Original Article

Effects and mechanisms of dexmedetomidine on apoptosis of hippocampal neurons in rats with cerebral ischemia-reperfusion injury

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Abstract: Objective: The aim of this study was to examine the effects of dexmedetomidine on apoptosis of hippocampal neurons in rats with cerebral ischemia-reperfusion injury (IRI). Methods: A total of 45 mice were randomly and equally (n = 15/each) divided into the sham (S) group, model (M) group, and dexmedetomidine (D) group. Nerve injuries were evaluated using Purdy scores and cerebral infarct area was detected by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The content of water and glutamate and γ-aminobutyric acid was detected using the wet-dry weighting method and HPLC-mass spectrometer, respectively. In addition, apoptosis of hippocampal neurons was detected by TUNEL staining and flow cytometry. Moreover, mRNA and protein expression of cleaved caspase-3, Bcl-2, and Bax was detected via real-time quantitative PCR and Western blotting, respectively. Results: Compared with Group S, Group M displayed significantly increased Purdy scores, cerebral infarct area, and water content (P < 0.01). These were significantly lower or smaller in Group D than those in Group M (P < 0.01). However, the content of γ-aminobutyric acid in Group M was significantly lower than that in Group S (P < 0.01), but significantly higher in Group D than in Group M (P < 0.01). In addition, apoptosis of hippocampal neurons was significantly decreased in Group D compared with Group M (P < 0.01). Furthermore, cleaved caspase-3 protein levels in hippocampal tissues in Group M were significantly increased (P < 0.01), with decreased Bcl-2/Bax mRNA and protein levels (P < 0.01). In Group D, cleaved caspase-3 protein levels were significantly lower than those in Group M (P < 0.01), with higher Bcl-2/Bax mRNA and protein levels (P < 0.01). Conclusion: Dexmedetomidine improves nerve function after cerebral IRI in rats, reduces cerebral infarct area, and decreases apoptosis of hippocampal neurons. These effects may be through upregulation of γ-aminobutyric acid, decrease of caspase-3 levels, and increase of Bcl-2/Bax ratios.

Keywords: Dexmedetomidine, cerebral ischemia-reperfusion, apoptosis

Introduction

Cerebrovascular disease can lead to disability and even death, seriously affecting the life quality of patients [1, 2]. After cerebral ischemia, insufficient blood and oxygen supply in brain tissues may lead to brain cell death, causing a series of serious nerve function impairment disorders [2]. A lot of research evidence has shown that the reperfusion process after ischemia can further lead to neuronal cell death, resulting in severe impairment of learning, memory, and motor function [3, 4]. A previous study [5] showed that nerve function impairment in patients is caused by the significant increase of neuronal apoptosis. Protecting nerves can significantly improve the nerve and motor function of patients with cerebral ischemia-reperfusion injury (IRI). Cerebrovascular thrombosis is a contributing factor to cerebral ischemia. Thrombolytic drugs are widely used in the treatment of cerebral ischemia, currently, but patients often miss the best thrombolytic treatment opportunity [6]. Therefore, identification of novel therapeutic drugs for treatment of IRI is required.

There are two general pathways of apoptosis. These are the intrinsic, which is originated from mitochondrial release of cytochrome c and associated with activation of caspase-3, and the extrinsic, which is originated from the acti-
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vation of cell surface death receptors, resulting in the activation of caspase-8 [7]. Over the last decade, experimental studies have provided considerable new information characterizing apoptotic processes occurring after cerebral ischemia, showing that both intrinsic and extrinsic pathways are involved in neuronal cell death [8, 9].

Dexmedetomidine is a selective agonist of epinephrine α2 receptor with strong sedative, analgesic, and anti-anxiety effects. Combined application of dexmedetomidine in clinic can reduce doses of anesthetics or analgesics. Moreover, its respiratory depression effects are very low, compared with the commonly-used epinephrine α2 receptor [10]. Samantha et al. [11] showed that dexmedetomidine is widely distributed in brain tissues through the blood brain barrier, thereby exerting a potent therapeutic effect. Administration of dexmedetomidine in advance can significantly decrease serum catecholamine levels and improve neuropathological changes [12]. In recent years, dexmedetomidine has been demonstrated to have potential neuroprotective effects. Douglas et al. [13] found that dexmedetomidine can significantly improve hippocampal neuron damage induced by hypoxia and reoxygenation. This might be through inhibition of calcium overload-mediated mitochondrial fission and mitochondrial apoptosis pathways. However, there are few in vivo studies investigating the correlation between dexmedetomidine and IRI.

In the current study, a rat model of middle cerebral artery occlusion (MCAO) was established to simulate the IRI of nerve cells in rats, aiming to study the effects of dexmedetomidine on neuronal apoptosis in rats with IRI, as well as explore possible mechanisms. The current study further aimed to provide a theoretical basis for the further development of dexmedetomidine.

Materials and methods

Animals and grouping

A total of 45 Sprague-Dawley (SD) rats, weighing 250-280 g, were purchased from the Laboratory Animal Center of Guangdong Province (Laboratory Animal Production License Number: SCXK2015-0012). Rats were fed in specific pathogen-free (SPF) cages for 1 week prior to experiments under a temperature of (25 ± 1)°C, humidity of (45 ± 2)%, and sunshine duration of 12 hours. They were given free access to food and water. Rats were randomly divided into the sham (S) group (n = 15), model (M) group (n = 15), and dexmedetomidine (D) group (n = 15). Rats in Group D were injected intraperitoneally with 60 μg/kg dexmedetomidine at 2 hours before the operation, while rats in Group S and Group M were intraperitoneally injected with 2 mL normal saline at 2 hours before the operation. All operations and protocols were approved by the Ethics Committee of Laboratory Animals, complying with relevant regulations in the International Laboratory Animal Protection Law.

Establishment of ischemia-reperfusion injury model

Rats in each group fasted for solids and liquids for 12 hours before modeling. During modeling, 4% chloral hydrate was injected intraperitoneally for anesthesia. The surface of skin was cut using surgical scissors along the neck midline. Muscle tissues of the neck were bluntly separated to find the common carotid artery and the internal carotid artery and external carotid artery were further separated. After the external carotid artery was ligated using the suture line, the internal carotid artery was clamped using the artery clamp. An incision was made in the proximal part of internal carotid artery using the syringe needle and the occlusion wire (Shenzhen RWD Life Science Co., Ltd., Shenzhen, China) matching the weight of rats was inserted. The occlusive effect was achieved when the black spot of occlusion wire reached the Y-shaped crotch. The internal carotid artery and common carotid artery were ligated to fix the occlusion wire, the neck skin was sutured, and about 0.5 cm-long occlusion wire was exposed outside the skin. At 2 hours after ischemia of rats, the occlusion wire was removed. This was followed by reperfusion for 24 hours. Considering the mortality rate during the modeling process, the number of model rats could be appropriately increased to ensure that there were 15 rats each group.

Nerve function evaluation and determination of cerebral infarct area

Nerve function was scored using the Zea-Longa scoring criteria at 24 hours after modeling: 0 points (No neurological impairment symptoms
and normal activity), 1 point (The contralateral forepaws cannot stretch freely), 2 points (Contralateral circles when walking), 3 points (The body inclines to the opposite side when walking), 4 points (Unable to walk spontaneously and loss of consciousness), and 5 points (death). After rats were sacrificed via cervical dislocation, the brains were isolated. This was followed by washing the blood from the brain surface. The brains were frozen in a refrigerator at -20°C for 20 minutes. Coronal sections of the brains were prepared (6 sections per brain). Brain sections of rats in each group were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) solution in an incubator under dark at 37°C for 30 minutes. Sections were gently flipped during staining, ensuring ample staining. After staining, TTC staining solution was removed and 4% paraformaldehyde was added to the sections for fixation in the refrigerator at 4°C overnight. Normal brain tissues showed red, while infarct regions showed white. The infarct region was analyzed and the cerebral infarct area of rats was calculated.

Determination of water content and amino acid content in brain tissues

Rats in each group were sacrificed at 24 hours after reperfusion and brains were taken. The ischemic hemisphere was then cut off and weighed using a one-ten-thousandth balance (Shanghai Yitai Electronic Science and Technology Co., Ltd., Shanghai, China). Wet weights were recorded. The hemisphere was dried in a drying oven at 110°C to constant weight (The deviation of weight measured for 3 times was not more than 0.2 mg), then dry weights were recorded. Water content in brain tissues in the ischemic area of rats in each group was calculated using the following formula: water content = (wet weight - dry weight)/wet weight * 100%. The content of glutamate and γ-aminobutyric acid in brain tissues of rats in each group was detected via liquid chromatography-mass spectrometry [14]. Changes in amino acid content in brain tissues of rats in each group were calculated using measurement methods of amino acids found in the literature.

Detection of cell apoptosis by flow cytometry

At 24 hours after reperfusion, the brains were extracted. Hippocampal tissues were isolated, cut into tissue fragments using surgical scissors, digested with collagenase, and dispersed into single cells, as previously described [15]. After cells were rinsed with pre-cooled phosphate buffered saline (PBS) 3 times, the single-cell suspension was collected. Apoptosis was detected using the apoptosis assay kit (BD Bioscience, San Jose, CA, USA), strictly according to manufacturer instructions. All samples were detected within 1 hour and apoptosis in the hippocampal tissues was statistically analyzed.

Detection of cell apoptosis levels via TUNEL staining

At 24 hours after reperfusion, paraffin sections of hippocampal tissues of rats were prepared. Apoptosis of hippocampal tissues was detected using the TUNEL apoptosis kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Paraffin sections were dewaxed, washed with PBS, dropwise added with 50 μL mixed solution of TdT, and DIG-d-UTP for labeling at 4°C for 2 hours (in a wet box to avoid volatilization). They were washed again with cleaning solution. Sections were sealed using 40 μL blocking solution at room temperature for 30 minutes, added dropwise with the biotin-labeled antibody (1:100) for incubation at 37°C for 40 minutes (in the wet box to avoid volatilization), and washed with cleaning solution. Next, streptavidin-biotin complex-fluorescein isothiocyanate (SABC-FITC) secondary antibody (1:100) was added dropwise for reaction at 37°C for 40 minutes. Sections were then washed with cleaning solution. Finally, sections were sealed using the anti-fluorescence quenching sealing agent, observed, and photographed under a confocal fluorescence microscope (Nikon). The number of TUNEL-positive cells (green fluorescence) in hippocampal tissues of rats in each group was calculated.

Detection of mRNA levels via quantitative qPCR

Total RNA was extracted from hippocampal tissues of rats in each group using TRIzol (Invitrogen, Waltham, Massachusetts, USA). Optical density (OD) values were detected. Isolated RNA was reversely transcribed into cDNA using the reverse transcription kit (TaKaRa) (42°C for 15 minutes and 95°C for 2 minutes). Primers were synthesized by Sangon (Shanghai) and sequences are shown in Table 1. The qPCR system was prepared with glyceraldehyde-3-phos-
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Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Sequence of PCR primer</th>
<th>Antisense Sequence of PCR primer</th>
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<tbody>
<tr>
<td>B-cell lymphoma-2 (Bcl-2)</td>
<td>GGTGGGGTCATGTGTGTGG</td>
<td>CGTTCAGGTACTCAGTCATCC</td>
</tr>
<tr>
<td>Bcl-2 associated X protein (Bax)</td>
<td>CCCAGAGCTTTTTTCCGAG</td>
<td>CAG GCCATGTTGTCTGAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TATCGAGGCGCTGTAC</td>
<td>TTCCCATCTCACGTTC</td>
</tr>
</tbody>
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Data are presented as mean ± standard deviation (SD) and were processed using Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc, Chicago, IL, USA). Chi-squared test was used for comparisons of enumeration data and one-way ANOVA with Bonferroni’s post hoc test was used for comparisons among multiple groups. P < 0.05 indicates statistical significance.

Results

Nerve function scores and determination of cerebral infarct area

Zea-Longa scores were used to evaluate behavioral changes of rats in each group after IRI. Results showed that the nerve function score of rats in Group M was significantly increased, compared with that in Group S (P < 0.01), but was significantly lower in Group D than in Group M (P < 0.01) (Figure 1A). The cerebral infarct area of rats in each group was determined via TTC staining. Results showed that the cerebral infarct area of rats in Group M was significantly larger than that in Group S (P < 0.01). However, it was significantly smaller in Group D, after treatment with dexmedetomidine, than that in Group M (P < 0.01) (Figure 1B, 1C).

Changes in water content and amino acid content in brain tissues

Changes in water content in brain tissues of rats in each group after IRI were calculated. Results revealed that the water content in ischemic brain tissues of rats in Group M was obviously higher than that in Group S (P < 0.01). The content was obviously lower in Group D than in Group M (P < 0.05) (Figure 2A). Amino acid content in brain tissues of rats in each group was detected via liquid chromatography-mass spectrometry, revealing no statistically significant
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However, the content of γ-aminobutyric acid in brain tissues of rats in Group M was significantly lower than that in Group S (P < 0.01), but was significantly higher in Group D than in Group M (P < 0.01) (Figure 2B).

Figure 1. Nerve function scores and determination of cerebral infarct area. (A) Nerve function scores of rats, (B) TTC staining graph of brain sections of rats in each group, (C) statistical graph of cerebral infarct area. Nerve function scores and cerebral infarct area of rats in Group M are significantly higher and larger than those in Group S. Compared with those in Group M, nerve function scores and cerebral infarct area of rats in Group D are significantly decreased (**P < 0.01 vs. Group M, ***P < 0.01 vs. Group S).

Figure 2. Changes in water content and amino acid content in brain tissues of rats. (A) Water content in brain tissues, (B) Changes in amino acid content. The water content in ischemic brain tissues of rats in Group M is significantly higher than that in Group S and it is significantly lower in Group D than that in Group M. The content of γ-aminobutyric acid in brain tissues of rats in Group M is significantly lower than that in Group S and it is higher in Group D than that in Group M (**P < 0.01 vs. Group M, ***P < 0.01 vs. Group S).

Neuronal apoptosis in hippocampal tissues

Flow cytometry and TUNEL staining were used to detect neuronal apoptosis in hippocampal tissues of rats. Results of flow cytometry showed that the apoptosis of hippocampal neurons of rats in Group M was significantly higher than that in Group S (P < 0.01) and was significantly decreased in Group D after treatment with dexmedetomidine (P < 0.01) (Figure 3). Results of TUNEL staining showed no obvious TUNEL-positive cells in hippocampal tissues of rats in Group S. However, the number of TUNEL-positive cells in hippocampal tissues of rats in Group M was significantly larger than that in Group S (P < 0.01) and significantly smaller in Group D than in Group M (P < 0.01) (Figure 4).

Detection of expression of apoptosis-related molecules

Changes in apoptotic molecule genes and protein levels in hippocampal tissues of rats in each group after IRI were detected via qPCR and Western blotting, respectively. Results of qPCR and Western blotting showed that, compared with Group S, Group M showed obviously decreased Bcl-2/Bax mRNA and protein levels (P < 0.01) (Figure 5A-C). Cleaved caspase-3 protein levels in hippocampal tissues of rats in Group D were lower than those in Group M after treatment with dexmedetomidine (P < 0.01), with increased Bcl-2/Bax mRNA and protein levels (P < 0.01) (Figure 5B and 5D).
Dexmedetomidine can act on the epinephrine α2 receptor, exerting strong sedative and analgesic effects. It has, therefore, been widely used in clinic [16]. Many studies have shown that dexmedetomidine can protect the myocardium, liver, and lung tissues from IRI. In this experiment, the protective effects of dexmedetomidine on hippocampal neurons of rats with IRI were investigated. Results showed that: (1) 60 μg/kg dexmedetomidine could effectively protect the behavior of rats from damage caused by IRI and reduce cerebral infarct size; (2) IRI could significantly increase the water content in ischemic brain tissues of rats and reduce the content of γ-aminobutyric acid, which could be significantly ameliorated by intraperitoneal injections of dexmedetomidine; (3) IRI could significantly increase the apoptosis of hippocampal neurons in rats and dexmedetomidine could significantly reduce neuronal apoptosis caused by IRI; (4) IRI could significantly increase levels of apoptosis protein and mRNA in hippocampal tissues and

Discussion

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dexmedetomidine could reduce changes of related proteins and mRNA. The above results suggest that dexmedetomidine can significantly improve damage to hippocampal neurons of rats caused by IRI. It can also reduce the water content in the ischemic area. Mechanisms may be related to the increase of γ-amino-butyric acid content in brain tissues, decrease of cleaved caspase-3 levels, and increase of Bcl-2/Bax ratios.

Ischemic strokes are a kind of acute cerebrovascular disease. They can lead to sudden weakness in the face, hands, or legs on the ischemic side and sudden fainting, even death in severe cases [17]. Reperfusion after thrombolysis will cause further damage to nerve cells. Thus, combined application of neuroprotective drugs is particularly important for patients with cerebral ischemia based on thrombolytic therapy. Zhang et al. [18] found that dexmedetomidine can activate mitogen-activated protein kinase (MAPK), thereby protecting brain injuries caused by subarachnoid hemorrhaging. Ischemia will cause brain tissue necrosis in the ischemic area, significantly increase the number of apoptotic neuronal cells, and lead to severe cell edema. At the same time, aggregation of many leukocytes will further lead to the increased release of oxygen free radicals, protein derivatives, pro-inflammatory factors, and other toxic substances, aggravating neuronal cell damage [19]. Moreover, γ-amino-butyric acid plays a neuroprotective role in the body, helping to repair injured cells. After cerebral IRI, the content of γ-amino-butyric acid in brain tissues of rats is significantly decreased and the number of apoptotic neuronal cells is further

Figure 5. Detection of expression of apoptosis-related molecules in rats in each group via qPCR or Western blotting. (A) Bcl-2/Bax mRNA expression levels. Compared with those in Group S, Bcl-2/Bax mRNA expression level is significantly decreased and Bcl-2/Bax mRNA expression level is significantly increased (\( ^* P < 0.01 \text{ vs. Group M, } ^{##} P < 0.01 \text{ vs. Group S} \)). (B): Band graph, (C) statistical graph of Bcl-2/Bax, (D) statistical graph of cleaved caspase-3. Compared with those in Group S, Bcl-2/Bax protein expression level in Group M is significantly decreased, but cleaved caspase-3 protein expression level is significantly increased. Bcl-2/Bax protein expression level in Group D is significantly higher than those in Group M, but cleaved caspase-3 protein expression level is significantly decreased (\( ^* P < 0.01 \text{ vs. Group M, } ^{##} P < 0.01 \text{ vs. Group S} \)).
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