Original Article
The in vitro protective effect of PNS in myocardial reperfusion injuries mediated through the JAK2/STAT3-SIRT1 signaling pathways

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Abstract: Background: The present study aimed to investigate the function of the JAK2/STAT3 and the SIRT1 signaling pathways in the normal myocardium, and the protective effect of PNS in myocardial ischemia injury. Material and methods: JAK2 siRNA was transfected, and the MTT assay was applied to detect the cardiomyocytes' viability, and Western blotting was employed to measure the protein expressions. Following this, the impacts of JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA SIRI on the protective effects of PNS on the expressions of p-JAK2, JAK2, p-STAT3, STAT3, SIRT1, and Ac-FOXO1 in SIRI cardiomyocytes were measured to explore the role of PNS within. Result: The experiment found that, in the normal myocardial cell model, JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA had no significant effect on myocardial viability. JAK2 siRNA and STAT3 siRNA can inhibit both the JAK2/STAT3 and SIRT1 pathways. SIRT1 siRNA can inhibit the SIRT1 pathway and reduce p-JAK2 and p-STAT3 expressions. In the PNS-administered SIRI cardiomyocyte model, JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA can reverse the protective effects of PNS on cardiomyocytes, through which PNS could significantly increase the p-JAK2, p-STAT3 and SIRT1 expressions. Both the JAK2/STAT3 and SIRT1 pathways activated by PNS were reversed by JAK2 siRNA and STAT3 siRNA, and likewise in the JAK2/STAT3 pathway, the p-JAK2 and p-STAT3 expressions decreased, while in the SIRT1 pathway, SIRT1 decreased and Ac-FOXO1 increased. Moreover, SIRT1 siRNA can reverse the SIRT1 pathway activated by PNS, in which SIRT1 decreased, Ac-FOXO1 increased, and the JAK2/STAT3 pathway was also affected as the p-JAK2 and p-STAT3 expressions increased. Conclusions: In the normal cardiomyocyte model, JAK2 siRNA, STAT3 siRNA, and SIRT1 siRNA have no significant effects on myocardial viability but can reverse the protective effects of PNS on cardiomyocytes.

Keywords: PNS, JAK2/STAT3-SIRT1 signaling pathways, myocardial ischemia and reperfusion, myocardial protection

Introduction

Ischemic heart disease (IHD), one of the deadliest threats to humans, can lead to cardiac arrhythmias, heart failure, and even death [1]. Coronary stenosis or obstruction is the main cause of IHD, and cardiopulmonary bypass surgery is another possible cause [2]. The key to IHD treatment is restoring the blood supply; however, after reperfusion, the myocardium may undergo more severe damage, which is called ischemia reperfusion injury (IRI), and IRI is the main cause of the poor prognosis of IHD patients.

Currently there are many drugs for the prevention of myocardial IRI, such as calcium antago-

nists [4], β-blockers [5], angiotensin converting enzyme inhibitors [6], etc. However, in recent years, a number of natural substances characterizing the advantages of safety and nontoxic, anti-inflammatory, anti-oxidative stress and anti-apoptotic properties have become the hot research topics of anti-myocardial IRI medicines.

PNS, one of these natural substances, can attend stasis and dredge blood vessels, inhibit platelet aggregation and increase cerebral blood flow and is presently used mainly for curing cerebrovascular sequelae, central retinal vein occlusion, and anterior chamber hemorrhage. PNS can significantly improve the performance of ischemic ECG, reduce myocardial

infarct size, reverse the left ventricular diastolic dysfunction of angina patients and even restore it back to normal.

Methods

The primary culture of neonatal rat cardiomyocytes and the establishment of the SIRI model

A group of 10 SD neonatal rats was obtained and disinfected. Up to the left side of the rat xiphoid, the rat chest was cut out, while instantly the rat body was squeezed to remove the heart. The heart was washed in PBS, and the atria was removed, while the ventricle tissues were retained. The ventricular myocardium was shredded, and then put into a HEPES solution of 1 mg/ml collagenase I to digest. The digestion was repeated 3 times, 5 min each time. Subsequently, the digestion was quenched with a solution of DMEM containing 10% serum. Following this, the solution was centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and 100 U/ml penicillin and 100 U/ml streptomycin were added into the DMEM solution containing 10% serum and 5-Brdu to resuspend the cells. The suspension cells were put into culture flasks, and then placed in a cell incubator for 1.5 h differential adhesion to remove cardiac fibroblasts.

The non-adherent cells were collected with a 200-mesh screen filter and the undigested tissues were removed. The filtrated cell suspension was collected and the number of cells was calculated. The cells were seeded into different types of flasks or plates. Following this, the cells were put into an incubator, and the medium was changed every 24 h. After 3-4 days, the cells were observed, and cells jumping in a film indicated model establishment. The cardiomyocyte SIRI process was simulated through classic hypoxia. Subsequently, the cells were placed in an incubator with 95% N_2 and 5% CO_2 for 2 h. While in re-oxygenation, the cells were placed in a normal culture medium and a normal incubator for 4 h.

Transfection of JAK2 siRNA, STAT3 siRNA and SIRT siRNA

The cells were seeded into 6 wells, with each well 2×10^5, and a fetal bovine serum DMED high glucose medium without antibiotics was added. The cells were placed in an incubator with CO_2 at 37°C for 18-24 h, and the incubation was discontinued when the cell fusion level reached 80%. Next, an AB solution was prepared. A solution: 20-80 pM JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA were added into 100 µl transfected medium, and mixed. B solution: 8 µl siRNA transfected reagent was added into 100 µl transfected medium, and mixed. The A solution and B solution were mixed and placed at room temperature for 45 min. The cells were washed once with a 2 ml siRNA-transfected culture. Following this, a 0.8 ml siRNA-transfected culture was added into the A + B mixture. The solution was gently agitated and then added into the washed cells. The cells were put into an incubator with CO_2 at 37°C for 7 h. To each well we added 1 ml of a medium containing fetal bovine serum (20%) and antibiotics. The plate was put in the incubator for 24 h. Subsequently, the culture medium was removed and replaced with a normal medium. The cells were used for further experiments after 24 h.

*Measuring cardiomyocyte viability using MTT assay*

The effects of JAK2 siRNA, STAT3 siRNA, and SIRT1 siRNA on the JAK2/STAT3 and SIRT1 pathways were observed. The experiment was conducted in 4 groups: A, the control siRNA + SIR group; B, the JAK2 siRNA + SIR group; C, the STAT3 siRNA + SIR group; and D, the SIRT1 siRNA + SIR group. Cell viabilities and the expressions of JAK2, p-JAK2, STAT3, SIRT1 and Ac-FOXO1 were measured. The effects of JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA on the JAK2/STAT3 and SIRT1 pathways during the process of PNS in the myocardium SIRI were observed.

The following experiment was conducted in each of the 5 groups: A, the Control siRNA SIR group; B, the Control + siRNA + PNS 50 µm + SIR group; C, the JAK2 siRNA + PNS 50 µm + SIR group; D, the STAT3 siRNA + PNS 50 µm + SIR group; and E, the SIRT1 siRNA + PNS 50 µm + SIR group. The cells from these groups were collected. A total of 100 µl phenol red-free DMEM culture medium and 10 µl 0.5% MTT solution were added. After 4 h incubation, the medium was discarded, and to each well we added 100 µl DMSO, and agitated it for 15 min. The OD value at wavelength 490 nm was measured with a microplate reader.

Figure 1. The impacts of JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA on normal cardiomyocytes.
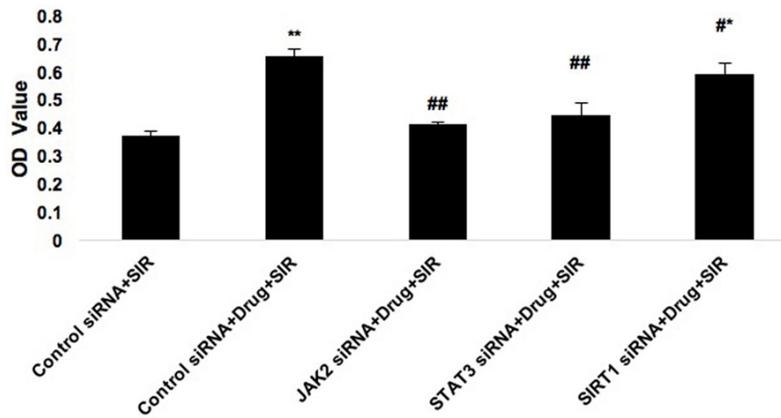
Figure 2. The impacts of PNS on myocardial viability. **P < 0.01 vs Control siRNA + SIR, *P < 0.05 vs Control siRNA + SIR, ##P < 0.01 vs Control siRNA + Drug + SIR, #P < 0.05 vs Control siRNA + Drug + SIR.

Detecting LDH, MDA, and SOD contents

The previously prepared medium was used to measure the LDH, MDA and SOD contents according to the kit's instructions.

Detecting PNS effects through a TUNEL assay

The administered cells were seeded into a 24-well plate at a density of 1×10^3 per well for complete adhesion. The liquid was abandoned. The cells were washed three times with 1×PBS, 5 min each time, and then were fixed with paraformaldehyde for 20 min at room temperature. Subsequently, the cells were washed three times with 1×PBS, 5 min each time, and then permeabilized with 0.5% Triton X-100 for 15 min. The cells were washed three times with 1×PBS, 5 min each time. Following this, Triton X-100 (0.1%) was added dropwise for 3 min, and the cells were agitated and washed three times with PBS, 5 min each time. The procedure was implemented in strict accordance with the Roche's TUNEL assay kit instructions. The cells were incubated in a dark room at

37°C for 1 h, and then they were agitated and washed three times with PBS, 5 min each time. The nuclei were stained with DAPI for 2 min, and then washed with PBS 3 times, 5 min each time. Subsequently, the cells were mounted with 50% glycerol and photographed using fluorescence microscopy.

Measuring the expressions of JAK2, p-JAK2, STAT3, p-STAT3, SIRT1 and Ac-FOXO1 proteins using Western blot

The cells from all the groups were collected and washed twice with PBS. 400 μl lysate and 40 μl PMSF were added to each flask. The flask was gently agitated and then placed on ice for 10 min to lyse the cells uniformly. The cells were repeatedly aspirated with a sterile syringe, and the lysate was added to an EP tube, which was ice-bathed for 30 min and then centrifuged at 12000 rpm for 15 min. The supernatant was decanted to a new EP tube. 20 μl buffer was added to each tube, then they were boiled for 5 min, mixed, and then stored at -80°C.

The above samples were obtained to conduct electrophoresis in 12% SDS-PAGE to separate the proteins. The protein bands were transferred to a PVDF membrane using the wet method and closed for 1 h at room temperature. The primary antibody (1:1000) was added and incubated at 4°C overnight. Subsequently, the primary antibody was eluted, and the secondary antibody (1:1000) was added and incubated for 1 h. The secondary antibody was eluted. Following this, chemiluminescence was applied to conduct color development and fixing. The expressions of JAK2, p-JAK2, STAT3, p-STAT3, SIRT1, and Ac-FOXO1 were measured.
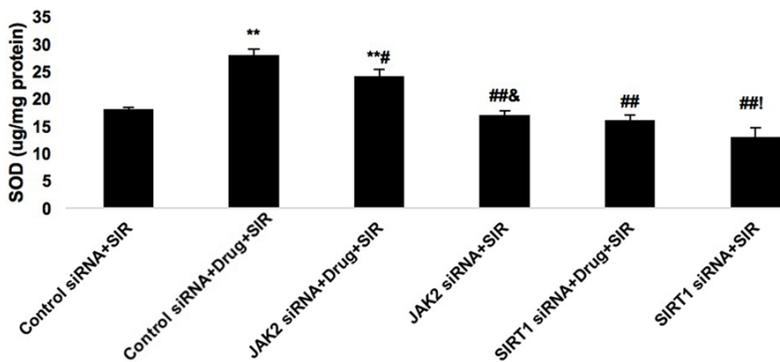
Statistical methods

All the data are expressed in the form of the mean ± SD. The comparisons between the two
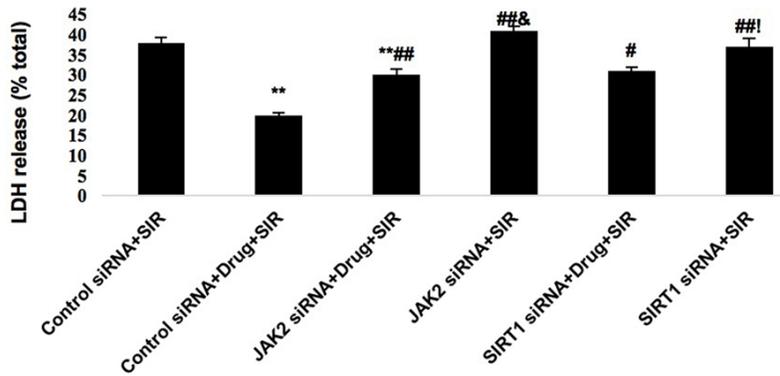
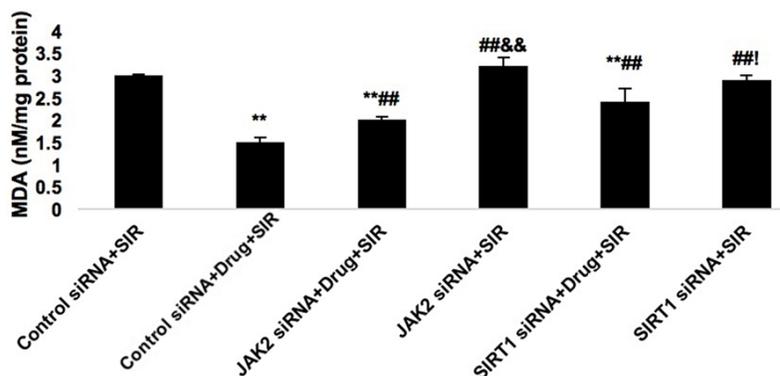**Figure 3.** The impacts of PNS on LDH levels in the cardiomyocyte medium. **P < 0.01 vs Control siRNA SIR ##P < 0.01 Control siRNA Drug SIR #P < 0.05 Control siRNA Drug SIR &P < 0.05 JAK2 siRNA Drug SIR !P < 0.05 SIRT1 siRNA Drug SIR.

Figure 4. The impacts of PNS on SOD viability in the myocardial medium. **P < 0.01 vs Control siRNA SIR ##P < 0.01 Control siRNA Drug SIR #P < 0.05 Control siRNA Drug SIR &P < 0.05 JAK2 siRNA Drug SIR !P < 0.05 SIRT1 siRNA Drug SIR.

Figure 5. The effects of PNS on MDA levels in the SIRI myocardial medium. **P < 0.01 vs Control siRNA SIR ##P < 0.01 Control siRNA Drug SIR #P < 0.05 Control siRNA Drug SIR &P < 0.05 JAK2 siRNA Drug SIR !P < 0.05 SIRT1 siRNA Drug SIR.

groups were conducted using a *t* test, and multiple sets of data (> 2) were compared using a one-way ANOVA, with P < 0.05 indicating statistical significance. All the data were analyzed with the Graph-Pad Prism 5.0 (GraphPad Software, San Diego, CA).

Results

The impacts of JAK2 siRNA, STAT3 siRNA and SIRT1 siRNA on normal cardiomyocytes

In the normal cardiomyocyte experiment, no significant impacts of JAK2 siRNA, STAT3 siRNA or SIRT1 siRNA on myocardial viability were found (**Figure 1**).

The impacts of PNS on myocardial viability

About 2-3 days after the successful culture of the cardiomyocyte model, the myocardial viability of each group was measured. As shown in **Figure 2**, PNS can significantly increase the SIRI myocardial viability (in comparison with the control siRNA + SIR group, P < 0.01). However, JAK2 siRNA, STAT2 siRNA, and SIRT1 siRNA can all reverse the protective effect of PNS on cardiomyocytes (**Figure 2**).

The impacts of PNS on LDH levels in the SIRI cardiomyocyte medium

After being administered by PNS, the release of cardiomyocyte LDH after SIR was effectively reduced. JAK2 siRNA can reverse the protective effect of PNS and increase LDH release. However, JAK2 siRNA + SIR had no significant effect on cell LDH, and the results of the experiment displayed identical expressions in both SIRT1 siRNA and JAK2 siRNA (**Figure 3**).
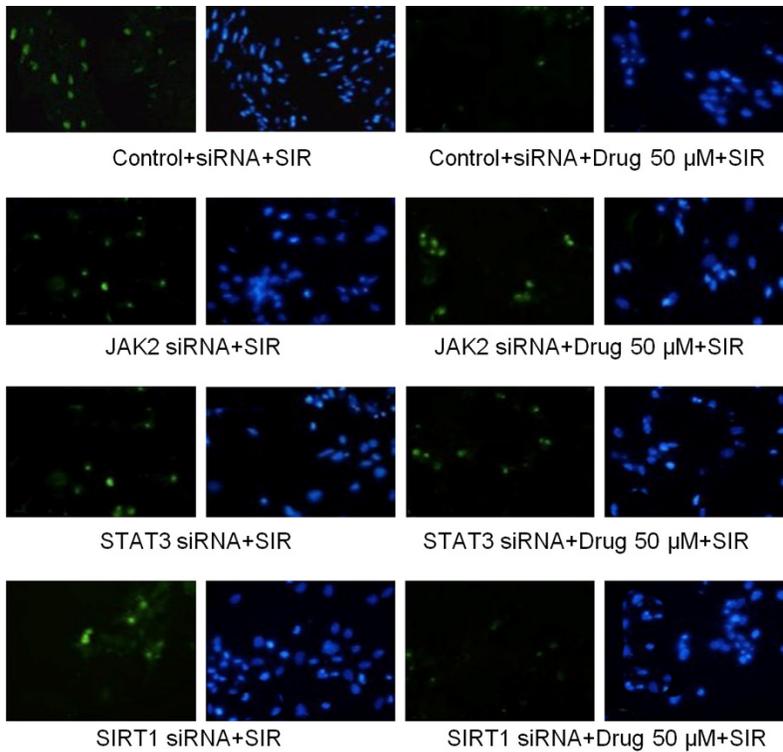
Figure 6. The effects of PNS by TUNEL assay.

The impacts of PNS on MDA levels in the myocardial medium

The MDA levels after SIR can be effectively reduced by PNS medication, but JAK2 siRNA can reverse the protective effect and increase MDA expressions. JAK2 siRNA + SIR had no significant effect on MDA. The expressions of SIRT1 siRNA and JAK2 siRNA were identical. See **Figure 5**.

Measuring PNS effects using TUNEL assay

Compared with the IR group, PNS medication can significantly reduce the myocardial apoptosis rate after cardiac reperfusion: D, STAT2 siRNA + 50 μm PNS + SIR; E, SIRT1 siRNA + 50 μm PNS + SIR. See **Figure 6**.
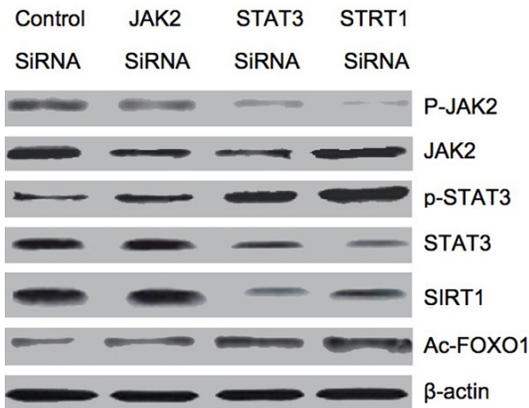

Figure 7. The impacts of JAK2 siRNA, STAT3 siRNA, and STRT1 siRNA on the p-JAK2, JAK2, p-STAT3, STAT3 and SIRT1 expressions.

The impacts of PNS on SOD levels in SIRI cardiomyocyte medium

Myocardial SOD viability after SIR can be increased by medication, but JAK2 siRNA can reverse the protective effect of PNS and reduce SOD viability. The expressions of SIRT1 siRNA and JAK2 siRNA are identical, as shown in **Figure 4**.

Measuring the expressions of p-JAK2, JAK2, p-STAT3, STAT3, SIRT1 and Ac-FOXO1 using Western blot

The Western blot results showed that, compared with the control siRNA group, JAK2 siRNA, STAT3 siRNA, and STAT1 siRNA can significantly reduce the expressions of p-JAK2, JAK2, p-STAT3 and SIRT1 and increase Ac-FOXO1 expression (**Figure 7**).
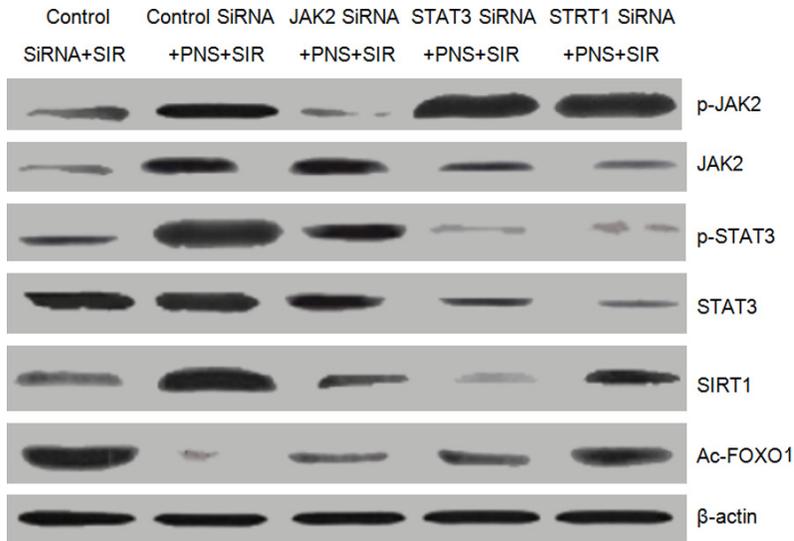
The impacts of JAK2 siRNA, STAT3 siRNA, and STRT1 siRNA on the expressions of p-JAK2, JAK2, p-STAT3, STAT3, SIRT1, and Ac-FOXO1 in PNS-medicated SIRI cardiomyocytes

The results showed that, compared with the control siRNA + SIR group, PNS can clearly increase the expressions of p-JAK2, p-STAT3 and SIRT1, and reduce Ac-FOXO1 expression. After JAK2 siRNA, STAT3 siRNA, and STRT1 siRNA were added, the effects of PNS on the protein expressions were reversed, and the expressions of p-JAK2, JAK2, p-STAT3, STAT3, and SIRT1 were decreased, as shown in **Figure 8**.

Figure 8. The impacts of JAK2 siRNA, STAT3 siRNA, and SIRT1 siRNA on the protein expressions in PNS-medicated SIRI cardiomyocytes.

Discussions

JAK2/STAT3 has become a hot topic in cardiovascular disease treatment. When a cytokine combines with a JAK2 receptor, JAK2 will be activated through self-phosphorylation. The activated JAK2 can phosphorylate one or more receptors, which are moved to STAT3 to phosphorylate and dissociate it, and then are transferred to the nucleus to regulate gene expressions. The JAK2/STAT3 signaling pathway plays an important role in the pathophysiological process of a variety of cardiovascular diseases. Myocardial ischemia can lead to a number of cell death activities, including apoptosis, necrosis and autophagy. The STAT protein family plays a substantial role in regulating cardiomyocyte apoptosis, in which STAT3 can protect myocardial cells [7, 8].

Hilfiker et al. found that, after being processed by IRI, the infarction areas in lab mice, which are eliminated by the cardiac-specific STAT3, are larger than those in the wild mice, and have a higher mortality [8]. The elimination of cardiac-specific STAT3 is sensitive to inflammatory injury. For instance, IRI-induced inflammation, especially the inflammatory injury after myocardial necrosis, is expressed more significantly in vivo in mice which is knocked out by STAT3 [9-11]. The JAK2/STAT3 pathway is an important part of the survivor activating factor en-

hancement (SAFE), playing a protective role in reperfusion injury through TNF-α activation [12-15]. Huffman et al. found that the blood flowing from the heart of IPC coronary can activate STAT3 in the receptor heart when transfused to the receiving heart, protecting the cardiomyocytes.

In recent years, the role of SIRT1 in ischemic diseases has attracted widespread attention. Studies have found that SIRT1 expression is decreased in the mononuclear cells of patients with stable coronary heart disease and acute coronary syndrome [16]. SIRT1 can inhibit cell apoptosis and senescence caused by ischemia, making it important in maintaining heart function in ischemia. Studies have also found that SIRT1 plays a protective role in myocardial ischemic disease and can enhance myocardial anti-oxidation and anti-apoptosis by activating or inhibiting related downstream molecules, inhibiting inflammation, and ameliorating injuries caused by ischemia. Therefore, SIRT1 might be a potential target for the treatment of ischemic heart disease [17-19].

Studies of normal cardiomyocytes have found that, JAK2 siRNA, STAT3 siRNA, and SIRT1 siRNA had no significant effects on normal myocardial viability. JAK2 siRNA and STAT3 siRNA can inhibit both the JAK2/STAT3 pathway (i.e. the expressions of p-JAK2, JAK2, p-STAT3 and STAT3 decreased) and the SIRT1 pathway (i.e. SIRT1 expression decreased, Ac-FOXO1 expression increased). SIRT1 siRNA can inhibit the SIRT1 pathway (i.e. SIRT1 expression decreased, Ac-FOXO1 expression increased), and reduce the expressions of p-JAK2 and p-STAT3. Therefore, there is an interaction between SIRT1 and the JAK2/STAT3 pathways in normal cardiomyocytes, which can jointly regulate the physiological functions of myocardial cells.

The study of the PNS-medicated SIRI cardiomyocyte model found that JAK2 siRNA, STAT3

siRNA, and SIRT1 siRNA can all reverse the protective effects of PNS on cardiomyocytes. The expressions of related molecules showed that PNS can significantly increase the expressions of p-JAK2, p-STAT3, and SIRT1.

JAK2 siRNA and STAT3 siRNA can reverse both the JAK2/STAT3 pathway activated by PNS (expressions of p-JAK2 and p-STAT3 decreased), and the SIRT1 pathway (SIRT1 expression decreased, Ac-FOXO1 expression increased). While SIRT1 siRNA can reverse the SIRT1 pathway (SIRT1 expression decreased, Ac-FOXO1 expression increased) and have a significant impact on the JAK1/STAT3 pathway (expressions of p-JAK2 and p-STAT3 decreased).

Conclusion

In summary, in the protective effect of PNS, there is a cross-talking interaction between the JAK2/STAT3 and SIRT1-FOXO1 pathways that mediates the PNS-medicated myocardial IRI effect.

Disclosure of conflict of interest

None.

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