Original Article

Panax notoginseng saponin reduces *IL-1β*-stimulated apoptosis and endoplasmic reticulum stress of nucleus pulposus cells by suppressing *miR-222-3p*

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Background: It is well documented that the malignant biological behaviors of nucleus pulposus cells (NPCs) could trigger intervertebral disc degeneration (IDD). Panax notoginseng saponin (PNS) is a traditional Chinese medicine that inhibits osteoclastogenesis. However, its effects on the phenotypes of NPCs in IDD remains largely unknown. This study sought to examine the role of PNS in IDD and its regulatory mechanism.

Methods: First, human NPCs (hNPCs) were treated with interleukin-1 beta (*IL-1β*) to induce an IDD cell model. Cell proliferation and apoptosis were estimated by Cell Counting Kit-8 (CCK-8) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. Western blot was employed to examine the levels of proteins related to apoptosis and endoplasmic reticulum (ER) stress. Enzyme-linked immunosorbent assays (ELISAs) were used to test inflammatory factors levels. Immunofluorescence (IF) assays were used to determine the nuclear translocation of nuclear factor-kappa beta (*NF-κB*) *p65*. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect *miR-222-3p* expression.

Results: We discovered that PNS enhanced the viability but reduced the apoptosis, inflammation, and ER stress response of *IL-1β*-induced hNPCs in a concentration-dependent manner. Additionally, PNS significantly reduced *miR-222-3p* expression in the *IL-1β*-induced hNPCs. Notably, these PNS effects were reversed by the upregulation of *miR-222-3p*.

Conclusions: In summary, PNS appears to facilitate the proliferation and attenuate the apoptosis, inflammatory response, and ER stress response of *IL-1β*-induced hNPCs by inhibiting *miR-222-3p* expression. Our findings provide a theoretical basis for a novel drug application in IDD research.

Keywords: Panax notoginseng saponin (PNS); interleukin-1 beta (*IL-1β*); *miR-222-3p*; nucleus pulposus cells (NPCs)

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Introduction

Intervertebral disc degeneration (IDD) is a major contributor of low back pain (1). IDD is a disc disease of adjacent vertebrae in which the degeneration of the discs and surrounding area is caused by structural damage (2). IDD has significant effects on human health; however, the mechanism underlying its onset and progression remain ambiguous (3). Nucleus pulposus cells (NPCs) are the major component of intervertebral discs, and changes in the behaviors of NPCs determine the function of intervertebral

discs (4). Ineffective conservative and surgical treatments have driven the emergence of cell-based therapies (5). The regulatory mechanism underlying the survival and apoptosis of NPCs needs to be determined to develop novel therapies for IDD.

Panax notoginseng (Burk) F. H. Chen, also known as Sanqi, is a traditional Chinese medicine with multiple pharmacological effects, including anti-inflammation, anti-oxidation, anti-insomnia, and anti-depression effects (6). As the main ingredient of *Panax notoginseng (Burk) F. H. Chen*, panax notoginseng saponin (PNS) has also been shown to have various biological roles in multiple human diseases. For example, Liu *et al.* have stated that PNS exacerbates cell apoptosis in retinoblastoma through phosphatidylinositol-3-kinase and protein kinase B signaling (7). PNS also confers skeletal muscle insulin sensitivity in diabetes (8). PNS eases the senescence and apoptosis of osteoarthritic chondrocytes (9). Further, PNS protects cortical neurons from oxidative stress stimulated by hypoxia/reoxygenation (10). More importantly, PNS has been reported to inhibit osteoclatogenesis and alleviate IDD by suppressing aberrant osteoclast activation in porous cartilaginous end plates (11). However, it is not yet known whether PNS also functions as an essential modulator in the progression of IDD.

MicroRNAs (miRNAs) are a series of small, endogenous RNAs without any protein-coding capacity (12). Numerous studies have identified the dysregulation of miRNAs as a potential hallmark of cancers and diseases (13-16). Additionally, there is emerging evidence that miRNAs are associated with proliferation, apoptosis, inflammation, and the extracellular matrix in IDD and are thus useful clinical biomarkers for IDD (17-20). *miR-640* serves as a promoter in IDD by mediating the nuclear factor-kappa beta (*NF-κB*) and *WNT* signaling pathway (21). *miR-150-5p* prevents the development of IDD via a competing endogenous RNA (ceRNA) mechanism (22). Additionally, *miR-222-3p* is upregulated and drives the progression of IDD by targeting *cyclin-dependent kinase inhibitor 1B* (23). Multiple studies shown that miRNAs may have a critical effect on PNS and its ingredients (24,25). Thus, miRNAs might be the potential target of PNS. Given this, we speculated that PNS may be implicated in IDD by modulating *miR-222-3p* expression.

This study sought to examine the effects of PNS on the biological activities of interleukin-1 beta (*IL-1β*)-induced NPCs and determine the interaction between PNS and *miR-222-3p* in IDD. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3203/rc).

Methods

Cell culture

The human NPCs (hNPCs) provided by Tongpai Biological Technology Co., Ltd. were maintained in Dulbecco's modified Eagle's medium (Gibco, NY, USA) with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and 1% antibiotics (Sigma-Aldrich, St. Louis, MO, USA), and kept in an atmosphere of 5% carbon dioxide at 37 ℃ for the culture. To establish the cell apoptosis model, 75 ng/mL of *IL-1β* (PeproTech, Rocky Hill, USA) was used to pretreat the hNPCs at 37 ℃ for 24 h (26). Afterwards, PNS (50, 100, 200, or 400 μg/mL) was added to the cells for 24 h of incubation (9,11,27,28).

Cell Counting Kit-8 (CCK-8)

To measure cell viability, the hNPCs were plated into 96-well plates for 24 h of cultivation at 37 ℃. Next, 10 μL of CCK-8 solution (APExBIO) was added into each well for an additional 2 h of incubation. Afterwards, a microplate reader (Molecular Devices, LLC, USA) was used to detect the absorbance (450 nm) value.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays

TUNEL assays were employed to examine cell apoptosis in the *IL-1β*-induced hNPCs. After treatment or transfection, the hNPCs were collected and washed with phosphate buffered solution, and then fixed with 1% paraformaldehyde (Solarbio) at room temperature for 15 min. Subsequently, 50 μL of the TUNEL kit (Elabscience, Wuhan, China) was added to the hNPCs and incubated for 1 h at 37 ℃ and 4',6-diamidino-2-phenylindole (DAPI) was used for the nuclei staining. The images from 3 fields of view were observed under a fluorescence microscope (BIO, Minneapolis, MN, USA; magnification, ×200).

Western blot assays

Total protein extracted from the hNPCs was prepared using ristocetin-induced platelet aggregation lysis buffer

(Epizyme Biotech, Shanghai, China). A bicinchoninic acid kit (Beyotime, China) was used to quantify the total protein. Next, 40 μg of the protein sample was subjected to electrophoresis in 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, MA, USA). Then, 5% non-fat milk was used to impede the membranes for 1 h at room temperature. Later, the membranes were treated overnight at 4 ℃ with the following primary antibodies: anti-B cell lymphoma-2 (*Bcl-2*; cat. no. ab32124; 1:1,000; Abcam, USA), anti-Bcl-2 associated X (*Bax*; cat. no. ab32503; 1:1,000; Abcam), anti-*cleaved caspase 3* (cat. no. ab32042; 1:500; Abcam), anti-*caspase 3* (cat. no. ab32351; 1:5,000; Abcam), anti-glucose regulating protein 78 (*GRP78*; cat. no. ab108615; 1:1,000; Abcam), anti-inositol-requiring protein 1 alpha (*IRE1α*; cat. no. #3294; 1:1,000; Cell Signaling Technology, USA), anti-X-box binding protein 1 (*XBP1*; cat. no. ab109221; 1:1,000; Abcam), anti-activating transcription factor 6 (*ATF6*; cat. no. ab227830; 1:1,000; Abcam), anti-C/EBP homologous protein (*CHOP*; cat. no. #5554; 1:1,000; Cell Signaling Technology), and anti-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; cat. no. ab9485; 1:2,500; Abcam), and anti-β-*actin* (cat. no. ab8227; 1:1,000; Abcam). Subsequently, the membranes were incubated with goat anti-rabbit *IgG* secondary antibody (cat. no. ab6702; 1:1,000; Abcam) for 1 h at room temperature. Protein signals were detected using an enhanced chemiluminescence reagent (Affinity Biosciences, OH, USA), and band intensity was determined using Image-Pro Plus software (Media Cybernetics, Inc., USA). GAPDH and β-actin were used as the loading control.

Enzyme-linked immunosorbent assay (ELISA)

Tumor necrosis factor-alpha (*TNF*-α; cat. no. ab181421) and interleukin 6 (*IL-6*; cat. no. ab178013) levels were detected by corresponding ELISA kits procured from Abcam in accordance with manufacturer's guidelines. A microplate reader (Molecular Devices, LLC) was used to examine the absorbance at 450 nm.

Immunofluorescence (IF)

After fixation with 4% paraformaldehyde for 15 min at 4 ℃ and permeabilization with 0.1% Triton X-100 for 10 min, the hNPCs were impeded by 1% bovine serum albumin (BSA) blocking solution for 1 h, and then cultivated with primary antibodies against *NF-κB p65* (cat. no. ab32536; 1:100; Abcam) overnight at 4 ℃. On the 2nd day, goat anti-rabbit *IgG* secondary antibody (cat. no. ab6702; 1:1,000; Abcam) was added and incubated for another 1 h at room temperature. After which, 1 mg/mL of DAPI was used to counterstain the cell nuclei for 10 min. Images were observed under a confocal microscope (CarlZeiss, Oberkochen, Germany; magnification, ×100).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was obtained from the hNPCs using TRIzol reagent (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Next, complementary deoxyribonucleic acid was generated by the reverse transcription of RNA through M-MLV Reverse Transcriptase (Promega, Wisconsin, Madison, WI, USA). RT-qPCR was conducted with the application of the GoTaq® qPCR Master Mix (Promega, Beijing, China) on the ABI 7500 real-time PCR system (Applied Biosystems, Hayward, CA, USA). The PCR thermocycling conditions were as follows: 10 min at 95 ℃, followed by 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 30 sec. The following primer sequences were used for PCR: *miR-222-3p*: forward, 5'-CGGCACGGGCCGAGGC-3' and reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; *U6*: forward, 5'-AAAGCAAATCATCGGACGACC-3', and reverse, 5'-GTACAACACATTGTTTCCTCGGA-3'. Relative *miR-222-3p* expression was normalized to *U6* using the $2^{-\Delta\Delta Ct}$ method (29).

Cell transfection

miR-222-3p mimic and miR-negative control (NC) were obtained from RiboBio Co., Ltd. (Guangzhou, China). In accordance with manufacturer's protocol, the aforementioned plasmids were transfected into the hNPCs at a final concentration of 50 nM with the aid of Lipofectamine 2000 (Thermo Scientific, Wilmington, DE, USA). The hNPCs were harvested for further analysis after 48 h of transfection.

Statistical analyses

All the data are presented as the mean ± standard deviation (SD). The statistical analysis was performed using SPSS software (version 220.2; IBM Corp., USA), and graphs were generated using GraphPad Prism 6.0 (San Diego, CA, USA). All experiments were independently repeated

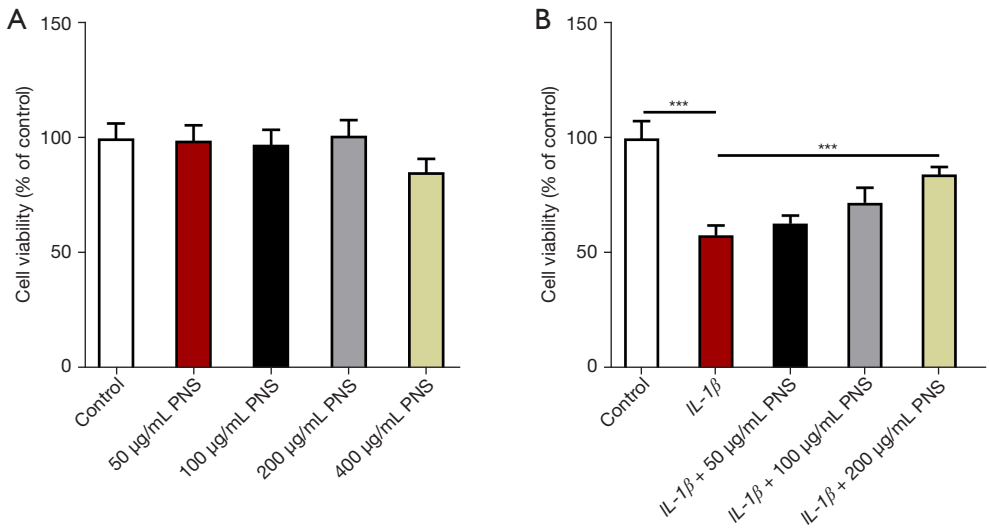

Figure 1 PNS enhances the viability of *IL-1β*-treated hNPCs in a concentration-dependent manner. (A) CCK-8 assays were used to estimate the viability of hNPCs after the administration of PNS. (B) The effect of PNS on the viability of *IL-1β*-treated hNPCs was assessed by CCK-8 assays. ***P<0.001. PNS, panax notoginseng saponin; *IL-1β*, interleukin-1 beta; hNPCs, human nucleus pulposus cells; CCK-8, Cell Counting Kit-8.

in triplicate and all experimental data were biologically repeated in triplicate. A one-way analysis of variance was used to perform variation comparisons among multiple groups followed by a Tukey's post-hoc test. Differences between two groups were compared using the Student's *t*-test. A P value <0.05 was considered statistically significant.

Results

PNS enhances the viability of IL-1β-treated hNPCs in a concentration-dependent manner

To determine the role of PNS in IDD, the hNPCs were treated with different concentrations of PNS (i.e., 50, 100, 200, and 400 μg/mL), and cell proliferation was then assessed using CCK-8 assays. As *Figure 1A* showed, PNS exerted no obvious effect on the viability of the hNPCs. Notably, the viability of the hNPCs was significantly reduced by *IL-1β*, but gradually enhanced by PNS in a concentration-dependent manner (see *Figure 1B*). In brief, PNS facilitated the proliferation of the *IL-1β*-induced hNPCs.

PNS weakens the apoptotic ability of IL-1β-exposed hNPCs in a dose-dependent manner

The experimental results of the TUNEL assays revealed that *IL-1β* stimulated a significant amount of apoptosis in the hNPCs, but this effect was abrogated by increasing concentrations of PNS (see *Figure 2A*,*2B*). Similar results were also observed in the western blot analysis. *Bcl-2* expression was downregulated while *Bax* and *cleaved caspase 3/caspase 3* expressions were upregulated in the *IL-1β*-induced hNPCs. After PNS was administrated to the *IL-1β*-stimulated hNPCs, elevated *Bcl-2* protein level and reduced *Bax* and *cleaved caspase 3/caspase 3* protein levels were observed (see *Figure 2C*,*2D*). In short, PNS appeared to have anti-apoptotic effects in the *IL-1β*-induced hNPCs.

PNS eases the IL-1β-evoked inflammatory response in hNPCs

The levels of proinflammatory cytokines, including *TNF*-α and *IL-6*, were detected using ELISA kits. Based on the experimental results, the increase in *TNF*-α and *IL-6* levels in the *IL-1β*-induced hNPCs decreased following the addition of PNS (see *Figure 3A*). Additionally, as the IF assays showed, *IL-1*β stimulated the nuclear translocation of *NF-κB p65*, which was impeded by PNS in a concentration-dependent manner (see *Figure 3B*). As 200 μg/mL of PNS had better effects in all groups, it was used in the subsequent experiments. In summary, PNS ameliorated the *IL-1β*-triggered inflammatory response and nuclear translocation

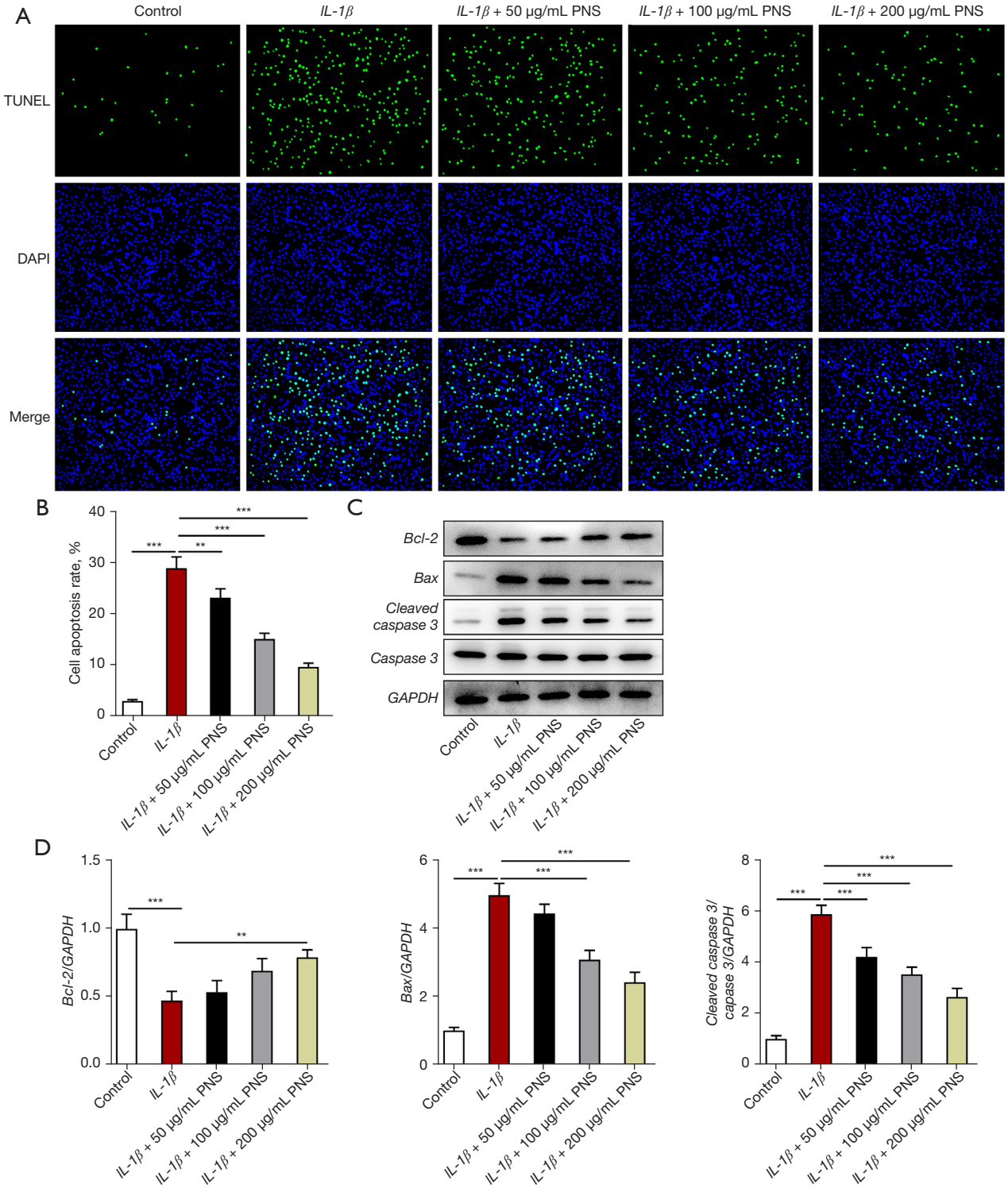

Figure 2 PNS decreases the apoptotic ability of *IL-1β*-exposed hNPCs in a dose-dependent manner. (A,B) TUNEL assays were applied to evaluate the effect of PNS on the apoptosis of *IL-1β*-stimulated hNPCs (×200). (C,D) The protein expressions of apoptosis-related factors were analyzed by western blot. **P<0.01; ***P<0.001. *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole; *Bcl-2*, B cell lymphoma-2; *Bax*, Bcl-2 associated X; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; hNPCs, human nucleus pulposus cells.

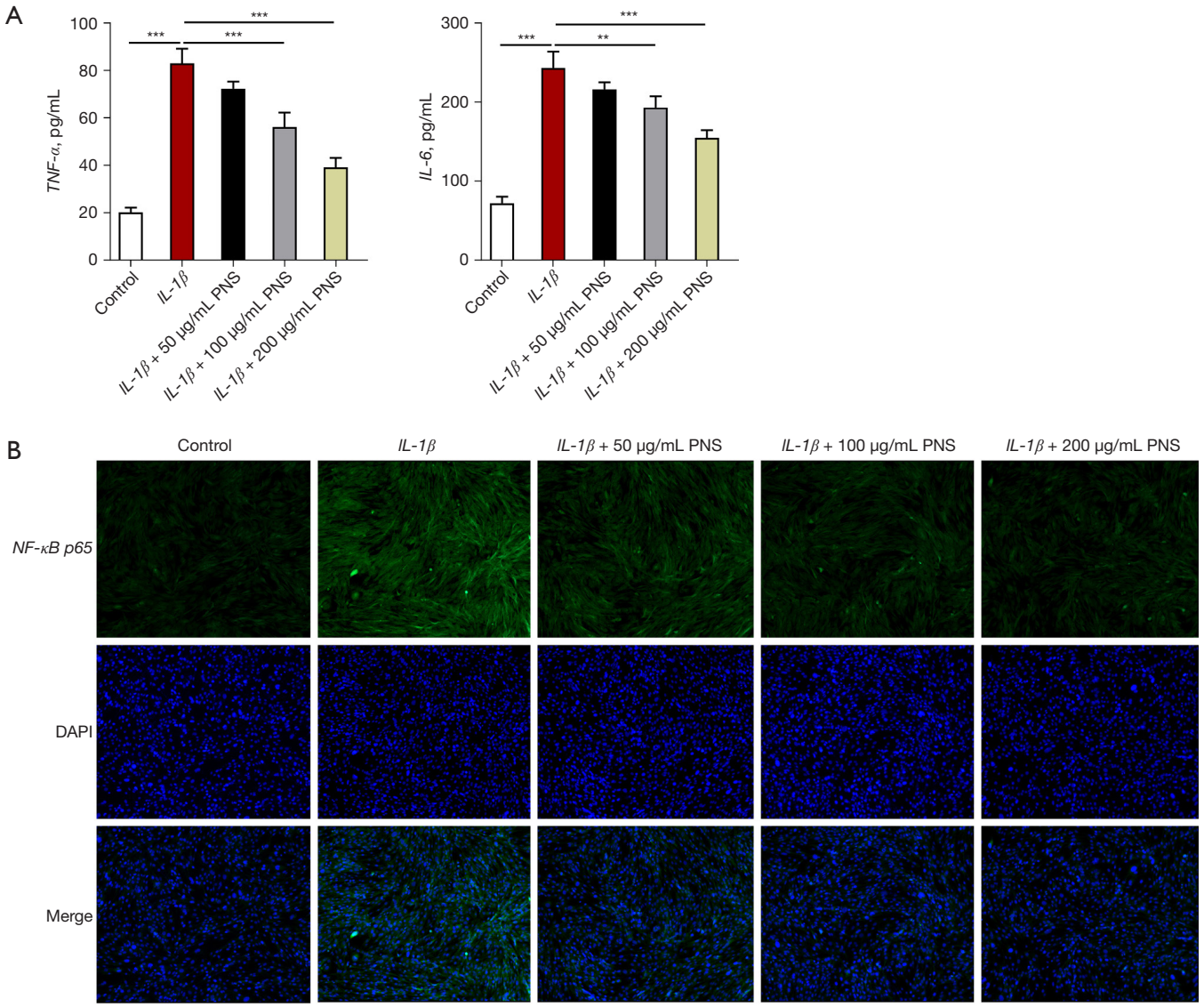

Figure 3 PNS eases the inflammatory response of hNPCs treated by *IL-1β*. (A) The levels of *TNF-α* and *IL-6* were examined by ELISA assays. (B) The nuclear translocation of *NF-κB p65* was detected by IF assays (×100). *P<0.05; **P<0.01; ***P<0.001. *TNF-α*, tumor necrosis factor-alpha; *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; *IL-6*, interleukin 6; *NF-κB*, nuclear factor-kappa beta; DAPI, 4',6-diamidino-2-phenylindole; hNPCs, human nucleus pulposus cells; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence.

of *NF-κB p65* in the hNPCs.

PNS alleviates IL-1β-stimulated endoplasmic reticulum (ER) stress in hNPCs

ER stress has been shown to play an enormous role in the onset and progression of IDD (30). Thus, the protein levels of ER stress-related factors, including *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*, were also analyzed by western blot. We found that *IL-1β* treatment distinctly increased the protein levels of *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*, but this effect was reduced by PNS (see *Figure 4*). In conclusion, PNS served as a suppressor in ER stress in the *IL-1β*-exposed hNPCs.

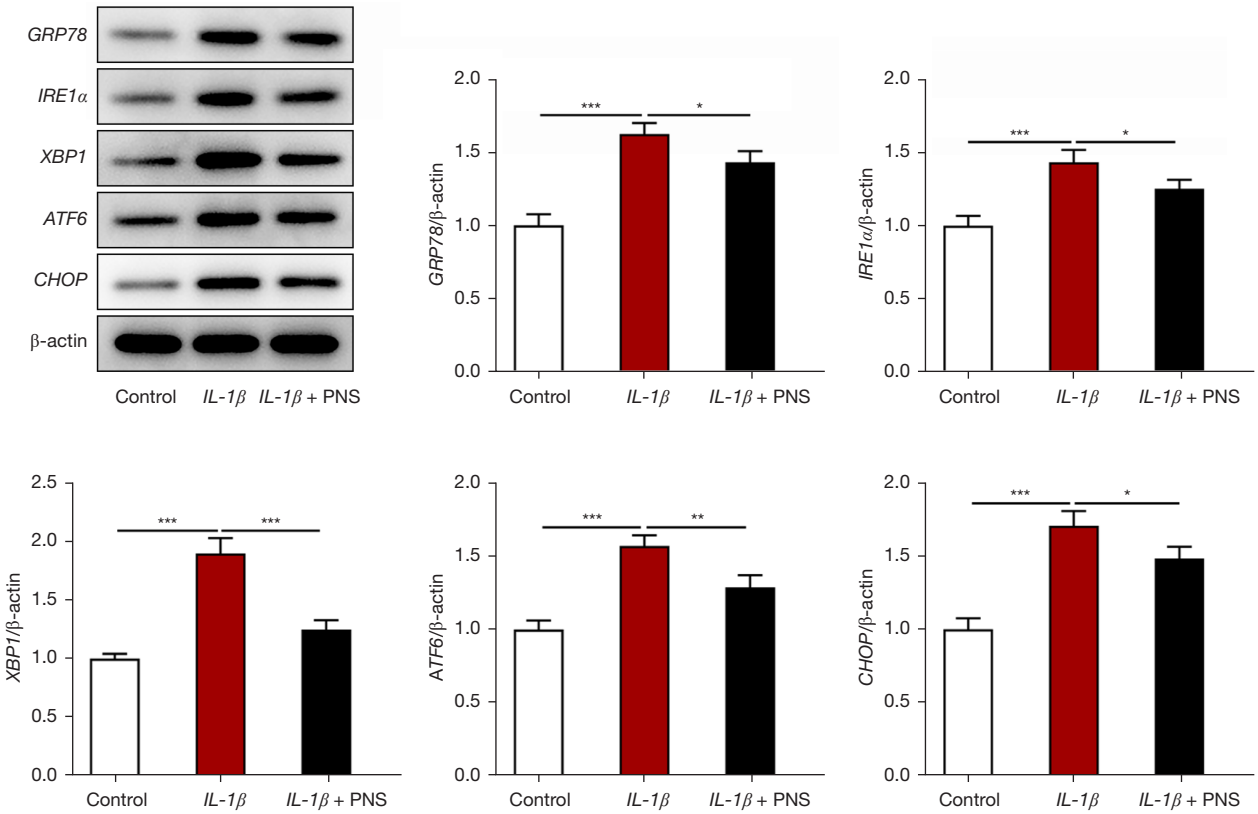

Figure 4 PNS alleviates *IL-1β*-stimulated ER stress in hNPCs. Western blot was adopted to analyze the protein levels of *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*. *P<0.05; **P<0.01; ***P<0.001. *GRP78*, glucose regulating protein 78; *IRE1α*, inositol-requiring protein 1 alpha; *XBP1*, X-box binding protein 1; *ATF6*, activating transcription factor 6; *CHOP*, C/EBP homologous protein; *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; ER, endoplasmic reticulum; hNPCs, human nucleus pulposus cells.

PNS lessens miR-222-3p expression in the IL-1β-induced hNPCs

Based on the RT-qPCR analysis, we also observed that *miR-222-3p* was highly expressed in the *IL-1β*-treated hNPCs, while PNS significantly reduced *miR-222-3p* expression (see *Figure 5A*). To upregulate *miR-222-3p*, *miR-222-3p* mimic was transfected into the hNPCs, and the overexpression efficiency was tested. We found that *miR-222-3p* expression was successfully enhanced in the *miR-222-3p* mimic group relative to the miR-NC group (see *Figure 5B*). Further, the CCK-8 assays revealed that *miR-222-3p* upregulation abrogated the viability of the hNPCs co-treated with both *IL-1β* and PNS compared to those in the *IL-1β* + PNS + mimic NC group (see *Figure 5C*). All the above results implied that *miR-222-3p* overexpression hampered the viability of the *IL-1β*- and PNS-cotreated hNPCs.

Overexpression of miR-222-3p exacerbates the apoptosis of hNPCs co-treated with both IL-1β and PNS

The transfection of *miR-222-3p* mimic appeared to boost the apoptosis of the hNPCs pretreated with both *IL-1β* and PNS (see *Figure 6A,6B*). As anticipated, the upregulation of *miR-222-3p* suppressed the *Bcl-2* protein level but promoted *Bax* and *cleaved caspase 3/caspase 3* protein levels compared to the *IL-1β* + PNS + mimic NC group (see *Figure 6C*). Taken together, *miR-222-3p* upregulation accelerated the apoptosis of the *IL-1β*- and PNS-co-treated hNPCs.

miR-222-3p elevation offsets the inhibitory effects of PNS on inflammation in IL-1β-induced hNPCs

The ELISA assays revealed that the inhibition of *IL-1β*-induced inflammation in the hNPCs by PNS was

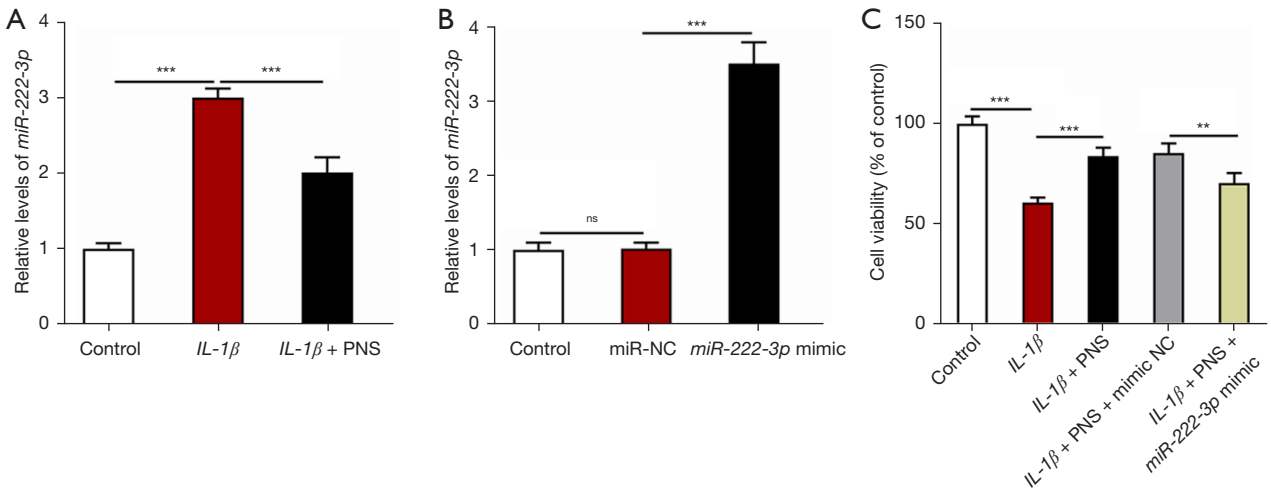

Figure 5 PNS decreases *miR-222-3p* expression in *IL-1β*-induced hNPCs. (A) *miR-222-3p* expression was tested by RT-qPCR. (B) RT-qPCR was employed to check the overexpression efficiency of *miR-222-3p*. (C) Cell viability was evaluated by CCK-8 assays. **P<0.01; ***P<0.001; ns, not significant. *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; NC, negative control; hNPCs, human nucleus pulposus cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8.

counteracted by *miR-222-3p*, as the downregulated *TNF-α* and *IL-6* levels in the *IL-1β*-treated hNPCs caused by PNS were both restored by *miR-222-3p* (see *Figure 7A*). Additionally, the suppressed nuclear translocation of *NF-κB p65* in the *IL-1β*- and PNS-co-treated hNPCs was stimulated by the overexpression of *miR-222-3p* (see *Figure 7B*). Overall, PNS appears to mitigate the *IL-1β*-evoked inflammatory response and nuclear translocation of *NF-κB p65* in hNPCs by downregulating *miR-222-3p*.

PNS relieves IL-1β-stimulated ER stress in hNPCs by suppressing miR-222-3p

The western blot analysis revealed that the levels of the ER stress-related proteins *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP* were all depleted in the *IL-1β*- and PNS-co-treated hNPCs, but this effect was counteracted after *miR-222-3p* was overexpressed (see *Figure 8*). Collectively, *miR-222-3p* upregulation offset the suppressive effect of PNS on *IL-1β*-stimulated ER stress in hNPCs.

Discussion

Given the complexity of IDD pathogenesis, a large number of in-depth studies have conducted and found that NPC dysfunction aggravates the progression of nucleus pulposus degeneration, which may trigger IDD (4). It has been argued that the excessive apoptosis of NPCs is a classical pathological change in IDD (31). Additionally, previous studies have illuminated that inflammation may be a risk factor for NPC apoptosis and is involved in the process of IDD (32,33). Additionally, ER stress has been determined to be responsible for normal cell metabolism and to provoke the apoptosis of NPCs (34,35). During the process of ER stress, unfolded or misfolded proteins are prone to accumulate in ER, which is referred to as an unfolded protein response (36). As major ER stress transduction factors, *ATF6* and *IRE1α* can modulate the ability of ER protein folding by upregulating *GRP78* and *XBP1* mRNA splicing (37-39). Further, the activation of *CHOP*, a determinant of ER-stress-induced apoptosis, is also a signaling event underlying ER stress-evoked cell apoptosis (40). Thus, the apoptosis, inflammation, and ER stress response of NPCs were investigated in the current study.

IL-1β, a known proinflammatory cytokine, is thought to be a mediator of apoptosis and extracellular matrix degradation in NPCs (41). Thus, most studies use *IL-1β* to induce NPCs to construct IDD cell models. For example, a previous study used 160 ng/mL of *IL-1β* to stimulate NPCs to establish an IDD cell model (42). HNPCs have also been treated with 10 μg/mL of *IL-1β* to maintain the degeneration of NPCs in IDD (43). In the previous study, 75 ng/mL of *IL-1β* was used to stimulate NPCs to establish an apoptosis model of IDD (26). Similarly, we used

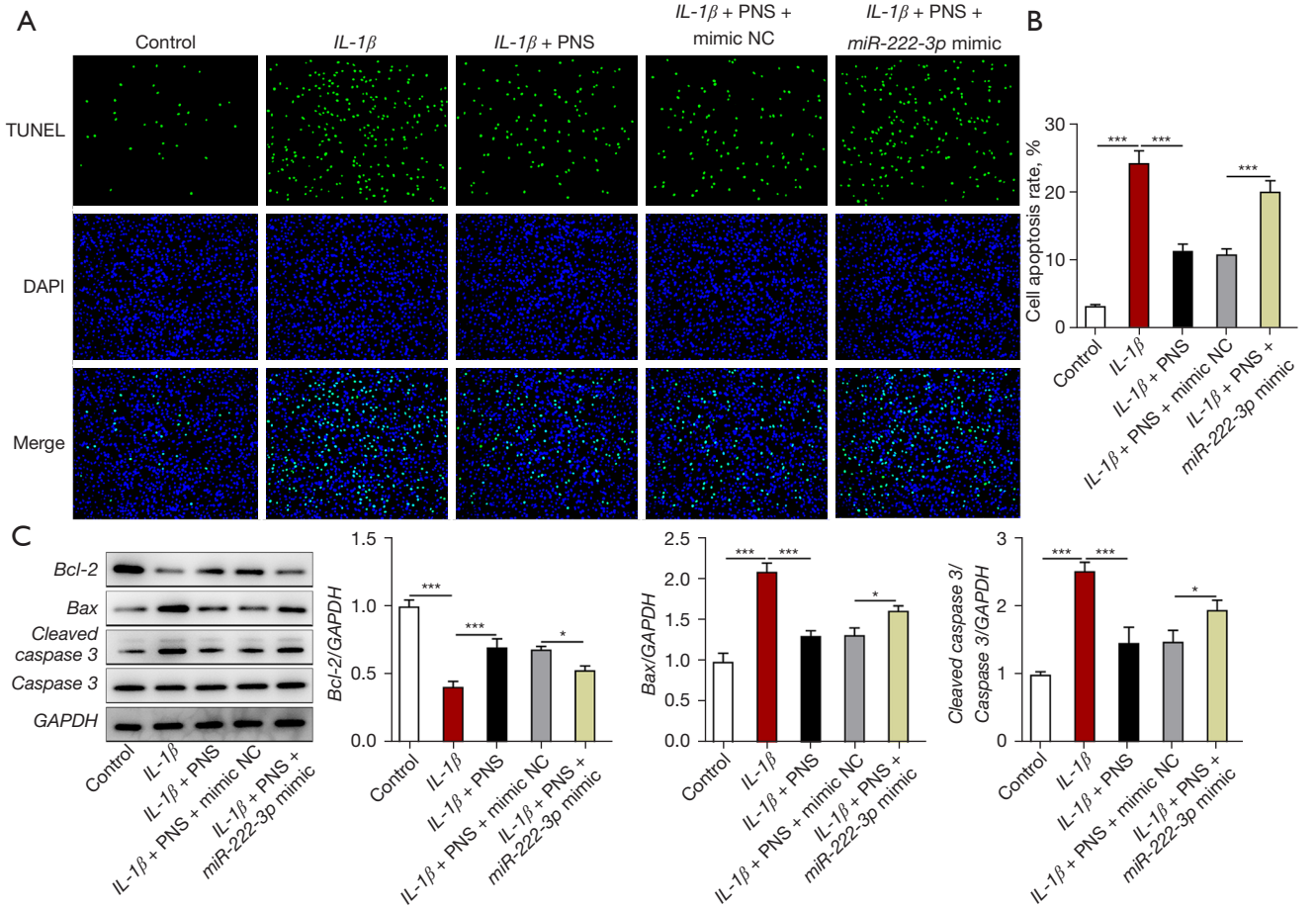

Figure 6 Overexpression of *miR-222-3p* exacerbates the apoptosis of hNPCs co-treated with both *IL-1β* and PNS. (A,B) TUNEL assays were used to evaluate cell apoptosis (×200). (C) The protein expressions of the apoptosis-related factors were analyzed by western blot. *P<0.05; ***P<0.001. *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; NC, negative control; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole; *Bcl-2*, B cell lymphoma-2; *Bax*, Bcl-2 associated X; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; hNPCs, human nucleus pulposus cells.

75 ng/mL of *IL-1β* to induce the apoptosis of NPCs. The experimental results revealed that *IL-1β* increased the levels of the proinflammatory cytokines, including *TNF-α* and *IL-6*, upregulated the expression levels of ER-stress-related factors, including *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*, and reduced the viability but increased the apoptosis of hNPCs. Additionally, the nuclear translocation of *NF-κB p65* was also promoted in hNPCs after stimulation by *IL-1*β.

As reported, the apoptosis of *IL-1β*-induced hNPCs can be influenced by multiple factors including higenamine (44), tyrosol (45), dezocine (46), Omentin-1 (47) and so on. PNS is a traditional Chinese medicine with important clinical value (6). Recent progress on the pharmacological effects of PNS has pointed out that PNS possesses anti-inflammatory, anti-oxidant, anti-aging, anti-insomnia and anti-depression pharmacological properties (6,9). A study has shown that PNS is of great value in treating cancers, diabetes, atherosclerosis, acute lung injury, and cardiovascular diseases (48). However, no previous study had explored the role of PNS in the phenotype of hNPCs in IDD. In the present study, we discovered that PNS strengthened cell viability but prohibited cell apoptosis in *IL-1β*-induced hNPCs, as evidenced by the increased *Bcl-2* protein level and the decreased *Bax* and *cleaved caspase 3* protein levels. Further, following the addition of PNS, the downregulated *TNF-α* and *IL-6* levels, *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP* expression levels, and the inhibited nuclear

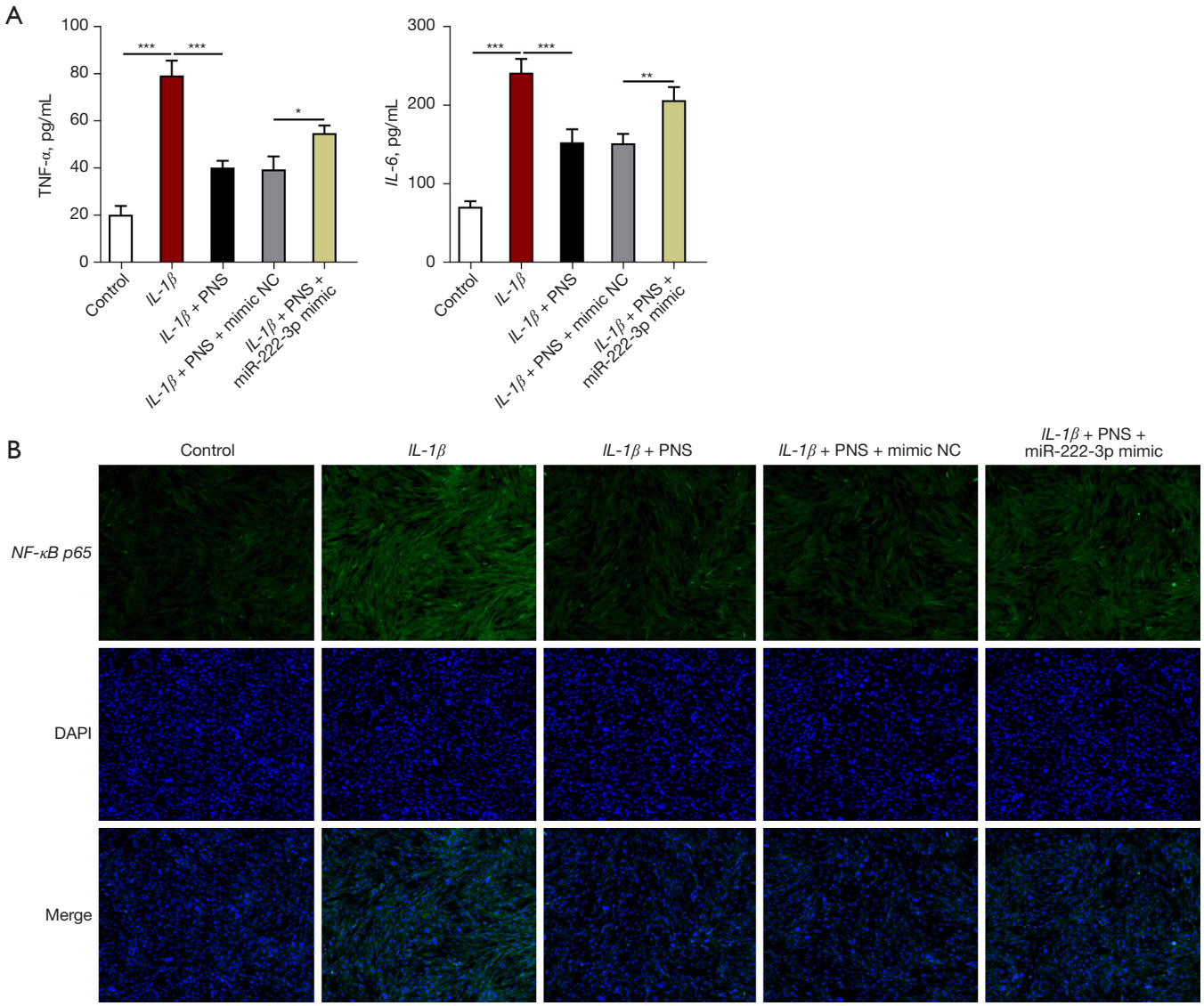

Figure 7 *miR-222-3p* elevation offsets the inhibitory effects of PNS on *IL-1β*-induced hNPCs. (A) Levels of *TNF-α* and *IL-6* were examined by ELISA assays. (B) The nuclear translocation of *NF-κB p65* was detected by IF assay (×100). **P<0.01; ***P<0.001. *TNF-α*, tumor necrosis factor-alpha; *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; *IL-6*, interleukin 6; NC, negative control; *NF-κB*, nuclear factor-kappa beta; DAPI, 4',6-diamidino-2-phenylindole; hNPCs, human nucleus pulposus cells; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence.

translocation of *NF-κB p65* implied that PNS was capable of protecting hNPCs against *IL-1β*-triggered inflammation, nuclear translocation of *NF-κB p65*, and ER stress.

It has been well established by recent studies that miRNAs widely participate in the process of IDD and are largely dependent on the modulation of the phenotypes of NPCs (17-20). For example, Chen *et al.* showed that *miR-150-5p* was involved in the development of IDD through a ceRNA mechanism (22). Lin *et al.* validated the role of the *miR-495-3p/IL5RA* axis in *TNF-α*-treated hNPCs (49). Additionally, the pro-apoptotic and pro-inflammatory roles of *miR-222* in hNPCs in IDD have also been highlighted (50,51). The upregulation of *miR-222-3p* has been found to be closely associated with colorectal cancer, osteosarcoma, non-small cell lung cancer, and diffuse large B-cell lymphoma (52-55). Our experimental results suggested

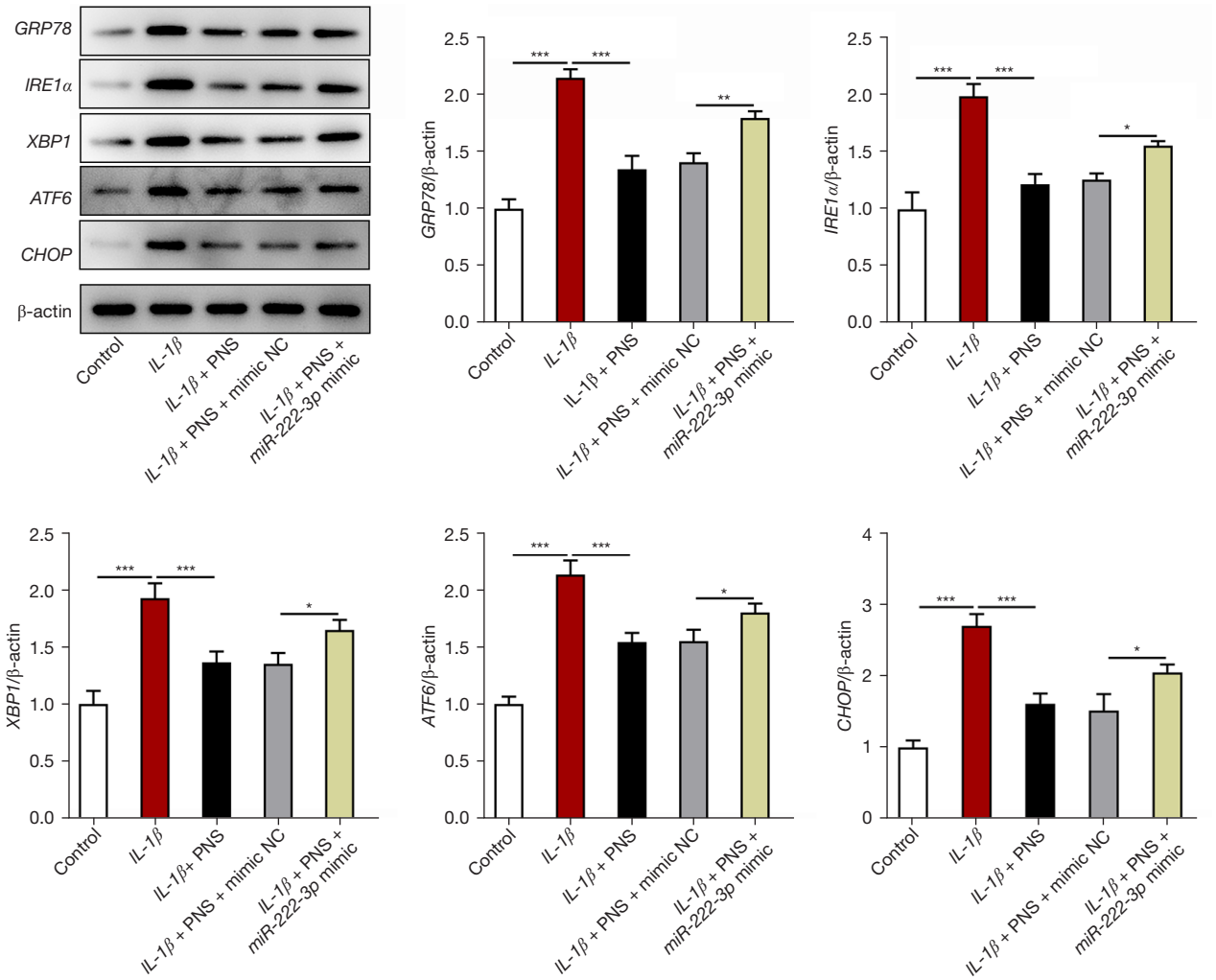

Figure 8 PNS relieves *IL-1β*-stimulated ER stress in hNPCs by suppressing *miR-222-3p*. Western blot was used to analyze the protein levels of *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*. *P<0.05; **P<0.01; ***P<0.001. *GRP78*, glucose regulating protein 78; *IRE1α*, inositol-requiring protein 1 alpha; *XBP1*, X-box binding protein 1; *ATF6*, activating transcription factor 6; *CHOP*, C/EBP homologous protein; *IL-1β*, interleukin-1 beta; PNS, panax notoginseng saponin; NC, negative control; ER, endoplasmic reticulum; hNPCs, human nucleus pulposus cells.

that the high expression of *miR-222-3p* in *IL-1β*-exposed hNPCs was decreased by PNS. Further, after *miR-222-3p* was overexpressed, the viability was inhibited but the apoptosis was stimulated in the *IL-1β*- and PNS-co-treated hNPCs, which was consistent with the findings of Liu *et al.* (23). Additionally, our investigation also revealed that *miR-222-3p* upregulation motivated the inflammatory response, nuclear translocation of *NF-κB p65*, and ER stress in *IL-1β*- and PNS-co-treated hNPCs, as evidenced by the elevated levels of *TNF-α* and *IL-6* and the expression levels of *GRP78*, *IRE1α*, *XBP1*, *ATF6*, and *CHOP*.

Conclusions

In summary, our study demonstrated that PNS appeared to exert pro-proliferation, anti-apoptosis, anti-inflammation, and anti-ER stress effects in *IL-1β*-pretreated hNPCs in IDD by downregulating *miR-222-3p*. These findings might provide novel insights into the therapy for IDD and implied the potential application to IDD treatment. However, it should be noted that this study has some limitations. First, other potential targets involved in the downstream of *miR-222-3p* need to be identified in following studies. Second, the function of PNS on IDD *in vivo* needs to be explored.

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Footnote

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