

Dexmedetomidine inhibits cerebral nerve cell apoptosis after cerebral hemorrhage in rats *via* the Nrf2/HO-1/NQO1 signaling pathway

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Abstract. – **OBJECTIVE:** To investigate the effect of dexmedetomidine on the apoptosis of cerebral nerve cells after cerebral hemorrhage (CH) in rats and its molecular mechanism.

MATERIALS AND METHODS: The rat model of CH was established by autologous blood injection. A total of 60 specific pathogen-free (SPF)-grade rats were randomly divided into sham-operation group, model group and dexmedetomidine group, and each group involved 20 rats. Rat brain water content was compared among the three groups. Besides, rat neurological function of the three groups was evaluated at 3, 5 and 7 d after operation by neurological function scoring. Western blotting assay was adopted to detect protein levels of apoptosis-related genes [B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax)] in rat brain tissues in the three groups. Moreover, the apoptosis level in the brain tissues in the groups was measured through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Biochemical tests were conducted to determine activities of reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) in the brain tissues among the three groups. Furthermore, the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/NAD(P)H quinone oxidoreductase 1 (NQO1) signaling pathway in the brain tissues of the three groups of rats was examined *via* Western blotting assay. An *in vitro* oxygen-glucose deprivation (OGD) model was prepared using SH-SY5Y cells. In addition, Nrf2 was intervened in SH-SY5Y cells by small hairpin ribonucleic acid (shRNA) transfection. Finally, flow cytometry and Annexin V/PI assay were performed to detect the response of cells to dexmedetomidine in OGD + dexmedetomidine + sh-Nrf2 group.

RESULTS: The brain water content and the neurological function score at 3, 5 and 7 d after operation were remarkably reduced in dexmedetomidine group compared with those in model group. The results of Western blotting and TUNEL assays indicated that dexmedeto-

midine group had a notably lowered apoptosis level in the brain tissues. Additionally, the biochemical test results manifested that activities of GSH and SOD were enhanced and that of MDA decreased in the brain tissues of dexmedetomidine group. Protein levels of Nrf2, HO-1 and NQO1 in the brain tissues were distinctly higher in dexmedetomidine group than those in model group. According to the results of flow cytometry, the apoptosis rate in OGD + dexmedetomidine + sh-Nrf2 group rose prominently compared with that in OGD + dexmedetomidine group.

CONCLUSIONS: Dexmedetomidine inhibits the nerve cell apoptosis in rat brain tissues by activating the Nrf2/HO-1/NQO1 signaling pathway in rat CH models.

Key Words:

Dexmedetomidine, Cerebral hemorrhage, Nrf2/HO-1/NQO1.

Introduction

Cerebral hemorrhage (CH) is a common type of cerebrovascular disease. According to the results of epidemiological studies, about 25 per 100,000 people suffer from CH every year around the world, and the morbidity rate of CH is significantly higher in Asian populations. CH is a primary non-traumatic lesion of cerebral parenchymal hemorrhage, which frequently occurs in middle-aged and elderly people (50-70 years old). Generally speaking, hypertension, type 2 diabetes mellitus, hypertensive arteriopathy and cerebral amyloid angiopathy are the major causes of CH. The mortality rate of CH is 30-50%, and approximately 75% survivors are unable to independently live within 1 year after treatment. Besides, it is predicted that the morbidity rate of CH is rising constantly with the aging popula-

tion. The long-term efficacy of operation for CH remains controversial, and at present, efficacious treatment methods for improving the prognosis of CH patients are still lacked.

Oxidative stress induced by intracerebral hematoma plays an important role in the pathological mechanism of CH, which poses significant effects on the prognosis and outcome of CH patients¹. After the onset of CH, microglial cells and neutrophilic granulocytes in the lesioned sites trigger the inflammatory response, thus releasing numerous free radicals. Abundant free radicals damages the antioxidant capacity in the body, finally causing cell death. Heme oxygenase-1 (HO-1) is the first rate-limiting enzyme in the degradation of heme to free iron, carbon monoxide (CO), and biliverdin, which is highly induced in oxidative stress conditions². Its synthesis is regulated mainly at a transcriptional level, and nuclear factor erythroid 2-related factor 2 (Nrf2) is considered a key regulator of HO-1 transcription. The Nrf2 signaling pathway is of great significance in inhibiting oxidative stress. Activated Nrf2 signaling pathway can prominently repress oxidative stress in CH cases³.

Dexmedetomidine, a highly selective α_2 -adrenoceptor agonist, is usually applied to anesthesia in clinical practices. It pharmacologically exerts sedative, hypnotic and anti-inflammatory functions⁴. Dexmedetomidine resists oxidative stress in various diseases by activating the Nrf2 signaling pathway^{5,6}. In the present study, we aim to explore the function of dexmedetomidine in inhibiting the apoptosis of cerebral nerve cells in CH rats by activating the Nrf2 signaling pathway.

Materials and Methods

Laboratory Animals

A total of 60 male Wistar rats aged 12-14 weeks old and weighing (279.78±9.94) g at specific pathogen-free (SPF) level were purchased from SIPPR-BK Laboratory Animal Co., Ltd. (Shanghai, China). Animal procedures were approved by the Animal Ethics Committee of Nantong University Animal Center. All rats were raised in a thermostatic (25°C) SPF-level animal room under a 12/12 h light/dark cycle, and they had free accesses to food and water. Following 1 week of adaptive feeding, the CH model was established *via* autologous blood injection as previously described⁷. Briefly, rats were first anesthetized by

3% isoflurane and fixed. Then, 0.3 mL of blood was drawn from the femoral artery and injected into the anterior fontanelle using a syringe (1 mL, 27 G needle) with the aid of a brain stereotaxic instrument. Using the brain stereotaxic instrument, the needle was adjusted to 2.5 mm away from the right paramedian area. Then, a puncture was made on the skull surface by rotating the syringe manually, and the arm of the stereotaxic instrument was carefully adjusted to place the needle above the puncture on the skull. The needle was moved downwards into the brain by 3.5 mm and withdrawn by 0.5 mm 2 min later, so that the tip depth was 3 mm. Subsequently, the blood of the rats was injected slowly. After 25-min indwelling, the needle was slowly withdrawn, and the wound was sutured. Finally, the rats returned to cages after resuscitation. In this research, all rats were assigned into sham-operation group, model group and dexmedetomidine group. Rats in dexmedetomidine group were intraperitoneally injected with 0.5 mg/kg dexmedetomidine at 2 h after operation once a day for 7 consecutive days. After that, the venous blood was drawn from the tail, and the brain tissues were collected after sacrifice.

Measurement of Protein Expressions Via Western Blotting Assay

Rat brain tissues were taken out from liquid nitrogen and thawed at 4°C, which were fully lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protein kinase inhibitor in a tissue homogenizer. Then, the tissue samples were centrifuged at 4°C and 12,000 rpm for 20 min. Subsequently, the protein supernatant was collected for quantification by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After denaturation, 40 µg of the proteins were loaded into each lane for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transfer onto a PVDF membrane (Millipore, Billerica, MA, USA). Next, the membrane was incubated with primary antibodies at 4°C overnight and then with horse radish peroxidase (HRP)-labeled secondary antibodies in the dark for 2 h. The enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used for color development. Finally, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the proteins were quantified using Image J software (NIH, Bethesda, MD, USA).

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Assay

Total ribonucleic acids (RNAs) in cells were extracted strictly according to the manufacturer's instructions of High-purity Total RNA Rapid Extraction Kit (Biotek Corporation, Beijing, China). After reverse transcription, cDNA was amplified at 95°C for 10-s denaturation, 60°C for 20-s annealing and 72°C for 30-s extension. The relative fold change of corresponding messenger RNAs (mRNAs) was calculated *via* $2^{-\Delta\Delta Ct}$ method with β -actin as the internal reference. The assay was performed for at least 3 times, and the primer sequences utilized were shown in Table I.

Cell Culture and Transfection

SH-SY5Y cells were used for establishing the oxygen-glucose deprivation (OGD) model, which were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) in a humidified incubator with 5% CO₂ at 37°C, and the transfection was performed when the cell density in 6-well plates reached 40-60%.

Nrf2 was intervened in SH-SY5Y cells by transfection of the small hairpin RNA (sh)-Nrf2, and the sh-Nrf2 sequences and lentiviral vectors were bought from Sigma-Aldrich (St. Louis, MO, USA). Cells were transfected in strict accordance with the manufacturer's instructions (MOI=3) and then cultured in medium containing virus particles for 12 h. Later, fresh medium was replaced for continuous culture. Finally, RT-qPCR assay was conducted to assess the transfection efficacy.

Apoptosis Assay

Apoptosis was examined using a Guava flow cytometer (Millipore, Billerica, MA, USA) and Annexin V-fluorescein isothiocyanate (FITC)/

propidium iodide (PI) (HaiGene, Harbin, China) staining. After treatment, the cells were trypsinized and adjusted to 1×10^5 cells/mL. Next, cells were stained with Annexin V-FITC for 15 min and PI for another 15 min at room temperature, followed by detecting apoptosis rate *via* flow cytometry and FlowJo software. All the experiments were performed in triplicate.

Evaluation of Neural Injury Severity and Brain Water Content

The neurological function score was graded to assess the protective role of dexmedetomidine against CH-induced brain injury in rats⁸. The severity of neural injury in rats was detected at 3, 5 and 7 d after operation. The intact brain tissues were obtained from 5 rats in each group at 7 d after operation and then weighed. Later, the brain tissues were placed in dry glass bottles for 48 h. The brain water content = (wet weight - dry weight) $\times 100\%$.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

The apoptosis level in rat brain tissues in each group was measured through TUNEL assay. Specifically, the brain tissues (2 mm thick) around the site of CH were prepared into paraffin-embedded sections and sliced to 5 μ m in thickness. After that, tissue sections were deparaffinized in xylene and treated with 100%, 95% and 70% ethanol, followed by incubation with proteinase K to increase the permeability of tissues. Subsequently, 30% hydrogen peroxide was added into the sections to decrease the activity of endogenous peroxidase. Afterwards, TUNEL assay was performed strictly as per the instructions of TUNEL assay kit (Life Technologies, Carlsbad, CA, USA). Finally, the sections were photographed under a fluorescence microscope (Zeiss, Oberkochen, Germany) after counterstaining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA).

Table I. Primer sequences used in the study.

Primer sequence		
Nrf2	Forward	3'-CACATCCAGTCAGAAACCAGTGG-5'
	Reverse	3'-GGAATGTCTGCGCCAAAAGCTG-5'
GAPDH	Forward	3'-AGGTCGGTGTGAACGGATTT-5'
	Reverse	3'-GGGGTCGTTGATGGCAACA-5'

Detection of Superoxide Dismutase (SOD), Malondialdehyde (MDA) and Reduced Glutathione (GSH) Levels in Rat Brain Tissues

In order to evaluate the severity of oxidative stress in rat brain tissues, we detected activities of GSH, SOD and MDA in the brain tissues using corresponding kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Specifically, the rat brain tissues were taken out from liquid nitrogen and thawed at 4°C. Then, the tissues were adequately lysed by 0.1 M phosphate-buffered saline and centrifuged at 4°C and 12,000 rpm for 20 min, and the protein supernatant was extracted. Finally, activities of GSH, SOD and MDA in rat brain tissues were determined according to the kit instructions.

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), and presented as mean \pm standard deviation. The *t*-test was performed for comparing differences between groups. *F*-test was used for comparing differences among multiple groups, followed by the Least Significant Difference (LSD) *t*-test for pairwise comparison. $p < 0.05$ suggested statistical significance.

Results

Dexmedetomidine Ameliorated Brain Water Content and Neurological Severity Score

The brain water content increased remarkably in model group compared with that in sham-op-

eration group, with a statistically significant difference ($p < 0.05$). Compared with model group, brain water content was significantly lower in dexmedetomidine group ($p < 0.05$). Besides, at 3, 5 and 7 d after operation, the neurological severity score was notably higher in model group than that in sham-operation group and dexmedetomidine group, and there were statistically significant differences ($p < 0.05$) (Figure 1).

Dexmedetomidine Prominently Lowered the Expression Levels of Apoptosis-Related Proteins in Rat Brain Tissues

The influences of dexmedetomidine on apoptosis-related proteins were examined using Western blotting assay. The anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) was down-regulated, whereas the pro-apoptotic proteins Bcl-2-associated X protein (Bax), Caspase-3 and Caspase-9 were upregulated in the brain tissues in model group than those of sham-operation group. However, the opposite results of these protein expressions were observed between dexmedetomidine group and model group, and all the differences were statistically significant ($p < 0.05$) (Figure 2).

Effect of Dexmedetomidine on Apoptosis Level in The Brain Tissues of CH Rats Examined by TUNEL Assay

To further determine the impact of dexmedetomidine on the CH-induced apoptosis level in rat brain tissues, the apoptosis in rat brain tissues was detected by TUNEL assay. As shown in Figure 3, model group exhibited an evidently larger number of apoptotic cells in the brain tissues than

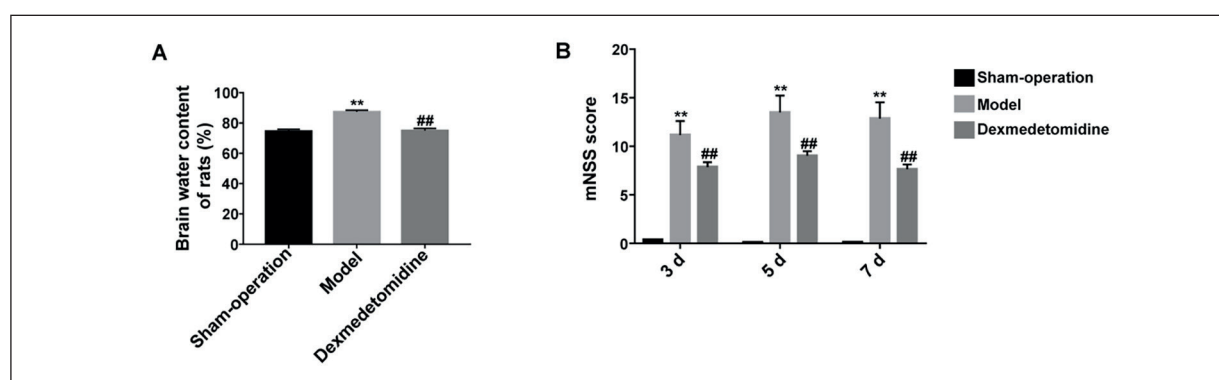


Figure 1. Dexmedetomidine ameliorated brain water content and neurological severity score. **A**, Effect of dexmedetomidine on brain water content in CH model. **B**, Effect of dexmedetomidine on CH-induced neural injury. # $p < 0.05$ vs. sham-operation group, ** $p < 0.05$ vs. model group.

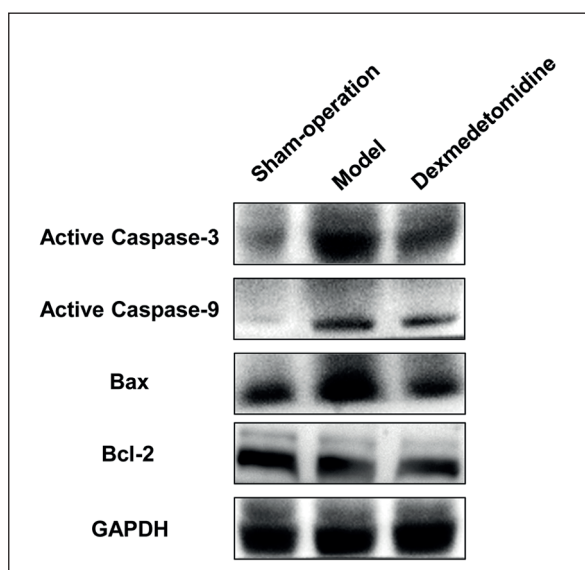


Figure 2. Dexmedetomidine prominently downregulated apoptosis-related proteins in rat brain tissues. The influences of dexmedetomidine on protein levels of apoptosis-related genes in CH model rats were detected *via* Western blotting assay. $^{##}p<0.05$ vs. sham-operation group, $^{**}p<0.05$ vs. model group.

sham-operation group. However, dexmedetomidine group displayed less apoptotic cells than model group.

Dexmedetomidine Had Apparent Effects on the Levels of Markers of Oxidative Stress in Rat Brain Tissues

Activities of GSH, SOD and MDA in the three groups of brain tissues were measured. The results manifested that activities of GSH and SOD in the brain tissues were reduced remarkably, while that of MDA was notably raised in model group in comparison with those in sham-operation group, showing statistically significant differences ($p<0.05$). However, compared with those in model group, increased activities of GSH and SOD, as well as decreased MDA activity were detected in dexmedetomidine group, and there were statistically significant differences ($p<0.05$) (Figure 4).

Dexmedetomidine Activated the Nrf2/heme Oxygenase-1 (HO-1)/NAD(P)H: Quinone Oxidoreductase 1 (NQO1) Signaling Pathway

Western blotting assay was adopted to examine the effect of dexmedetomidine on the Nrf2/HO-1/NQO1 signaling pathway. It was revealed that the protein levels of Nrf2, HO-1 and NQO1 in the brain tissues declined clearly in model group compared with those in sham-operation

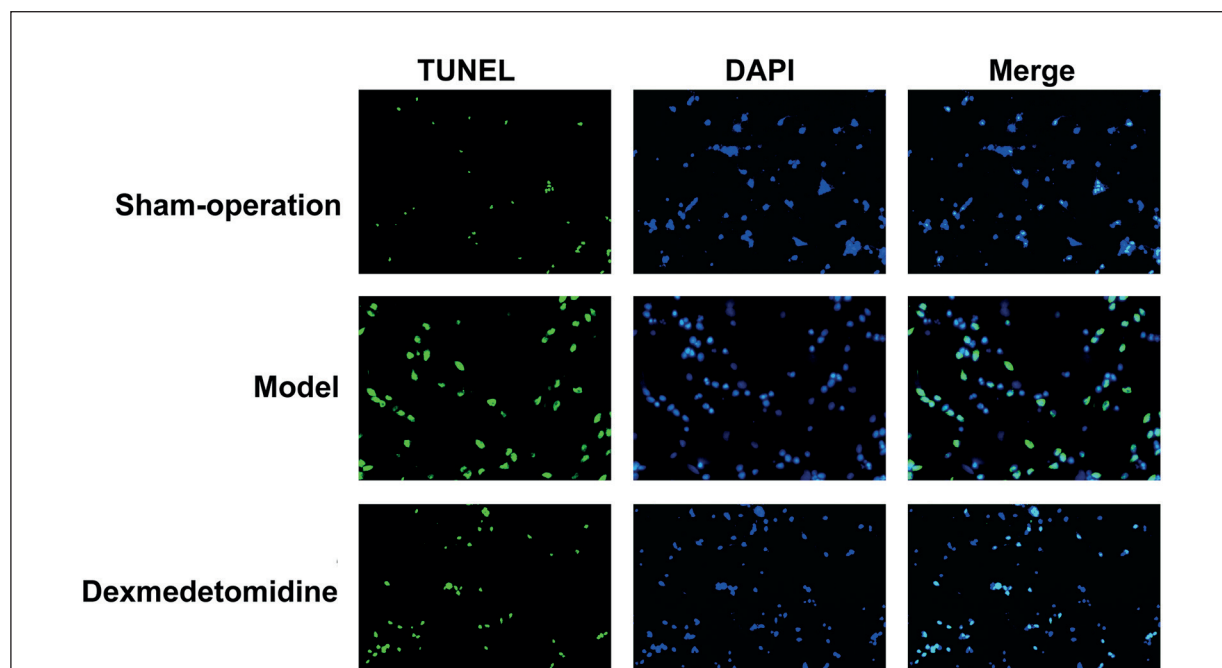


Figure 3. Effect of dexmedetomidine on apoptosis level in the brain tissues of CH rats examined through TUNEL assay. TUNEL assay was performed to detect the effect of dexmedetomidine on CH-induced apoptosis level in rats (magnification: 400 \times).

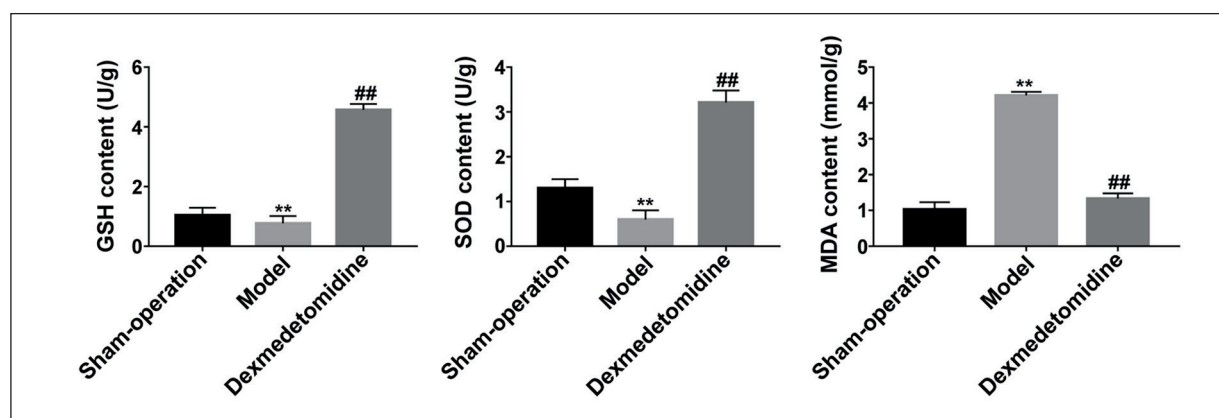


Figure 4. Dexmedetomidine had apparent effects on activities of oxidative stress markers in rat brain tissues. Activities of GSH, SOD and MDA in the brain tissues among the three groups were determined using biochemical tests. ## $p < 0.05$ vs. sham-operation group, ** $p < 0.05$ vs. model group.

group, and the differences were statistically significant ($p < 0.05$). Moreover, dexmedetomidine group had significantly higher protein levels of Nrf2, HO-1 and NQO1 than model group, showing statistically significant differences ($p < 0.05$) (Figure 5).

Knockdown of Nrf2 Abrogated the Protective Effect of Dexmedetomidine on Nerve Cells

An *in vitro* OGD model was established in SH-SY5Y cells to further validate our findings. First of all, the results of RT-qPCR assay indicated that the mRNA level of Nrf2 in SH-SY5Y cells was prominently lower in OGD group than that in control group and OGD + dexmedetomidine group. Moreover, OGD + dexmedetomidine + sh-Nrf2 group exhibited a significantly lower mRNA level of Nrf2 in cells than OGD + dexmedetomidine group. All the differences were statistically significant ($p < 0.05$) (Figure 6A and 6B).

According to the results of apoptosis assay, the apoptosis rate was raised evidently in OGD group compared with that in control group (5.7% vs. 47.3%), and there was a statistically significant difference ($p < 0.05$). Furthermore, the apoptosis rate in OGD + dexmedetomidine group (21.2%) was distinctly lower than that in OGD group ($p < 0.05$) and OGD + dexmedetomidine + sh-Nrf2 group (37.6%) ($p < 0.05$). These results suggested that the knockdown of Nrf2 abrogates the protective effect of dexmedetomidine on nerve cells (Figure 6C).

Discussion

Dexmedetomidine, a highly selective α_2 -adrenoceptor agonist, can prevent various kinds of organ injuries, such as neural injury in brain tissues⁹, hepatic ischemia/reperfusion injury¹⁰ and lung injury¹¹. Currently, dexmedetomidine has a protective effect against CH, but its exact mechanism of action remains unknown¹². In this research, the rat model of CH was established using autologous blood injection, and the influences

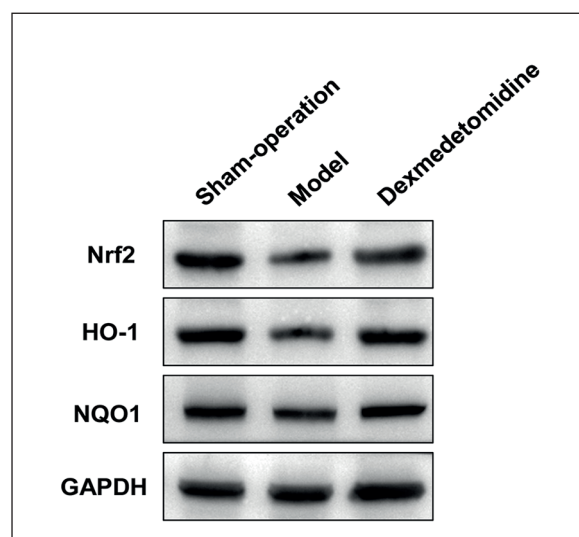


Figure 5. Dexmedetomidine activated the Nrf2/HO-1/NQO1 signaling pathway. The effect of dexmedetomidine on the Nrf2/HO-1/NQO1 signaling pathway in rat brain tissues was determined *via* Western blotting assay. ## $p < 0.05$ vs. sham-operation group, ** $p < 0.05$ vs. model group.

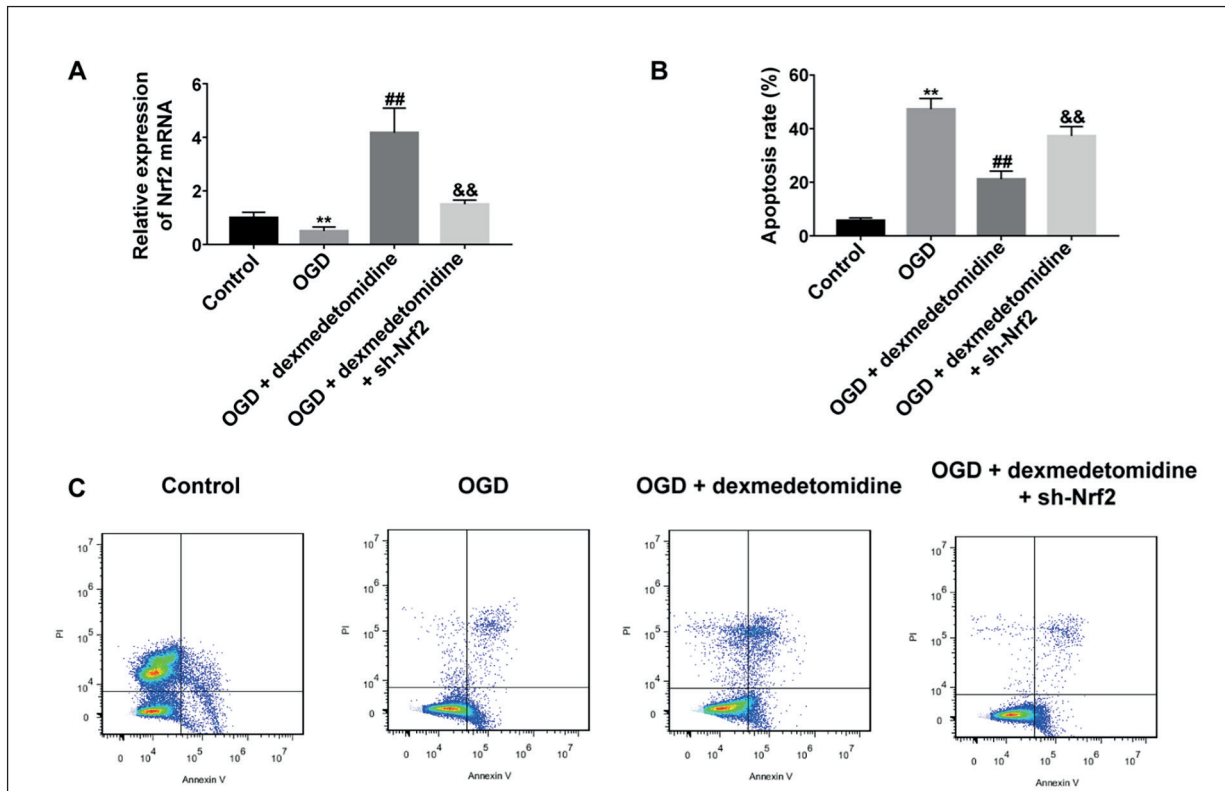


Figure 6. Knockdown of Nrf2 abrogated the protective effect of dexmedetomidine on nerve cells. **A**, Protein level of Nrf2 in different groups detected by RT-qPCR assay. **B, C**, OGD-induced apoptosis in each group examined using flow cytometry. ** $p < 0.05$: OGD group vs. control group, ## $p < 0.05$: OGD + dexmedetomidine group vs. OGD group, and && $p < 0.05$: OGD + dexmedetomidine + sh-Nrf2 group vs. OGD + dexmedetomidine group.

of dexmedetomidine on oxidative stress and cell apoptosis in the pathological process of CH in rats were investigated. The results indicated that dexmedetomidine was able to remarkably inhibit oxidative stress and apoptosis in rat brain tissues by activating the Nrf2/HO-1/NQO1 signaling pathway.

CH immediately causes mechanical injury and hematoma. Hematoma-induced secondary injury is more severe than hemorrhage-induced mechanical injury in the pathological processes of CH. Multiple mechanisms are involved in secondary brain injury, among which excessive oxidative stress plays an important role and results in neuronal death in the brain tissues. Under physiological conditions, the oxidative stress level in the brain tissues can be effectively restrained by SOD and GSH. Besides, uncontrolled oxidative stress will cause the depletion of antioxidant system in the brain tissues in pathological states. The free radicals, especially strongly bioactivated reactive oxygen species (ROS), can easily react with biomacromolecules to destroy a wide range

of biological structures through redox reactions, and thus cause cell death. Based on the results in this research, dexmedetomidine was capable of markedly enhancing activities of SOD and GSH and reducing that of MDA. It is indicated that dexmedetomidine can increase the level of antioxidant proteases in the brain tissues of CH model rats, thereby protecting their brain tissues. Huang et al¹³ denoted that dexmedetomidine is able to prevent neurological dysfunction in mice with CH by repressing oxidative stress and mitochondrial dysfunction, which is consistent with the findings in this research.

Cell apoptosis, also known as programmed cell death, occurs under a variety of pathological conditions, such as hypoxia, inflammatory response and mechanical injury in the pathological model of CH. In addition, excessive oxidative stress injury will also cause cell apoptosis. The precise mechanism of cell apoptosis induced by oxidative stress injury has not been clarified yet. It is generally believed that the JNK and p38 MAPK signaling pathways are one

of the key links of oxidative stress injury and cell apoptosis¹⁴. Besides, oxidative stress injury is prominently associated with the Caspase protein family. ROS can increase the mitochondrial membrane permeability to trigger the release of cytochrome C, ultimately enhancing the release and activation of Caspase proteins. Once CH occurs, the apoptosis of nerve cells results in neurological dysfunction. It was revealed in this research that dexmedetomidine could significantly suppress cell apoptosis in the brain tissues of CH model rats by downregulating Bax/Bcl-2, Caspase-3 and Caspase-9. Moreover, dexmedetomidine was able to markedly inhibit OGD-induced cell apoptosis in SH-SY5Y cells. Similar to the results in this research, Hwang et al¹⁴ manifested that in the CH model, dexmedetomidine is capable of repressing apoptosis in hippocampal tissues of rats evidently, thus alleviating CH-induced memory dysfunction.

In the state of oxidative stress, the Nrf2 signaling pathway is a critical cytoprotective pathway¹⁵. Additionally, the activation of the Nrf2 signaling pathway can distinctly inhibit the oxidative stress, so as to restrain the neuronal apoptosis in the case of cerebral ischemia. Shi et al¹⁶ found that monomethyl fumarate can relieve CH-induced neural injury *via* activating the miR-139/Nrf2 signaling pathway. Furthermore, Wei et al¹⁷ discovered that nicotinamide mononucleotide can activate alleviate the CH-induced brain tissue injury through the Nrf2/HO-1 signaling pathway. Based on the results in this research, dexmedetomidine could notably upregulate Nrf2/HO-1/NQO1 in the CH model. Besides, the knockdown of Nrf2 in OGD-induced SH-SY5Y cells could significantly abrogate the anti-apoptotic effect of dexmedetomidine. All the aforementioned findings elucidated that dexmedetomidine inhibits the nerve cell apoptosis in rat brain tissues by activating the Nrf2/HO-1/NQO1 signaling pathway in rat CH models.

Conclusions

Dexmedetomidine inhibits the nerve cell apoptosis lesioned brain tissues by activating the Nrf2/HO-1/NQO1 signaling pathway in rat CH models.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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