CST) and smooth muscle actin (SMA) (1:200, Sigma) overnight. Upon washing, they were stained with Hoechst 33342 and sealed.

Western Blot Analysis

Total proteins were extracted from the cell lysate, and the bicinchoninic acid (BCA) method completed the protein concentration measurement. The proteins were separated through electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was probed with primary antibodies (human anti-rabbit 1:1,000, Sigma) overnight and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:2,000, Shanghai Huzhen Industrial Co., Ltd.) and secondary antibodies (1:10,000, Sigma) for 1 h. The samples were washed with phosphate-buffered saline (PBS), developed, and detected by ECL Plus Western Blotting Substrate (Thermo Scientific).

Statistical Analysis

Data were processed with Statistical Package for the Social Sciences (SPSS) 27.0 software and presented as $\bar{x} \pm s$. The comparison between groups was performed by analysis of variance (ANOVA). $p < .05$ indicates a significant difference.

Results

Administration with PVAX-NPY3-36 Reduces Mouse Mortality

Compared to the mortality of the Vehicle group (4/16, 25%), mice treated with PVAX-NPY3-36 exhibited decreased mortality (4/22, 18%), as no deaths appeared in the sham group. Among the four deaths in the Vehicle group, 1, 2, and 1 appeared on the 3rd, 5th, and 6th day after the operation, respectively. In the PVAX-NPY3-36 group, there was one death on the 3rd day, 1 on the 4th day, and 2 on the 5th day.

Assessment of Cardiac Function and Hemodynamics

As revealed by transthoracic echocardiography, PVAX-NPY3-36 caused a decline in the infarction area compared to normal saline treatment ($p = .01$). The particles significantly improved cardiac injury ($p = .05$) (Table 1).

As for heart function, echocardiography of the mouse heart was carried out 2 weeks after treatments. The results demonstrated that the PVAX-NPY3-36 group exhibited improved contractile force based on ejection fraction ($p = .01$). The heart expanded, accompanied by an increase in systolic and diastolic volumes. Until the presence of the nanoparticles, the heart size, end-systolic and end-diastolic volume were significantly improved with a functional enhancement in compliance and diastole and elevation of ventricular stroke, ejection fraction, stroke volume, and cardiac output (Table 2).

PVAX-NPY3-36 Enhances Angiogenesis

Relative to the ischemic tissue in the Vehicle group, the PVAX-NPY3-36 group presented a higher level of Ang-1 and vascular endothelial growth factor (VEGF). The highest level of transforming growth factor-beta (TGF-β) was noticed in the PVAX-NPY3-36 group. The PVAX-NPY3-36 group had a higher level of platelet-derived growth factor-beta (PDGF-β) than the sham group, and its receptors increased significantly. VEGF level increased in the PVAX-NPY3-36 group and the sham group relative to the vehicle group (Figure 2).

Mitochondrial Function

Increased levels of PGC-1α appeared in the ischemic tissues of PVAX-NPY3-36 and Vehicle groups, indicating the tissues responded to elevated mitochondrial reactive oxygen species

Table 1. Echocardiographic Parameters of Three Groups After Myocardial Ischemia/Reperfusion.

Measurement	Sham	Vehicle	PVAX-NPY3-36	*p* Value
Fractional shortening (%)	28.63 (3.36)	7.34 (0.6)	13.90 (1.9)	<.01
LVID, systole (mm/m^2)	2.6 (0.22)	5.07 (0.3)	4.24 (0.5)	<.01
LVID, diastole (mm/m^2)	3.65 (0.24)	5.47 (0.3)	4.92 (0.5)	.06
LV vol systole (μL)	25.02 (5.38)	122.60 (15.4)	81.46 (20.1)	<.01
LV vol diastole (μL)	56.97 (8.55)	146.48 (20.4)	115.45 (26.8)	<.01
Heart weight (g)	0.11	0.17	0.152	<.01
Infarction size (%)	N/A	3.78	2.81	.01
Body weight pre-MI (g)	24.01	24.58	24.49	.052
Body weight post-MI (g)	25.29	25.86	25.62	.06

Note: LVID: Left ventricular diameter; LV vol: Left ventricular volume; MI: Myocardial ischemia/reperfusion.

Table 2. Cardiac Hemodynamic Parameters of Three Groups.

Measurement	Sham	Vehicle	PVAX-NPY3-36	*p* Value
SV (μL)	15.2 (3.0)	12.4 (5.8)	19.1 (3.3)	.01
Cardiac output (mL/min)	8.1 (1.4)	4.7 (2.0)	9.3 (1.5)	<.01
Ejection fraction (%)	34.6 (4.3)	18.8 (8.4)	29.0 (4.4)	<.01
Ventricular stroke work (mL × mmHg)	1,914.3 (433.1)	1,075.9 (495.9)	1,576.3 (409.6)	<.01
Heart rate (bpm)	532.6 (45.5)	400.8 (78.0)	490.4 (72.5)	.004
dp/dT max (mmHg/s)	13,342.1 (1,900.9)	5,227.3 (1,272.6)	8,160.7 (2,137.7)	<.01
dp/dT min (mmHg/s)	−9,907.1 (1,703.1)	−4,384.0 (803.3)	−6,114.5 (1,287.6)	<.01

Note: LVID: Left ventricular diameter; LV vol: Left ventricular volume; MI: Myocardial ischemia/reperfusion; SV: Stroke volume.

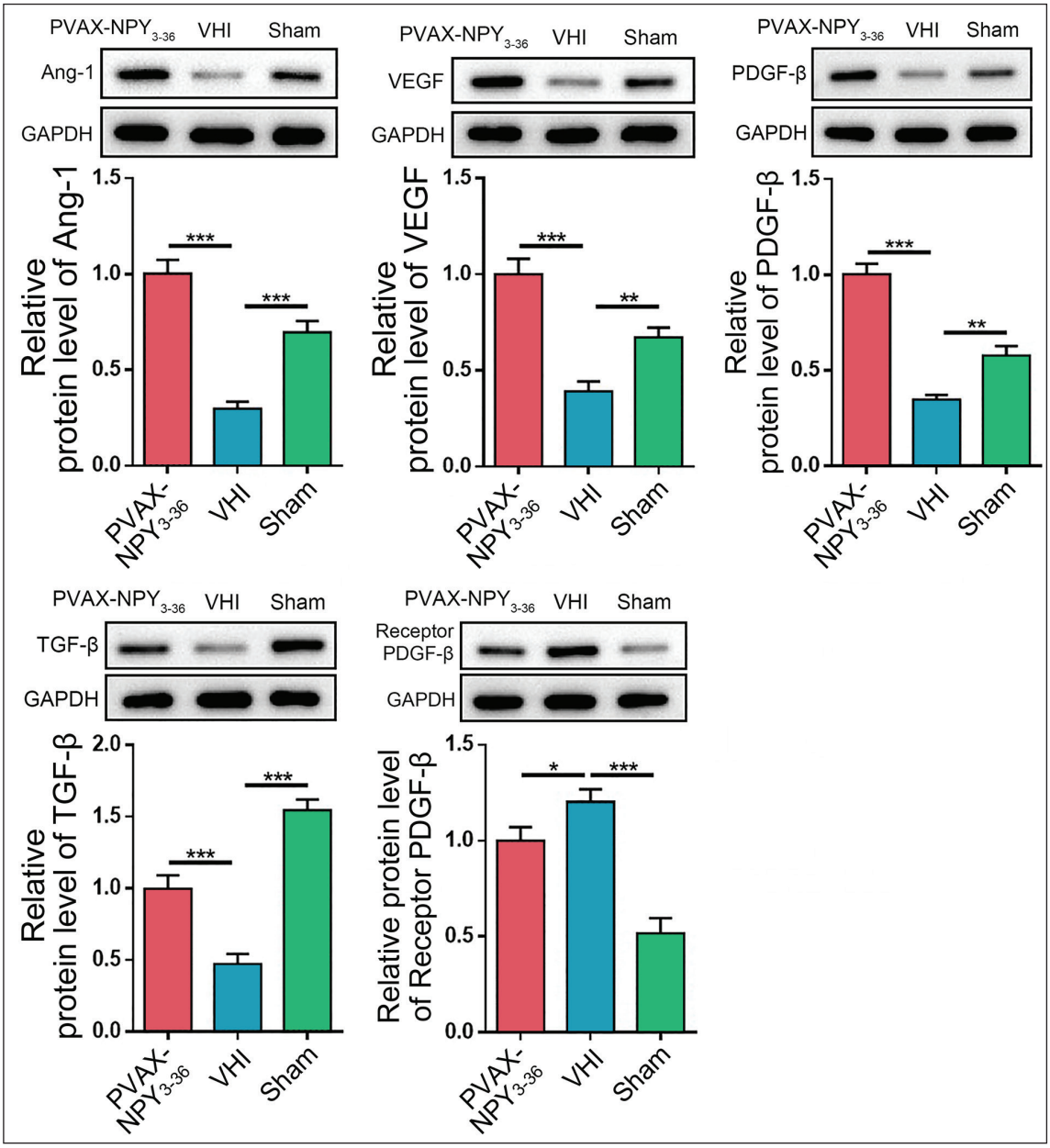


Figure 2. Markers of Angiogenesis. Western Blot Analysis of Ang-1, Vascular Endothelial Growth Factor (VEGF), Platelet-derived Growth Factor-beta (PDGF-β), PDGF-β receptor, and Transforming Growth Factor-beta (TGF-β) Expression upon Treatments. *$p < .05$.

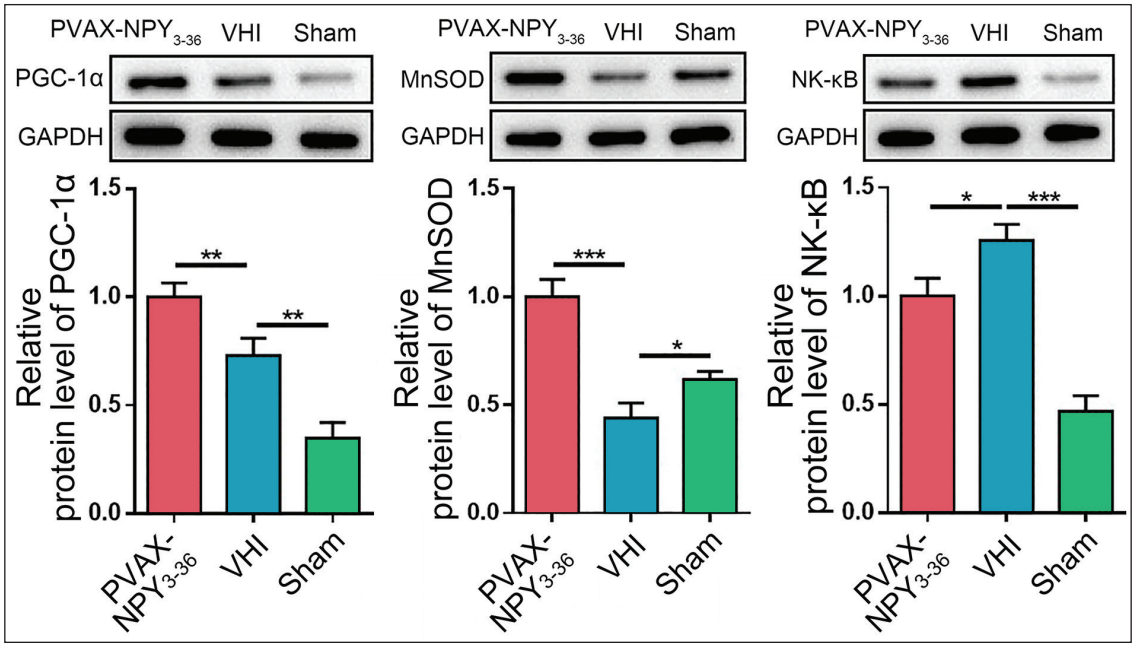


Figure 3. Western Blot Analysis of PGC-1α, MnSOD, and NK-κB Expression in Three Groups. *$p < .05$.

(ROS). The PVAX-NPY3-36 group and the sham group presented increased MnSOD levels. The NF-κB level of the Vehicle group was higher than that of the sham group ($p < .05$) (Figure 3).

PVAX-NPY3-36 Treatment Decreases Cell Apoptosis

Results from Western blot analysis showed that compared with the Vehicle group, the PVAX-NPY3-36 group had decreased expression of pro-apoptotic factor Smad5, with no difference in Bcl-XL between the two groups. Meanwhile, PVAX-NPY3-36 also decreased the expression of P-Akt compared to normal saline. Of note, the operation affected the level of anti-apoptotic factor Smad4, as demonstrated by the higher levels of Smad4 in Vehicle and PVAX-NPY3-36 than in the sham group (Figure 4).

PVAX-NPY3-36 Promotes Angiogenesis in the Myocardial Ischemic Area

Compared with the Vehicle group and the sham group, the PVAX-NPY3-36 group resulted in higher capillary density and increased arterioles (Figure 5).

Discussion

In this study, we elucidated the mechanism underlying PVAX nanoparticles carrying NPY3-36, and the results demonstrated a protective effect of the PVAX-NPY3-36 nanoparticles on the small blood vessels. We also proved that angiogenesis is linked to metabolism and apoptosis in myocardial tissue. Apart from the known impact of matrix metalloproteinases and cathepsins on angiogenesis (Matyal et al., 2012; Merz et al., 2006), our work depicts that the improved mitochondrial function in the experimental group might be attributed to the increased angiogenesis, inducing myocardial cell perfusion. The presence of NPY3-36 improved left ventricular remodeling and cardiac function, resulting in an elevated survival rate of mice with myocardial I/R. We confirmed the significance of PVAX to therapeutic drug delivery, as the composite particles specifically targeted the oxidative stress area secondary to I/R injury. Finally, the therapeutic potential of NPY in myocardial I/R injury was characterized.

With the PVAX nanoparticles, NPY3-36 was delivered directly to ischemic tissues, producing ROS. The role of nerves in regulating angiogenesis has been frequently reported (Mukouyama, 2014; Mukouyama et al., 2002, 2005; Pan et al., 2016). NPY3-36 is one of the neurotransmitters and is indicated to promote the formation of new blood vessels (Panting et al., 2002). In addition to its cardioprotective function, the function of NPY3-36 in myocardial growth was highlighted in this study. Our results indicated neuropathological loss associated with diseases like diabetes may decrease coronary microvessels or induce pathogenesis.

The activity of the sympathetic nerve in the cardiovascular system affects heart remodeling. Administration with β-blockers and other drugs is administered to patients with heart diseases, considering their anti-hypertensive effect and relieving effect on sympathetic nerve activity (Reis et al., 2001). However, the lack of sympathetic nerve activity also triggers resistance and disorders (Robich et al., 2010; Safdar et al., 2018). The mechanism underlying the decreased loss of

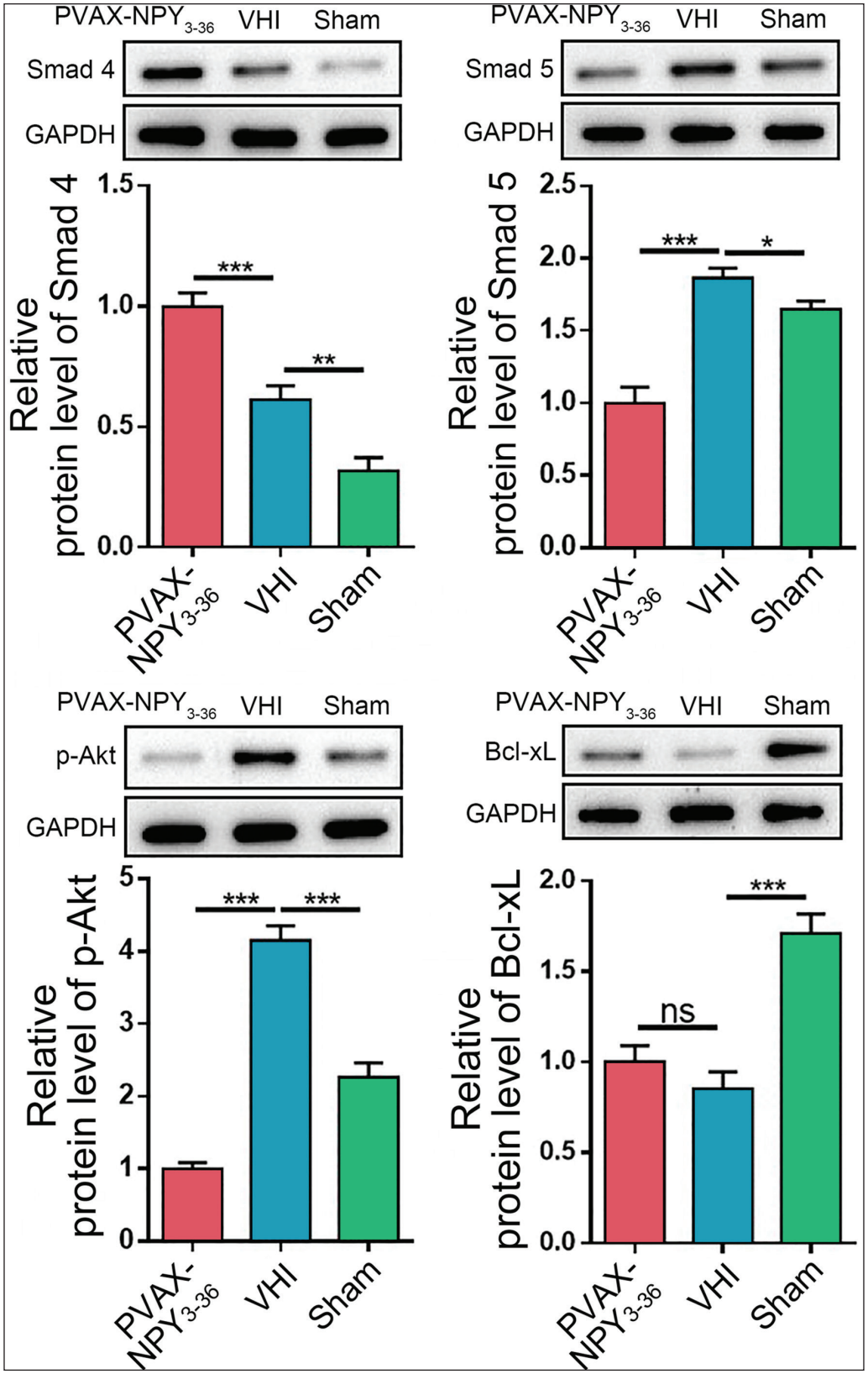


Figure 4. Western Blot Analysis of Smad4, Smad5, p-Akt and Bcl-xL Expression, and Apoptosis Analysis. *$p < .05$.

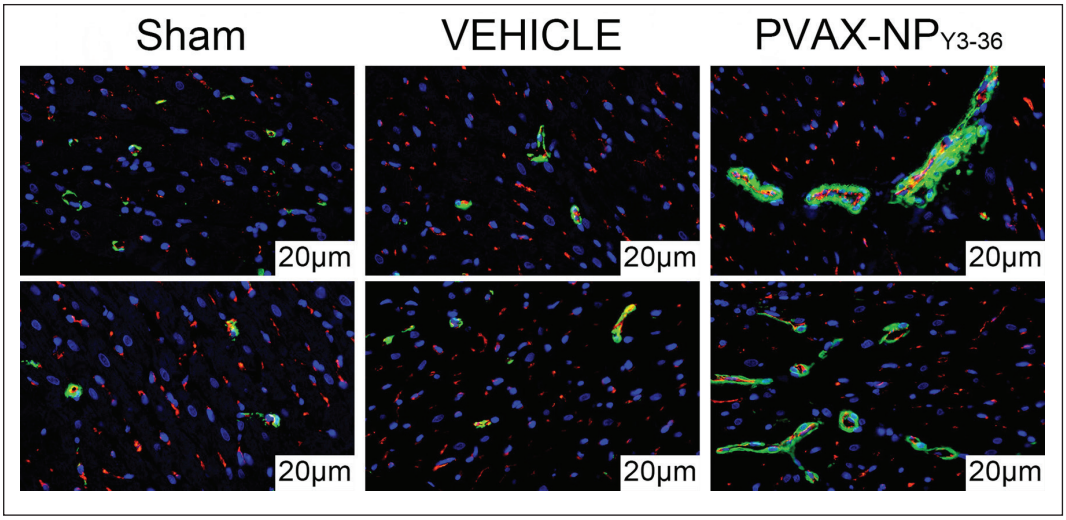


Figure 5. Immunohistochemical CD31 and Smooth Muscle Actin (SMA) Staining were used to Detect Capillary Density (Upper) and the Number of Small Arteries (Lower) in Three Groups (Red: Artery, Green: Capillary).

nerve-related growth factors or drug resistance affecting the cardiovascular system must be further identified. Although we have explored the mechanism *in vitro*, whether we can show the same results *in vivo* needs more verification. Next, we will elaborate on the cell line.

Conclusion

This study demonstrates that PVAX nanoparticles carry the neurotransmitter NPY3-36 to the myocardium, promoting angiogenesis, restoring myocardial function, and attenuating the injury caused by myocardial ischemia. Our following research will continue to identify the mechanism of PVAX-NPY3-3 by examination of the activities of matrix metalloproteinases, cathepsins, and anti-apoptotic and pro-apoptotic proteins in heart tissue. These findings might lay a foundation for the treatment of myocardial ischemia.

Abbreviations

I/R: Ischemia/reperfusion; NPY: Neuropeptide Y; PVAX: Neuropeptide Y3-36-loaded copolyoxalate-containing vanillyl alcohol.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval was obtained from the relevant ethics committee or Institutional Review Board (IRB).

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Informed Consent

The participant has provided informed consent for the submission of the article to the journal.

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