

Nischarin attenuates apoptosis induced by oxidative stress in PC12 cells

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Abstract. Nischarin (NISCH) is a cytoplasmic protein known to serve an inhibitory role in breast cancer cell apoptosis, migration and invasion. Recently, NISCH has been reported to be involved in the regulation of spinal cord injury (SCI). However, the molecular mechanism is still unclear. Oxidative stress contributes to tissue injury and cell apoptosis during the development of various diseases, including SCI. The aim of the present study was to investigate the role of NISCH in the regulation of apoptosis induced by oxidative stress in PC12 cells. H₂O₂ was used to establish an oxidative stress model in PC12 cells. Apoptosis levels were examined using flow cytometry analysis, and the expression of NISCH, Bcl-2, Bcl-2-associated X (Bax) and caspase-3 were examined using western blot and immunofluorescence staining analyses. The results demonstrated that treatment with 100 μ M H₂O₂ significantly increased the apoptotic rate and expression of NISCH in PC12 cells. At 48 h following incubation with 100 μ M H₂O₂, NISCH downregulation partially inhibited apoptosis of PC12 cells. In addition, the expression of Bcl-2 was significantly reduced and the expression of Bax and caspase-3 were significantly increased by H₂O₂ treatment. These effects were also partially inhibited by the downregulation of NISCH. The authors of the present study therefore hypothesize that NISCH may function as a pro-apoptotic protein that participates in the regulation of oxidative stress, and NISCH downregulation may protect cells from oxidative stress-induced apoptosis.

Introduction

Spinal cord injury (SCI) occurs as a result of a multifactorial process, involving primary injury subsequently followed by a secondary injury (1). Primary injury, caused by an initial

physical impact, is characterized by acute bleeding and ischemia (2). Secondary injury may be induced by several mechanisms, including inflammation, oxidative stress, ion balance dysregulation and excitotoxicity (3-5). It has been reported that allicin may protect rats against SCI via regulation of oxidative stress and inflammatory response pathways (6), while inhibition of reactive oxygen species (ROS) production may improve outcomes following SCI by mediating acute reductions in oxidative stress and inflammation (7). Apoptosis has been suggested as a potential therapeutic target for secondary SCI (8), as one of the most damaging processes of secondary SCI is neuronal apoptosis (9). Motor neuron apoptosis is the main cause of dysfunction following SCI, as it is one of the primary obstacles for locomotor functional recovery (10).

The generation of ROS, such as superoxide anions, H₂O₂ and hydroxyl radicals, is a normative response to disease or injury, including SCI (11). The increased formation of ROS may exceed the capacity of the antioxidant defense systems and subsequently lead to oxidative stress (12,13). Oxidative stress serves an important role in apoptosis induction under physiologic and pathologic conditions (14,15). A previous study demonstrated that myricetin may protect cells against H₂O₂-induced cell damage via its anti-apoptotic effects (16).

Nischarin (NISCH) is a cytosolic protein that is found anchored to the inner layer of the plasma membrane, and has been demonstrated to interact with cytosolic and intermembrane proteins (17). NISCH is expressed in various organs, and a previous study demonstrated that NISCH serves an inhibitory role in cell migration, invasion and the carcinogenesis of breast cancer cells (18). NISCH is highly expressed in the brain of adult rats, and has been confirmed to be expressed in PC12 and Neuro-2A cell lines (19). It has been reported that overexpression of NISCH may induce human breast cancer apoptosis and inhibit cell migration and invasion (20), while inhibition of NISCH expression promotes neurite outgrowth (18). Recently, it has been shown that NISCH downregulation by small-interfering RNA (siRNA) accelerates rat motor function recovery following SCI (21), indicating that NISCH may be involved in the pathological mechanisms of SCI. However, the exact molecular mechanism remains unclear.

The authors of the present study hypothesized that NISCH downregulation attenuates oxidative stress-induced apoptosis in PC12 cells. Therefore, the apoptotic rate of PC12 cells and

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the expression of Bcl-2/Bcl-2-associated X (Bax) signaling pathway members was examined in the present study.

Materials and methods

Cell culture. The PC12 rat pheochromocytoma tumor cell line was purchased from PeproTech Inc. (Rocky Hill, NJ, USA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂.

Flow cytometric analysis of cell apoptosis. Cells were treated with 100 µM H₂O₂ for 0, 24, 48, 72 and 96 h. Following H₂O₂ treatment, cells were harvested by trypsinization and washed twice with cold phosphate-buffered saline (PBS). Cell apoptosis was measured following drug treatment using the Annexin V-FITC/PI Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Cells were resuspended and mixed with 500 µl binding buffer containing 5 µl Annexin V-fluorescein isothiocyanate (FITC) and 5 µl propidium iodide (PI). Following incubation for 15 min at room temperature in the dark, cell apoptosis was detected using fluorescence-activated cell sorting (FACS) flow cytometers from BD Biosciences (Franklin Lakes, NJ, USA) or Beckman Coulter, Inc. (Brea, CA, USA) using BD Cell Quest research software (version 5.2.1; BD Biosciences).

NISCH siRNA preparation and cell transfection. The NISCH siRNA and negative control sequences were as follows: NISCH siRNA sense, 5'-GCAAGCACUGACCACUCUATT-3' and anti-sense, 5'-UAGAGUGGUCAGUGCUUGCTT-3'; Negative control sense, 5'-UUCUCCGAACGUGUCACGUTT-3', and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'. NISCH siRNA was diluted to 20 µM with a universal buffer (Beijing Huaxia Ocean Technology Co., Ltd., Beijing, China). Cells were divided into control, vehicle and NISCH-siRNA groups. PC12 cells in the logarithmic growth phase were seeded at a density of 2.5 × 10⁵ cells/well in a 12-well plate (1 ml/well) and incubated at 37°C and 5% CO₂ overnight. At 2 h prior to transfection, the culture medium was replaced with serum-free RPMI-1640 culture medium. To prepare cells for transfection, 5 µl siRNA was first diluted in 200 µl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) without serum. Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was gently mixed with the diluted siRNA solution (total volume 400 µl) and the samples were incubated at room temperature for 20 min. A total of 200 µl of this mixture was added into each well, and the cell culture plate was gently shaken to mix the culture solution. The transfection groups were as follows: Control group, no transfection; vehicle group, transfected with negative control sequences; NISCH-siRNA group, transfected with siRNA sequences targeting the rat NISCH gene.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using a TRIzol reagent kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) and reverse transcribed into cDNA using the HiScript® Reverse Transcriptase

(RNase H) kit (Vazyme, Piscataway, NJ, USA), according to the manufacturer's protocol. Real-time PCR was performed using the 7900HT Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. The mRNA expression levels of NISCH were normalized to the endogenous expression of β-actin. Primers were purchased from Tsingke (Beijing, China). The following primer pairs were used for qPCR analyses: NISCH, forward, 5'-TAT GTTGTGGCACAGAAGATGG-3' and reverse, 5'-TTCAGG CAATGGATAGTGGAT-3'; β-actin forward, 5'-CACGATGGA GGGGCCGGACTCATC-3' and reverse, 5'-TAAAGACCT CTATGCCAACACAGT-3'. The PCR reaction was performed in a total volume of 20 µl, consisting of 4 µl cDNA (5 ng/µl), 0.8 µl primer mix (10 µM), 10 µl SYBR-Green PCR Master Mix (Vazyme Biotech Co., Ltd.), 0.4 µl ROX Reference Dye 2 (50X; ShenZhen Hui Nuo Biotechnology Co., Ltd., Shenzhen, China) and 4.8 µl H₂O. Thermal cycling conditions were as follows: 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min. β-actin was used as an internal control and the expression levels of target genes were calculated using the 2^{-ΔΔC_q} method (22).

Western blot analysis. Total protein was extracted from H₂O₂-treated cells using lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the samples were incubated on ice for 50 min. Protein concentration was measured using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). An equal quantity of protein for each sample was separated via SDS-PAGE on a 12% gel and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 10% skim milk in PBS and 0.1% Tween-20 (pH 7.2) for 2 h at room temperature and incubated at 4°C overnight with an appropriate quantity of the following primary antibodies: Mouse monoclonal anti-β-actin (dilution, 1:200; cat. no. BM0627; Wuhan Boster Biological Technology, Ltd., Wuhan China), mouse polyclonal anti-NISCH (dilution, 1:500; cat. no. 558262; BD Biosciences), rabbit polyclonal anti-Bcl-2 (dilution, 1:500; cat. no. 12789-1-AP; Wuhan Sanying Biotechnology, Wuhan, China), rabbit polyclonal anti-Bax (dilution, 1:5,000; cat. no. ab32503; Abcam, Cambridge, UK) and rabbit polyclonal anti-caspase-3 (dilution, 1:800; cat. no. 19677-1-AP; Wuhan Sanying Biotechnology). Immunoblots were incubated with the horseradish peroxidase-labeled secondary antibodies goat anti-rabbit Ig (dilution, 1:50,000; cat. no. BA1054) or goat anti-mouse Ig (dilution, 1:50,000, cat. no. BA1051; both Wuhan Boster Biological Technology, Ltd.) for 2 h at 21°C, and bands were detected using the Novex™ ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.). Densitometry analysis was performed using ImageJ software (version 1.48u; National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence analysis. Cells mounted on slides were placed in a culture plate and washed with PBS three times. The cells were then fixed with 4% paraformaldehyde for 15 min at 4°C and incubated with 0.5% Triton X-100 at room temperature for 20 min. Subsequently, cells were blocked with 5-10% goat serum (Wuhan Boster Biological Technology, Ltd.) at room temperature for 30 min. Cells were subsequently incubated overnight at 4°C with primary antibodies against NISCH

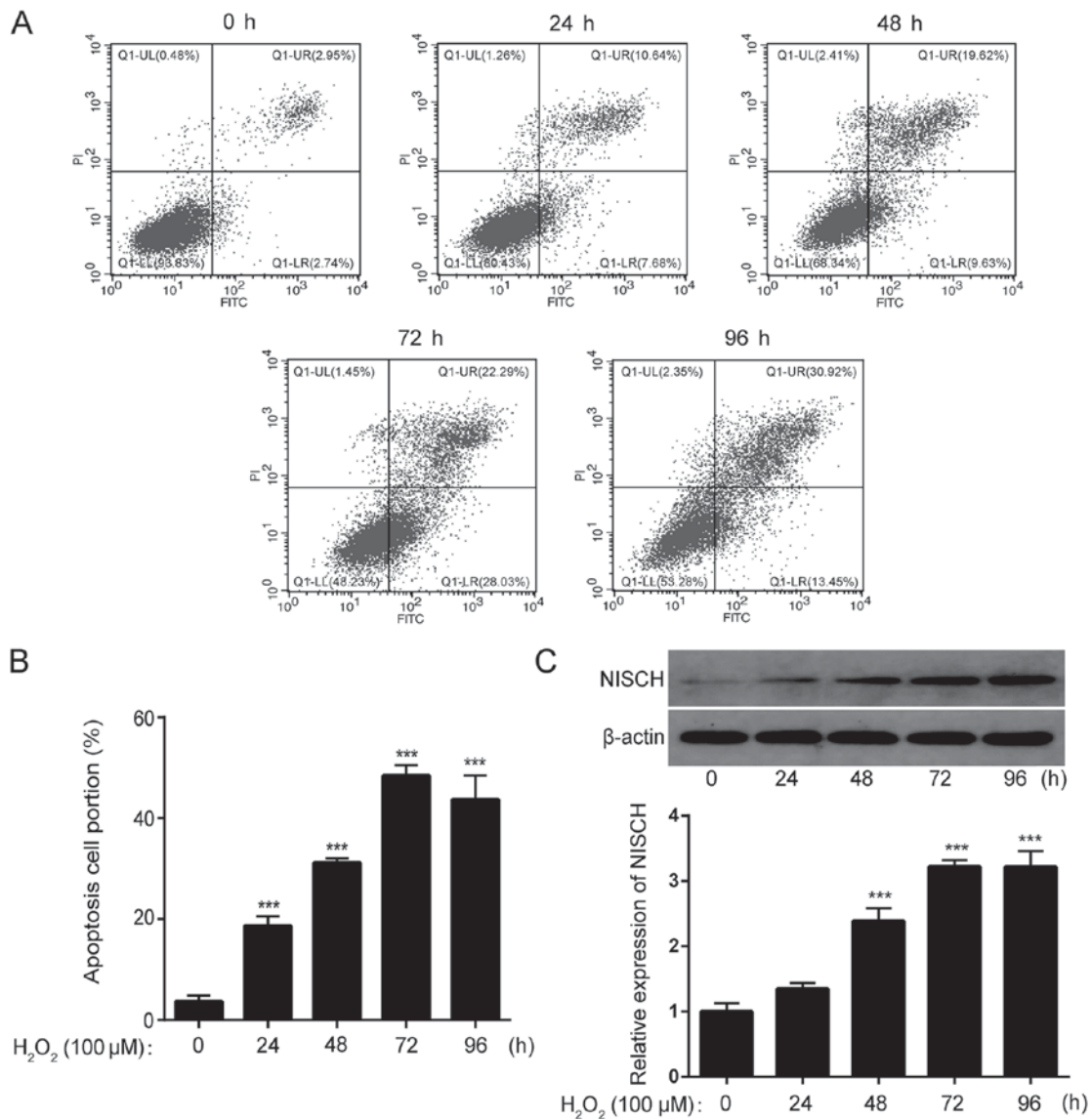


Figure 1. Apoptosis levels and NISCH expression induced by H₂O₂ in PC12 cells. (A) Apoptosis levels in PC12 cells following treatment with 100 μM H₂O₂ for 0, 24, 48, 72 and 96 h and (B) quantitative evaluation of the results. (C) The expression of NISCH protein in PC12 cells following treatment with 100 μM H₂O₂ for 0, 24, 48, 72 and 96 h. The results are presented as the mean ± standard error of the mean and are representative of three independent experiments. ***P<0.001 vs. 0 h. NISCH, nischarin; PI, propidium iodide; FITC, fluorescein isothiocyanate.

(dilution, 1:50; cat. no. 558262; BD Biosciences), Bcl-2 (dilution, 1:50; cat. no. 12789-1-AP; Wuhan Sanying Biotechnology), Bax (dilution, 1:250; cat. no. ab32503; Abcam) and caspase-3 (dilution, 1:50; cat. no. 19677-1-AP; Wuhan Sanying Biotechnology). Immunofluorescence was generated by incubating samples with the Cy3-conjugated secondary antibodies goat anti-rabbit IgG (dilution, 1:100; cat. no. BA1032) or goat anti-mouse IgG (1:100; cat. no. BA1031; both Wuhan Boster Biological Technology, Ltd.) for 1 h at 37°C in the dark. DAPI (5 μg/ml) was added to counterstain the nuclei, and the slides were incubated for 5 min at room temperature in the dark before being washed with PBS to remove excess DAPI. Images were obtained using a fluorescence microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using GraphPad 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data are expressed as the mean ± standard error of

the mean and are representative of three independent experiments. Comparisons among multiple groups were performed by one-way analysis of variance followed by a Bonferroni post hoc multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

H₂O₂-induces cell apoptosis and increases NISCH expression in PC12 cells. Cells were treated with 100 μM H₂O₂ for 0, 24, 48, 72 and 96 h, respectively. By staining cells with Annexin V-FITC and PI, the apoptotic rate was analyzed by FACS. The results demonstrated that the proportion of apoptotic cells was significantly increased by H₂O₂ treatment of PC12 cells at all time points (Fig. 1A and B). Furthermore, the expression of NISCH in PC12 cells was significantly increased by H₂O₂ treatment in a time-dependent manner (Fig. 1C).

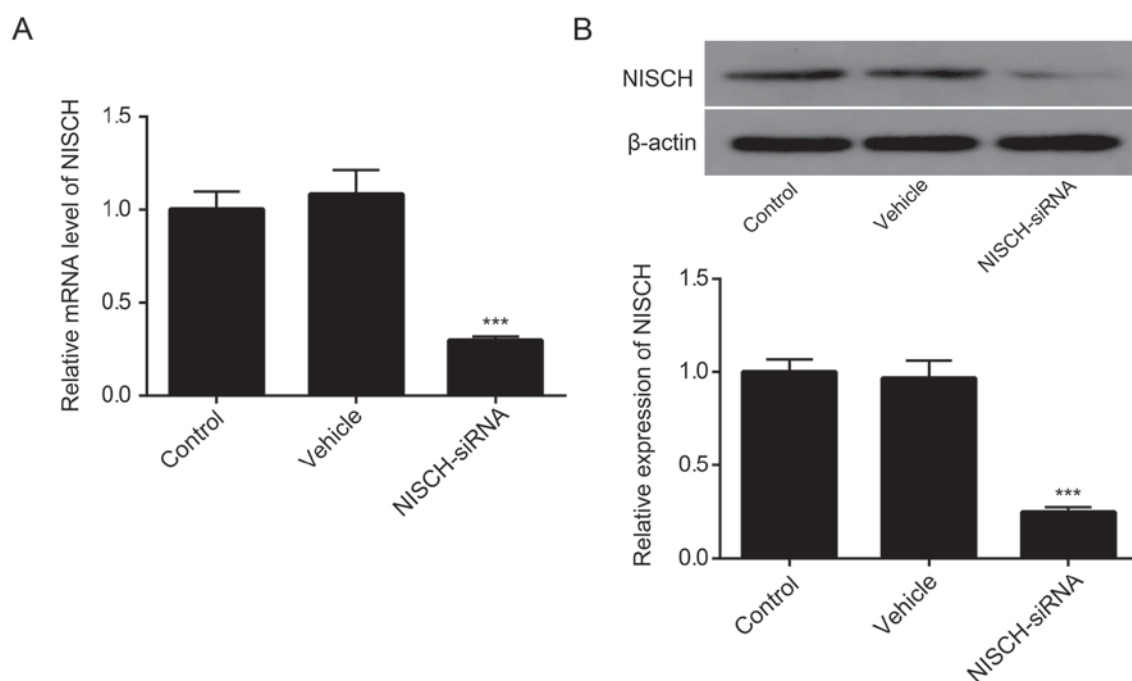


Figure 2. Expression of NISCH following transfection of NISCH-siRNA in PC12 cells. At 48 h following NISCH-siRNA transfection, the (A) mRNA and (B) protein levels of NISCH were significantly decreased. The results are presented as the mean \pm standard error of the mean and are representative of three independent experiments. *** P <0.001 vs. Vehicle group. NISCH, nischarin; siRNA, small-interfering RNA.

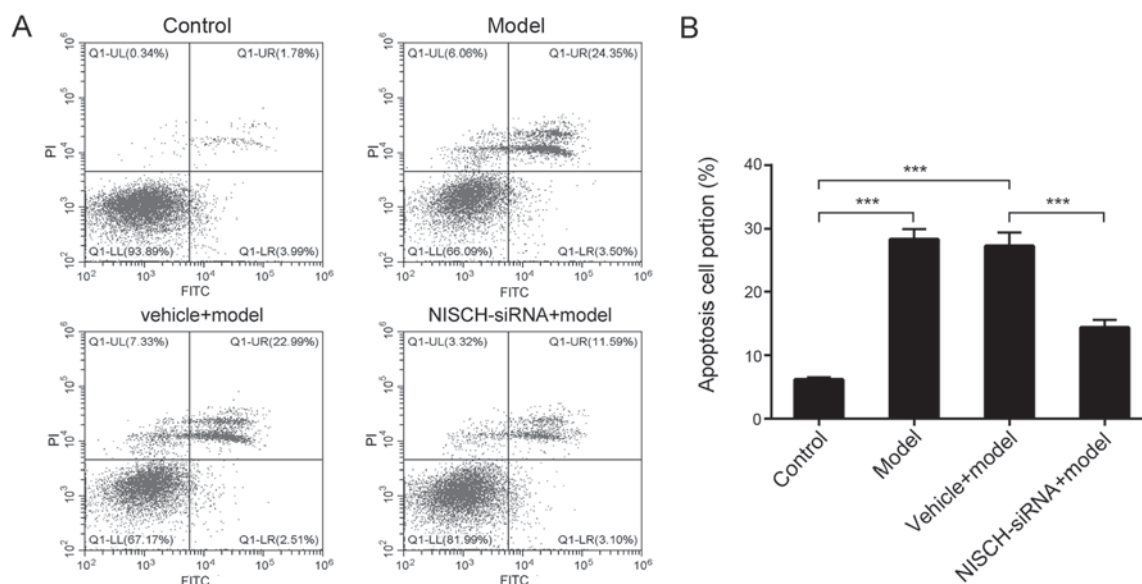


Figure 3. Effects of NISCH downregulation on apoptosis levels induced by H_2O_2 . (A) Apoptosis levels following treatment with H_2O_2 for 48 h and (B) quantitative evaluation of the results. The results are presented as the mean \pm standard error of the mean and are representative of three independent experiments. *** P <0.001 as indicated. NISCH, nischarin; PI, propidium iodide; FITC, fluorescein isothiocyanate; siRNA, small interfering RNA.

NISCH-siRNA reduces NISCH expression in PC12 cells. As demonstrated in Fig. 2A, the level of NISCH mRNA was significantly decreased in PC12 cells at 48 h following transfection with NISCH-siRNA. Consistent with these results, western blot analysis indicated that the expression of NISCH was reduced in PC12 cells at 48 h following NISCH-siRNA transfection (Fig. 2B).

NISCH downregulation attenuates H_2O_2 -induced apoptosis in PC12 cells. As demonstrated in Fig. 3A and B, H_2O_2

significantly increased the level of apoptosis in PC12 cells, while NISCH downregulation significantly reduced apoptosis levels at 48 h following H_2O_2 treatment. These results suggested that NISCH may function as a pro-apoptotic protein during H_2O_2 -induced apoptosis, and downregulation of NISCH may serve a protective role in H_2O_2 -treated PC12 cells.

NISCH downregulation alters Bcl-2/Bax signaling induced by H_2O_2 in PC12 cells. The Bcl-2/Bax signaling pathway serves an important role in apoptosis (23). The pro-survival

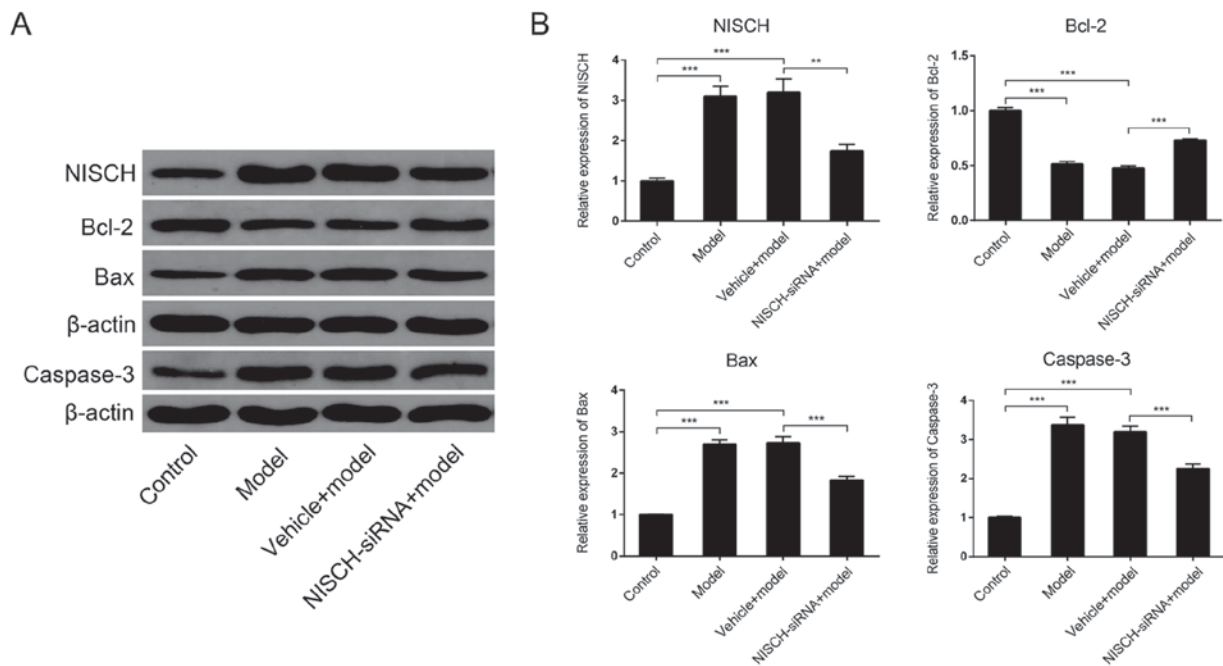


Figure 4. Effects of NISCH downregulation on the Bcl-2/Bax signaling pathway induced by H_2O_2 . (A) At 48 h following H_2O_2 treatment, the protein expression levels of NISCH, Bcl-2, Bax and caspase-3 in PC12 cells were evaluated by western blotting analysis and (B) the results were semi-quantified. The results are presented as the mean \pm standard error of the mean and are representative of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ as indicated. NISCH, nischarin; Bax, Bcl-2-associated X; siRNA, small interfering RNA.

Bcl-2 and pro-apoptotic Bax are the two most extensively studied members of the Bcl-2 family. These proteins function as the primary regulators of the mitochondrial apoptosis signaling pathway (24). Caspase enzymes are crucial effectors of the cell death signaling pathway and are activated by almost all apoptosis-inducing stimuli within neurons and non-neuronal cells (25). Caspase-3 appears to serve a pivotal role in this pathway and has been demonstrated to be necessary for the regulation of development-associated cell death in the brain (25). Therefore, the expression of Bcl-2 and Bax in PC12 cells at 48 h following H_2O_2 treatment was examined in the present study. As demonstrated in Fig. 4, H_2O_2 significantly increased NISCH expression, while NISCH downregulation significantly reduced H_2O_2 -induced NISCH expression. In addition, Bcl-2 expression was significantly reduced, while Bax and caspase-3 expression levels were significantly increased by H_2O_2 treatment (Fig. 4). These H_2O_2 -induced expression alterations were partially inhibited by NISCH downregulation. The results indicate that NISCH downregulation may protect against the apoptosis of PC12 cells by inhibiting the transduction of Bcl-2/Bax signaling pathways. Immunofluorescence analysis demonstrated the same alternations in NISCH, Bax, Bcl-2 and caspase-3 expression in response to H_2O_2 treatment with or without transfection with NSCH-siRNA (Fig. 5).

Discussion

Oxidative stress and the generation of free radicals, as primary or secondary events, have been implicated in a number of diseases (26). Recent studies have demonstrated that oxidative stress may serve a role in the pathogenesis of SCI (27,28). It has been demonstrated that markers of

oxidative stress, such as malondialdehyde, and advanced oxidation protein products are significantly increased in rats with SCI, while antioxidants such as glutathione peroxidase and catalase are significantly decreased (27). In addition, a previous study demonstrated that a significant reduction of the expression of lipid peroxidation factors malondialdehyde may contribute to the reported neuroprotection of the spinal cord from oxidative damage, likely induced by the increased SOD (28). NISCH is reportedly involved in regulating the pathological mechanisms of SCI (21). In the present study, the role of NISCH in oxidative stress-induced apoptosis was investigated using a model of H_2O_2 -induced oxidative damage in PC12 cells. The results indicated that treatment of cells with 100 μM H_2O_2 significantly induced apoptosis and increased NISCH expression at different time points. By contrast, NISCH downregulation significantly attenuated apoptosis levels and inhibited the expression of Bcl-2/Bax signaling induced by H_2O_2 .

Apoptosis is important for the development of neuronal and non-neuronal cells in both the peripheral and central nervous system (29). Aberrant apoptosis contributes to the pathogenesis of a variety of disease states, such as SCI, while inhibition of apoptosis in the pathological state may improve the pathological injury. For instance, it has been demonstrated that treadmill exercise can promote the recovery of motor function by suppressing apoptosis in the injured spinal cord (30), and overexpression of neuroglobin can improve functional recovery by suppressing neuronal apoptosis following SCI (31). In the present study, the expression of NISCH and levels of apoptosis were significantly increased in PC12 cells following treatment with H_2O_2 in a time-dependent manner, indicating that NISCH may be involved in H_2O_2 -induced apoptosis in PC12 cells.

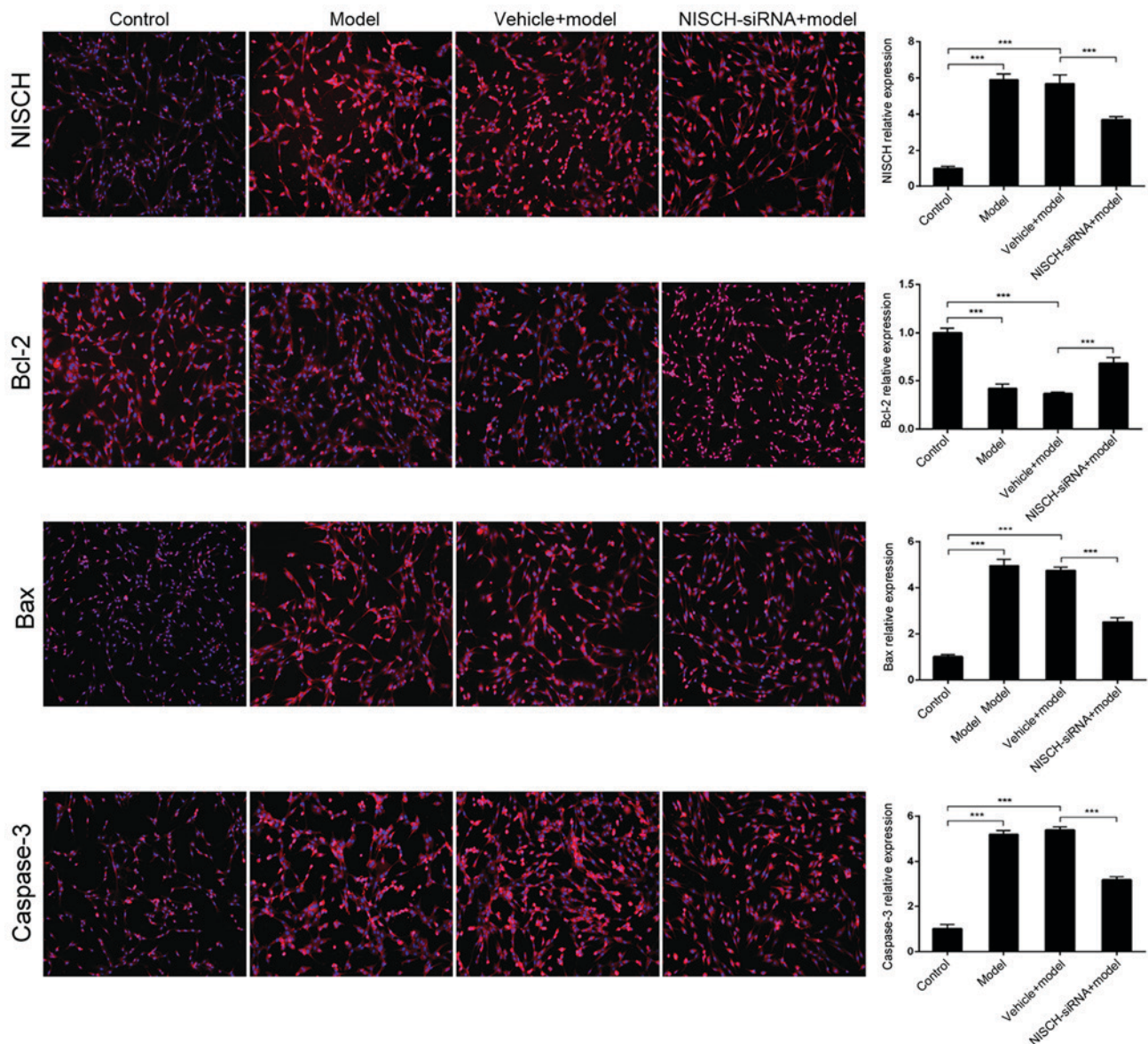


Figure 5. Immunofluorescence analysis of the effects of NISCH downregulation on the Bcl-2/Bax signaling pathway induced by H_2O_2 . At 48 h following H_2O_2 treatment, the expression levels of NISCH, Bcl-2, Bax and caspase-3 in PC12 cells was analyzed by immunofluorescence staining and quantified. The results are presented as the mean \pm standard error of the mean and are representative of three independent experiments. *** $P < 0.001$ as indicated. NISCH, nischarin; Bax, Bcl-2-associated X; siRNA, small interfering RNA.

Bcl-2 and Bax are two discrete members of a gene family involved in the regulation of apoptosis. Bcl-2 inhibits cell death in response to various stimuli, while overexpression of Bax exerts a pro-apoptotic effect, and antagonizes the anti-apoptotic activity of Bcl-2 (32,33). Furthermore, the Bcl-2/Bax signaling pathway reportedly participates in the pathogenesis of SCI (34-36). Caspase-3 is a crucial mediator of apoptosis and a frequently activated cell death protease that catalyzes the specific cleavage of numerous key cellular proteins (37). A previous study demonstrated that caspase-3 is activated following SCI (38), and prevention of caspase-3 activation reduces apoptosis levels (39). In the current study, NISCH downregulation was demonstrated to inhibit the H_2O_2 -induced reduction in Bcl-2 and increase in Bax expression in PC12 cells, indicating that NISCH downregulation inhibited transduction of the Bcl-2/Bax signaling pathway. In addition, NISCH downregulation inhibited the activation of caspase-3 by H_2O_2 , which further demonstrated that

NISCH downregulation may attenuate H_2O_2 -induced apoptosis in PC12 cells via inhibition of the Bcl-2/Bax apoptotic signaling pathway.

NISCH has been previously identified as a novel protein that selectively binds to the proximal transmembrane region of the integrin $\alpha 5$ subunit cytoplasmic tail (40). Overexpression of the $\alpha 5$ subunit may protect cells against apoptotic stimuli by modulating the expression of the anti-apoptotic protein Bcl-2 via activating the phosphoinositide-3-kinase/Akt signaling pathway (41). NISCH selectively binds to the integrin $\alpha 5$ subunit and negatively regulates the expression of integrin $\alpha 5$ subunit (40). In addition, a previous study demonstrated that overexpression of NISCH may induce apoptosis in human breast cancer cells (20). Therefore, NISCH may exert pro-apoptotic activities by regulating the activity of the Bcl-2/Bax signaling pathway via interaction with the integrin $\alpha 5$ subunit. However, additional apoptosis pathways may be

involved in the pathological mechanism induced by oxidative stress, which requires further investigation in future studies.

In conclusion, the current study provides evidence for the role of NISCH in oxidative stress-induced apoptosis, and suggests a potential mechanism by which NISCH downregulation attenuates cell apoptosis induced by oxidative stress. The authors of the present study hypothesize that NISCH downregulation may inhibit cell apoptosis by inhibiting transduction of the Bcl-2/Bax signaling pathway. These results may provide a therapeutic candidate for protecting against oxidative stress-induced apoptosis.

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZG, YY, and YG performed the experiments. ZG, HW, and CS collected the data and prepared the manuscript. ZG and MH designed the study and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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