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Original Article

Panax notoginseng saponins protects H9c2 cardiomyocytes against hypoxia/reoxygenation injury via activating the JAK2/STAT3 pathway

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Abstract: Panax notoginseng saponin (PNS) is isolated from Panax notoginseng, a traditional chinese herbal medicine with cardioprotective effects. Accumulating evidence has confirmed that the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway plays a vital role in mediating cardioprotection against ischemia/reperfusion (I/R) injury. Herein, the purpose of this study was to investigate the role of the JAK2/STAT3 pathway in the cardioprotective effects of PNS during hypoxia/reoxygenation (H/R) injury in H9c2 cardiomyocytes. The results showed that PNS pretreatment significantly blocked H/R-induced down-regulation of cell viability and the up-regulation of LDH release in H9c2 cells. Meanwhile, PNS obviously reversed H/R-induced decrease in the ratio of p-JAK2/JAK2 and p-STAT3/STAT3, indicating that PNS activates the JAK2/STAT3 pathway. Subsequently, the results revealed that, JAK2 siRNA transfection reduced JAK2 miRNA and protein levels as well as p-STAT3/STAT3, and further remarkably abolished PNS-inhibited H/R-induced cytotoxicity and apoptosis as revealed by the decrease in cell viability and the increases in LDH release, apoptosis ratio, caspase-3 activity and Bax/Bcl-2 ratio. In addition, PNS reversed H/R-induced increase in ROS generation and MDA content, and decreased the SOD and GSH-PX activities, while these effects were blocked by JAK2 miRNA. These results suggest that PNS ameliorates H/R-induced apoptosis and oxidative stress by activating the JAK2/STAT3 pathway in H9c2 cardiomyocytes.

Keywords: Panax notoginseng saponins, myocardial ischemia-reperfusion injury, JAK2/STAT3 pathway, apoptosis, oxidative stress

Introduction

Ischemia/reperfusion is often associated with angioplasty, thrombolysis and coronary bypass surgery which improve myocardial ischemia, but simultaneously induce further damage to myocardial tissue; this is known as myocardial ischemia/reperfusion (MI/R) injury [1]. So far, MI/R injury still remains the leading cause of mortality and disability worldwide in various cardiovascular diseases [2]. Although there is a lot of research is about the defense of MI/R injury, there is not a very effective means to treat this disease. It has been widely accepted that apoptosis plays a pivotal role in the progress of MI/R injury and the reduction of cardiomyocyte apoptosis may be a potential effective therapy for attenuating MI/R injury [3, 4]. On the other hand, accumulating experimental evidence reveals that the development of oxidative stress induced by the production of reactive oxygen species (ROS) during the acute reperfusion phase contributes to the pathogenesis of MI/R injury [5, 6], indicating that protecting cardiomyocytes from ROS damage could be another rational method for ameliorating MI/R injury. Therefore, it is necessary to investigate novel strategies and the possible regulatory mechanism related to decreasing apoptosis and inhibiting oxidative stress which may result in protective effects against MI/R injury.

Panax notoginseng saponins (PNS) is one of the most important compounds isolated from a traditional Chinese herbal medicine: Panax notoginseng (Burkill) F.H. Chen (Araliaceae), has been frequently-used in treating hemorrhagic disorders, ischemic cerebrovascular disease

and coronary heart disease in China over hundreds of years [7, 8]. Emerging evidence suggests that PNS has significant therapeutic effects and multiple pharmacological functions in a variety of cardiovascular diseases (CVDs), resulting in four clinical trials completed and/or currently underway [9]. PNS exhibits beneficial effect against anti-apoptosis, anti-oxidative stress, anti-inflammation, anti-atherosclerosis, and promotes angiogenesis [10, 11]. In the last decade, the versatile effects of PNS against MI/R injury has attracted much attention, and there's plenty of research showing that PNS may be therapeutically useful for ameliorating MI/R injury [12, 13]. Research from Li X et al, shows that PNS prevents cardiac ischemia induced by ischemia and reperfusion in a rat model [14]. Another study elicits that ginsenoside Rg1, a major active ingredient of PNS, prevents rat cardiomyocytes from hypoxia/reoxygenation (H/R) oxidative injury [15]. Nevertheless, the underlying protective mechanisms of PNS on MI/R injury have not been elucidated.

Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway is a stress-responsive mechanism that has been implicated in variety of cardiac pathophysiologic processes including hypertrophy, apoptosis, oxidative stress, myocardial interstitial fibrosis, and myocardial infarction signaling [16-19]. Recent studies have demonstrated that JAK/STAT, particularly JAK2/STAT3 signaling, plays a vital role in mediating cardioprotection against ischemia/reperfusion (I/R) injury [20-22]. Many studies report that JAK/STAT pathway activation is essential for multiple myocardial protective effects such as opioid and isoliquiritigenin-induced cardioprotection *in vivo* and *in vitro* [23, 24]. However, our understanding of the functional consequences of JAK2/STAT3 activation in MI/R injury is still incomplete and it is unknown whether PNS induces cardioprotection through regulating the JAK/STAT pathway.

Herein, the present study, a rat cardiomyocyte cell line (H9c2 cardiomyocytes) treated by hypoxia/reoxygenation (H/R) was used to simulate MI/R injury *in vivo*. On this foundation, we investigated the protective effects of PNS on H/R-induced apoptosis and oxidative stress, and the functions of JAK2/STAT3 pathway in these processes. We first confirm that PNS ameliorates H/R-induced H9c2 cardiomyocytes injury through the activation of JAK2/STAT3 pathway.

Materials and methods

Cell culture

Embryonic rat myocardium-derived cells (H9c2 cardiomyocytes) were purchased from Shanghai Tiancheng Technology Co (Shanghai, China) which are a well-characterized cell line used to study myocardial ischemic and reperfusion injury *in vitro* [25]. H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (both from GIBCO Grand Island, NY), 100 U/mL of penicillin and streptomycin (Beyotime, China) at 37°C in a humidified atmosphere with 5% CO_2. Cell-culture medium was changed every 2 to 3 days.

Hypoxia/reoxygenation (H/R) injury model and drug treatment

To induce H/R injury model, H9c2 cells were incubated in serum-free DMEM and placed in hypoxic conditions containing 0.1% O_2, 5% CO_2 and approximately 95% N_2 at 37°C for 6 h. Then, H9c2 cells were reoxygenated for 4 h by incubation under normoxic conditions in serum-containing DMEM and a humidified atmosphere with 5% CO_2 and 95% air. In order to investigate the effects of PNS (Sigma-Aldrich, St. Louis, MO, USA) on H/R injury, PNS at different concentrations (5, 25, and 50 mg/L) was added to H9c2 cells for 1 h prior to H/R treatment.

JAK2 siRNA transfection

The siRNA transfection was carried out using Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The target sequence for JAK2 siRNA was as follows: sense, 5'-GCUCAAAUGAAAGUAGAAUTT-3', antisense, 5'-AUUCUACUUUCAUUUGAGCTT-3'. Briefly, H9c2 cells were seeded into a 6-well plate in 2 mL of antibiotic-free DMEM supplemented with FBS overnight. After growing to 70-90% confluence, the pre-prepared complexes containing siRNA transfection reagents and siRNA transfection medium were added to the cells. After 4-6 h of incubation, cells were placed in normal DMEM medium with FBS. In order to determine the

role of JAK2/STAT3 signaling pathway in the protective effects of PNS on H/R-induced injury, H9c2 cells were transfected with JAK2 siRNA and were treated with PNS (25 mg/L) followed by H/R treatment.

MTT assay for cell viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to evaluate the viability of cells according to manufacture's instructions. In brief, H9c2 cells in a 96-well plate were exposed to different interventions as described earlier and were incubated with MTT reagent (Sigma, 5 mg/mL) for 4 h at 37°C. Then, dimethyl sulfoxide (DMSO, 100 μl/well) was added to terminate the reaction and dissolve the formazan crystals. The optical density (OD) value at 490 nm was detected using the epoch microplate reader (Biotek, CA, USA). The viability of cells was expressed as percentage of OD value in groups with reference relative to that of control group.

LDH release assay for cytotoxicity

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme which is released into the supernatant resulting from cell membrane damage and cell lysis. The level of LDH in the culture supernatant was proportional to the degree of cells necrosis [26]. The cytotoxicity was evaluated using LDH release assays kits according to manufacture's instructions (Sigma, USA). Briefly, the culture supernatant (50 μL) romf each group was transferred to a 96-well plate and mixed with reaction regents (50 μL) and incubated for 30 min at room temperature in the dark. Then, a stop solution (50 μL) was added to each well for 30 min. The absorbance at 490 nm was read using an epoch microplate reader. Each experiment was performed in triplicate.

Real-time PCR analysis

To evaluate whether the level of JAK2 mRNA was effectively reduced after JAK2 siRNA transfection, total RNA in H9c2 cells undergoing different treatments was extracted with TRIzol reagent (Invitrogen, USA). Then RNA was reverse transcripted to cDNA and amplified using SuperScript One-StepRT-PCR system (Invitrogen, USA). After cDNA synthesis, PCR was performed at 95°C for 30 s, alternating with 60°C for 30 s and 72°C for 45 s for 40 cycles. The final step was performed at 72°C for 10 min. The primers and probes (Takara Bio, Dalian, China) used were as follows: JAK2 forward, 5'-TTTGAAGACAGGGACCCTACACAG-3' and reverse, 5'-TCATAGCGGCACATCTCCACA-3'; and β-actin forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3' and reverse, 5'-GACTCATCGTACTCCTGCTTGCTG-3'. β-actin was used as an internal control. The relative ratio of JAK2/β-actin was quantified by the $2^{-\Delta\Delta CT}$ method. The result was calculated and used as an indication of the relative expression level in the experimental group to control group.

Hoechst 33258 staining

The morphological changes of apoptosis were observed by Hoechst 33258 (Beyotime, China) according to manufacture's instructions. After treatment as discussion above, the H9c2 cells were fixed with cold 4% formaldehyde for 10 min at room temperature and then washed with cold phosphate buffered saline (PBS) three times. Subsequently, cells were incubated with Hoechst 33258 staining solution for 10 min in the dark. They were then washed with PBS three times again The nuclear morphology of the H9c2 cells was detected under a fluorescence microscope. Five independent fields (magnification, × 200) were randomly assessed from each well to calculate the average of apoptosis ratio. The result was expressed as the ratio of apoptotic cells versus total cells.

Caspase-3 activity assay

The activity of caspase-3 in H9c2 cells incubated with different intervention was detected using a commercially available kit (Institute of Jiancheng Bioengineering, Nanjing, China). Cells in the logarithmic phase of growth were seeded into 6-well plates at a density of 1×10^6 cells/well overnight. At the end of incubation, total protein was collected by application of lysis buffer supplied within the kit. The proteins were incubated with a substrate of caspase-3 (N-acetyl-DEVD-p-nitroaniline) for 2 h at 37°C. Finally, the absorbance at 405 nm was measured using a microplate reader. The results were expressed by values of the experimental group relative to control group.

Western blot analyses

The protein from cultured H9c2 cells was extracted in RIPA buffer (Beyotime, China) at 4°C for 30 min and quantified using the BCA

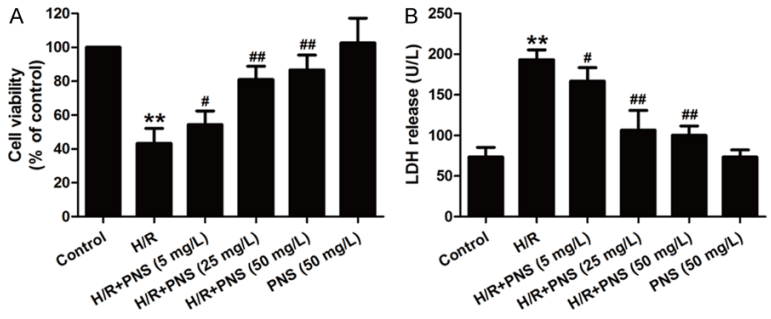

Figure 1. PNS effectively reduced cytotoxicity in H/R-treated H9c2 cells. H9c2 cells pretreated with PNS at the concentrations of 5, 25 and 50 mg/L for 1 h were subjected to hypoxia for 6 h followed by reoxygenation for 4 h. A. The viability of H9c2 cells was evaluated by CCK-8 assay. The result was expressed as a percentage of control group. B. The lactate dehydrogenase (LDH) release was calculated by LDH release assay which was positively correlated with the cell death. The result was expressed as fold of control group. Data are expressed as the mean ± SD of three separate experiments. $^{**}P < 0.01$, vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs H/R treatment group. Panax notoginseng saponins: PNS, Hypoxia/reoxygenation: H/R.

protein assay kit (Merck Millipore Technology, Darmstadt, Germany). Equal amounts of protein from each group were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, MA, USA). After blocking for 2 h at room temperature with 5% skim milk in Tris-buffered saline containing Tween 20 (TBST), the membranes were incubated with primary antibodies against Bax (1:1,000 in TBST, Proteintech), Bcl-2 (1:2,000 in TBST, Abcam), p-JAK2, JAK2, p-STAT3, STAT3 (1:1,000 in TBST, Santa Cruz Biotechnology) or GAPDH (1:2,000 in TBST, Cell Signaling) at 4°C overnight. After washing with TBST three times, the membranes were incubated with secondary antibodies in TBST at 37°C for 2 h, and then washed as described above. Protein bands were analyzed using an enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China) and quantified using an image analyzer Quantity One System (Bio-Rad, Richmond, CA, USA). The results were normalized to GAPDH and expressed as fold of control group.

Measurement of intracellular ROS production

Reactive Oxygen Species (ROS) generation was estimated using a ROS sensitive dye 2',7'-dichloruoresceindiacetate (DCFH-DA, Sigma) following recommended protocols. Intracellular ROS levels were determined via measurement of the oxidative conversion of cell-permeable DCFH-DA to fluorescent dichlorofluorescein (DCF) by a flow cytometry sorter (BD Biosciences, San Jose, CA, USA). Briefly, cells were seeded in 6well plates (1 × 10^6 cells/well) and exposed to different treatments. After washing with PBS, cells were incubated with DCFH-DA at 37°C for 20 min. The DCF fluorescence was detected using flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Determination of malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activities

MDA content, SOD and GSH-PX activities were detected using commercial kit reagents (JianCheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. In briefly, after treatment with different regent, cells were lysed for 30 min on ice, and centrifuged at 12,000 × g for 10 min at 4°C. Protein concentration was quantified using the BCA protein assay kit and the supernatant was collected for detection of MDA content, SOD and GSH-PX activities.

Statistical analysis

All experiments were carried out in triplicate and the data were showed as mean ± standard deviation (SD). Statistical analyses were performed using a 2-tailed Student t-test for unpaired observations or a one-way ANOVA followed by least significant difference (LSD) test for multiple comparisons. The value of $P < 0.05$ was considered as significant.

Results

Panax notoginseng saponins (PNS) attenuates hypoxia/reoxygenation (H/R)-induced cytotoxicity in H9c2 cardiomyocytes

First, in order to evaluate the effect of PNS on H/R injury in H9c2 cells, 3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Lactate Dehydrogenase (LDH) release assay were used to detect the cytotoxicity. As shown in **Figure 1**, compared with H/R treatment group, PNS co-treatment significantly increased the viability of H9c2 cells in a con-

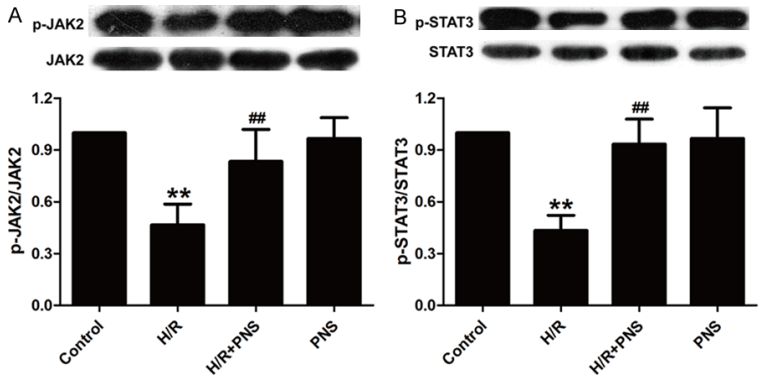

Figure 2. PNS significantly activated the JAK2/STAT3 pathway in H/R-treated H9c2 cells. H9c2 cells pretreated with PNS at the concentrations of 25 mg/L for 1 h were subjected to hypoxia for 6 h followed by reoxygenation for 4 h. The expressions of p-JAK2, JAK2 (A) p-STAT3 and STAT3 (B) proteins were measured by Western blot analysis. Data are expressed as the mean ± SD of three separate experiments. $^{**}P < 0.01$, vs control group; $^{\#\#}P < 0.01$, vs H/R treatment group.

centration-dependent manner (**Figure 1A**). The result from the LDH assay also showed that PNS pretreatment dose-dependently reversed H/R-induced increase in LDH release in H9c2 cells (**Figure 1B**). Notably, PNS treatment alone had no effect on cell viability and LDH release. According to the above results, 25 mg/L of PNS markedly increased cell viability and decreased LDH release in H9c2 cells in the follow-up experiment. Therefore, 25 mg/L was chosen as the best protective concentration of PNS under H/R injury. These results indicate that PNS prevents H/R-induced injury in H9c2 cardiomyocytes.

PNS activates the JAK2/STAT3 pathway during H/R injury in H9c2 cardiomyocytes

To further understand the underlying cardioprotective molecular mechanism of PNS, we investigated the effects of the JAK2/STAT3 pathway under H/R condition. As shown in **Figure 2**, H/R treatment markedly down-regulated the expressions of p-JAK2 and p-STAT3 proteins, as well as p-JAK2/JAK2 and p-STAT3/STAT3 in H9c2 cells compared with control group (**Figure 2A** and **2B**), indicating H/R-induced the inhibition of the JAK2/STAT3 pathway. However, these effects were blocked by pretreatment with PNS (25 mg/L) in H9c2 cells. PNS treatment alone had no effect on the expressions of p-JAK2 and p-STAT3 proteins (**Figure 2A** and **2B**). These results suggested that the activation of the JAK2/STAT3 signaling pathway may be involved in the protective effects of PNS against myocardium H/R injury.

Inhibition of JAK2/STAT3 pathway effectively ameliorates cytoprotective action of PNS in H/R-induced H9c2 cardiomyocyte injury

To confirm the role of the JAK2/STAT3 pathway in PNS-exhibited beneficial effects on H/R-induced injury, H9c2 cells were transfected with JAK2 siRNA to down-regulate the JAK2/STAT3 pathway. We found that compared with control group, the H9c2 cells transfected with JAK2 siRNA significantly reduced the level of JAK2 mRNA and protein (**Figure 3A-C**) as well as the expression of p-STAT3 protein (**Figure 3B** and **3C**). In addition, compared with co-treatment of H/R and PNS groups, the level of JAK2 mRNA and the expression of p-STAT3 protein in the H/R+PNS+JAK2 siRNA group was also reduced (**Figure 3A** and **3B**). These results showed that JAK2 siRNA repressed the JAK2/STAT3 pathway activation. In addition, JAK2 siRNA obviously blocked PNS-induced reversal of H/R-induced decrease in the viability of H9c2 cells (**Figure 3C**). The down-regulation of LDH release induced by PNS was also abolished by JAK2 siRNA (**Figure 3D**). Notably, although JAK2 siRNA treatment remarkably decreased the level of JAK2 mRNA, it alone had little effect on cell viability and LDH release (**Figure 3A-C**). All in all, these results suggested that PNS prevents cardiomyocytes from cytotoxicity induced by H/R via activation of the JAK2/STAT3 pathway.

Blockage of the JAK2/STAT3 pathway blocks PNS-induced decrease in apoptosis in the H9c2 cardiomyocytes subjected to H/R

Next, we determined the role of the JAK2/STAT3 pathway in the protective effect of PNS on H/R-induced apoptosis in H9c2 cells. Hoechst 33258 staining results showed that PNS pretreatment clearly ameliorated H/R-induced phenomenon of nuclear condensation and fragmentation with bright blue fluorescence in H9c2 cells, whereas this effect was attenuated by JAK2 siRNA (**Figure 4A**), indicating that PNS

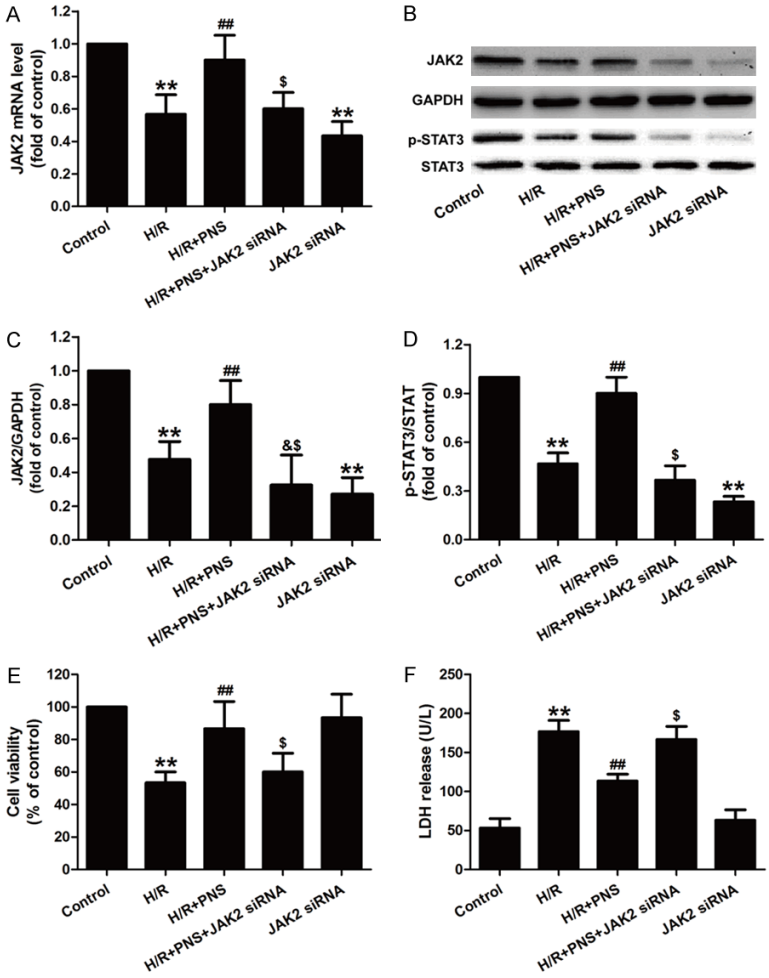

Figure 3. JAK2 siRNA blocked the inhibitory effects of PNS on cytotoxicity in H/R-treated H9c2 cells. H9c2 cells were transfected with JAK2 siRNA and then exposed to PNS (25 mg/L) for 1 h followed by H/R treatment. (A) The level of JAK2 mRNA was determined by QRT-PCR. Result was expressed as $2^{-\Delta\Delta CT}$. (B) The expressions of JAK2, p-STAT3 and STAT3 proteins were measured by Western blot analysis. Quantitative analysis of JAK2 expression (C) and p-STAT3/STAT3 (D). (E) The viability of H9c2 cells was evaluated by CCK-8 assay. (F) The LDH release was calculated by LDH release assay. Data are expressed as the mean ± SD of three separate experiments. $^{**}P < 0.01$, vs control group; $^{\#\#}P < 0.01$, vs H/R treatment group, $^{\$}P < 0.05$, vs H/R and PNS co-treatment group.

improves morphological changes of the apoptotic nuclei. Consistently, statistics showed that JAK2 siRNA reversed the PNS-inhibited apoptosis rate induced by H/R treatment. In addition, we found that PNS treatment remarkably blocked the H/R-induced increase in the activity of caspase-3 in H9c2 cells. However, this inhibitory effect was abolished by JAK2 siRNA transfection (**Figure 4B**). Furthermore, we demonstrated the effects of JAK2 siRNA on apoptosis-related proteins and found that JAK2 siRNA transfection evidently alleviated the reversal effect of PNS on H/R-exhibited increase in the ratio of Bax/Bcl-2 in H9c2 cells (**Figure 4C**). The JAK2 siRNA alone did not affect cellular apoptosis. These results indicated that PNS protects against H/R-induced apoptosis through enhancement of the JAK2/STAT3 pathway.

Inhibition of the JAK2/STAT3 pathway reverses the inhibition of PNS on H/R-induced oxidative stress in H9c2 cardiomyocytes

In the process of ischemia and reperfusion, oxidative stress is thought to play an important role in the genesis of tissue and cell injury [5, 6]. Therefore, we further investigated the effect of the JAK2/STAT3 pathway on oxidative stress in PNS-induced cardioprotection against H/R injury. Results presented in **Figure 5** showed that PNS pretreatment significantly reversed the H/R-induced increase in the ROS production (**Figure 5A**) and MDA content (**Figure 5B**) in H9c2 cells, while these effects were blocked by the inhibition of the JAK2/STAT3 pathway induced by JAK2 siRNA. In addition, PNS obviously increased the activity of the antioxidant enzymes including SOD (**Figure 5C**) and GSH-PX (**Figure 5C**) under H/R injury conditions. However, the inhibition of PNS was also reversed by JAK2 siRNA. The JAK2 siRNA alone had no effect on these indicators of oxidative stress. These results indicated that the JAK2/STAT3 pathway mediates the protection of PNS against H/R-induce oxidative stress in H9c2 cells.

Discussion

In the current study, we utilized *in vitro* models to investigate the protective effect of panax

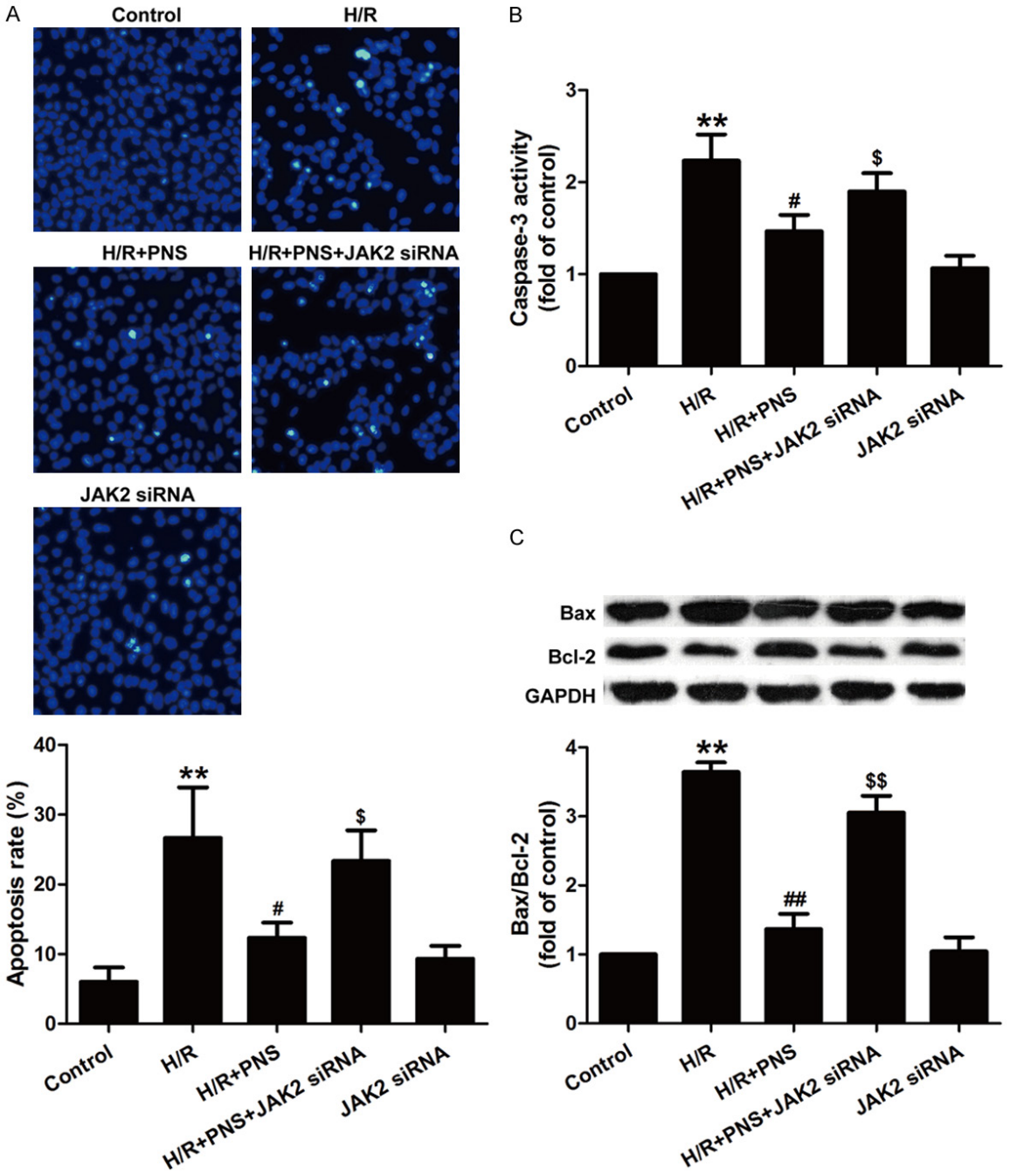

Figure 4. JAK2 siRNA abolished the reversal effects of PNS on apoptosis in H/R-treated H9c2 cells. H9c2 cells were transfected with JAK2 siRNA and then exposed to PNS (25 mg/L) for 1 h followed by H/R treatment. A. The morphological characteristics of apoptosis was detected by Hoechst 33258 staining (× 200). The result was expressed as the ratio of apoptotic cells to the total cell. B. The activity of caspase-3 was measured by commercially available kit (Caspase-3 assay kit). C. The ratio of Bax/Bcl-2 was evaluated by western blot assay. Data are expressed as the mean ± SD of three separate experiments. $^{**}P < 0.01$, vs control group; $^{\#\#}P < 0.05$, $^{\#\#}P < 0.01$, vs H/R treatment group, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs H/R and PNS co-treatment group.

notoginseng saponins (PNS) on hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocyte injury and further demonstrate the underlying protective mechanisms. We found that PNS markedly ameliorated the H/R-induced oxidative stress, thereby reducing apoptosis, leading

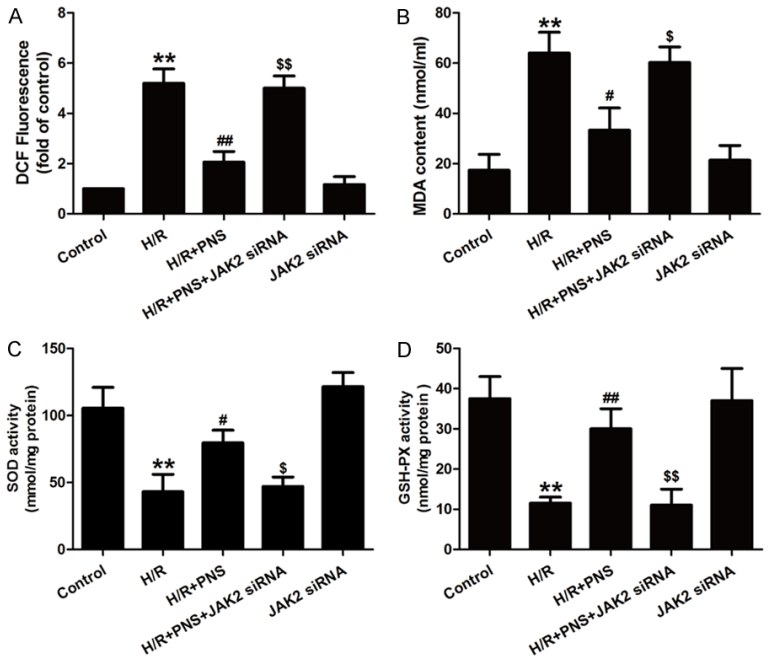

Figure 5. JAK2 siRNA reversed the inhibition of PNS on oxidative stress in H/R-treated H9c2 cells. H9c2 cells were transfected with JAK2 siRNA and then exposed to PNS (25 mg/L) for 1 h followed by H/R treatment. (A) The ROS production was measured by 2',7'-dichloruoresceindiacetate (DCFH-DA) followed by flow cytometry sorter. The MDA content (B), SOD activity (C) and GSH-PX activity (D) was determined by commercial kit reagents, respectively. Data are expressed as the mean ± SD of three separate experiments. $^{**}P < 0.01$, vs control group; $^{\#\#}P < 0.05$, $^{\#\#}P < 0.01$, vs H/R treatment group, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs H/R and PNS co-treatment group.

to cardioprotection in H9c2 cardiomyocytes. Importantly, it has proven the critical role of JAK2/STAT3 signaling in this protective process. This study gives better understanding of the pharmacology of PNS in the treatment of heart ischemic disease and expedites the development of a novel strategy against MI/R injury.

In recent years, emerging evidence revealed that natural products have been used clinically worldwide for treatment of cardiovascular diseases (CVDs) as a result of their antihypertensive and vasodilatory actions with good effects [27, 28]. Importantly, the cardioprotective activity of PNS has been also widely recognized [10, 29, 30]. Yang BR et al, prove that saponins separated from flower buds of panax notoginseng attenuates acute myocardial infarction via pro-angiogenesis and anti-apoptosis [30]. In our current study, we found that PNS pretreatment ameliorates H/R-induced decrease in the viability of H9c2 cells and increases the LDH released, resulting in protective effects on cardiomyocyte cytotoxicity. Ge ZR et al, also confirm that notoginsenoside R1 (NG-R1), a phytoestrogen isolated from PNS, reduces myocardial cell injury in ischemia-reperfusion (IR)-induced myocardial injury in a rabbit model, leading to cardioprotective properties [29]. With these conclusions, we present that PNS provides beneficial effects against cytotoxicity in cardiomyocytes exposed to H/R injury, indicating the cardioprotective role and potential therapeutic effects of PNS in MI/R injury.

Over the past decade, it has been proven that a complex signaling network consisting of multiple kinases and transcription factors are involved in the impacts of ischemia on the heart [31, 32]. Among these signaling pathways, Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway, a stress-responsive mechanism that transduces signals from cell surface to the nucleus, thereby modulating gene expression, wonderfully contributes to the mediation of cardioprotection against ischemia/reperfusion injury [20]. So far, four JAKs (JAK1, 2, 3, and Tyk2) and seven STATs (STAT1, 2, 3, 4, 5a, 5b, and 6) have been discovered [33]. Notably, cumulating evidence has specifically implicated the key role of JAK2/STAT3 signaling in ameliorating the MI/R-induced cardiac injury [34, 35]. Previous studies also exert that STAT3 plays a role in reducing apoptotic cell death of cardiomyocytes [36]. Herein, we surmised that JAK2/STAT3 signaling is involved in the protective effects of PNS on H/R-induced cytotoxicity and apoptosis in H9c2 cells. We found that H/R treatment significantly decreased the phosphorylation of JAK2 and STAT3, while theses effects were reversed by pretreatment with PNS, indicating the activation of JAK2/STAT3 signaling by PNS under H/R injury. Notably, knocking down the level of JAK2 by using JAK2 siRNA resulted in the inhibition of the JAK2/STAT3 pathway. In addition, in rat acute myocardial infarction, inhibition of JAK2,

causes the suppressed phosphorylation of STAT3, and increased activity of caspase-3 and expression of Bax, indicating the protective effects of JAK/STAT activation against acute myocardial infarction through inhibition of apoptosis [37]. Similarly, additional research reported that JAK2/STAT3 signaling was effectively up-regulated by berberine in H9c2 cells exposed to simulated ischemia/reperfusion (SIR), and JAK2 siRNA blocked berberine-down-regulation of myocardial apoptosis [38]. Thus, intracellular JAK2/STAT3 activity might be protective during the ischemia-reperfusion period. In this study, we further found that inhibition of JAK2/STAT3 signaling by JAK2 siRNA abolished PNS-induced decreases in cell viability and apoptosis. Based on these studies, the results proved that JAK2/STAT3 plays a key role in PNS's cardioprotective actions.

A growing number of studies have intensively investigated reactive oxygen species (ROS) to reveal their role in MI/R injury, ranging from beneficial to inimical [39, 40]. When the balance between the production and scavenging of ROS is destroyed, irreversible damage to cells may occur, eventually leading to cell apoptosis [41]. Therefore, antioxidant agents have been proposed to treat MI/R. Many studies confirm that PNS elicits protective effect against multiple stimuli-induced injuries through reducing oxidative stress [29, 42, 43]. However, it is unknown whether PNS also suppresses oxidative stress in MI/R injury. The research from Fan Y et al, reveals that PNS reduces ROS production and malondialdehyde (MDA) levels, and also increased the endogenous antioxidant system including total superoxide dismutase (SOD), MnSOD, catalase (CAT), and glutathione peroxidase (GSH-PX) activation in high glucose-treated rat retinal capillary endothelial cells [42]. In addition, it also proved that PNS inhibits the production of 8-hydroxydeoxyguanosine (8-OHdG), enhanced the expressions and activities of SOD, CAT, and GSH-PX in the brains of SAMP8 mice, inhibiting oxidative stress [43]. Consistent with these findings, our experimental findings found that PNS remarkably reversed H/R-induced increases in the ROS generation and MDA content, and decreased the SOD and GSH-PX activity, indicating that the inhibition of oxidative stress may contribute to the protection of PNS against MI/R injury. In addition, emerging evidence shows that activating JAK2/STAT3 signaling protects the heart against myocardial injury through ROS production [38, 44]. Zhao GL et al, proved that transfection with JAK2 siRNA abolishes berberine-induced protection against MI/R-induced cell apoptosis and oxidative stress [38]. Similarly, the present study also reveals that blockage of the JAK2/STAT3 pathway induced by JAK2 siRNA increases the ROS and MDA levels, and reduces the activities of SOD and GSH-PX, indicating that the JAK2/STAT3 pathway mediates the inhibition of PNS on H/R-induced oxidative stress.

In conclusion, the present study demonstrated the novel roles of the JAK2/STAT3 pathway in the protection of PNS against MI/R. This study confirmed that PNS reduced myocardial apoptosis and oxidative stress via activation of the JAK2/STAT3 signaling pathway, thus ameliorating the H/R injury. These findings may provide a novel mechanistic insight into the cardioprotective roles of PNS in MI/R.

Disclosure of conflict of interest

None.

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