Int J Clin Exp Pathol 2021;14(6):786-793
www.ijcep.com /ISSN:1936-2625/IJCEP0118403

Original Article

Effects of interventional therapy on AQP4 gene expression and neuron apoptosis in rabbits with ischemic brain injury caused by carotid artery stenosis

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Received July 17, 2020; Accepted January 7, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: *Objective:* To explore the effects of interventional therapy (IT) on Aquaporin-4 (AQP4) gene expression and neuron apoptosis in rabbits with ischemic brain injury caused by carotid artery stenosis, and to further optimize the therapeutic regime of clinical ischemic brain injury (IBI). *Methods:* 30 New Zealand rabbits were randomly divided into three groups: Sham group (n=10), IBI group (n=10) and IBI+IT group (n=10). A rabbit model of carotid artery stenosis was established to induce IBI. 12 hours later, interventional therapy was achieved through percutaneous transluminal coronary angioplasty (PTCA) by pumping up the balloon to widen the catheter. The expression of AQP4 mRNA and protein were determined by reverse transcription polymerase chain reaction (RT-PCR) and western blot, respectively. The distributions of rabbit hippocampus and cerebral cortex were detected by Nissl staining. The neuronal function of rabbits in the three groups after surgery was assessed with mNSS score. TUNEL (Terminal Transferase-mediated dUTP Nick End-labelling) staining was used to observe neuron apoptosis in rabbit brain tissue. The expressions of Bax and Bcl-2 protein were detected by western blot. Markers of oxidative stress in rabbit brain tissues were detected by Reactive Oxygen Species ELISA Kits. Morphology of the organelles in rabbit brain tissues was observed with transmission electron microscopy (TEM). *Results:* The protein and mRNA expressions of AQP4 in rabbit brain tissues were significantly decreased in the IBI group. Nissl staining results showed that IT improved IBI in rabbit hippocampus. In addition, IT significantly ameliorated the neuronal function of rabbits, and reduced the apoptosis and oxidative stress level of neurons in brain tissues. Furthermore, we found that after IT, organelle damage was significantly reduced in rabbit neurons. *Conclusion:* After receiving IT, AQP4 gene level and neuron apoptosis were significantly reduced in rabbit brain tissues with ischemic brain injury from carotid artery stenosis.

Keywords: Interventional therapy, carotid artery stenosis, brain injury, apoptosis, AQP4

Introduction

Carotid atherosclerosis is one of the main reasons for ischemic stroke [1]. Since most carotid artery stenosis does not directly cause stroke, carotid artery stenosis is often considered "asymptomatic". In population-based studies, the incidence of asymptomatic carotid atherosclerotic disease is as high as 5.7%, which is relatively high in both males and older populations [2]. However, the effects of cerebral ischemia induced by moderate to severe carotid stenosis on the structure and cognitive function of cerebral neurons have attracted much attention [3, 4]. Therefore, researchers have been further exploring the mechanisms of brain injury caused by carotid stenosis to prevent and treat ischemic brain injury (IBI).

Studies have shown that carotid endarterectomy and carotid artery stenting have a certain therapeutic effect on both symptomatic and asymptomatic carotid stenosis [5]. Nevertheless, the results of randomized clinical trials show that carotid artery stenting has better efficacy than traditional carotid endarterecto-

Table 1. Primer sequences of each gene in RT-PCR

Target Gene		Primer Sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAACCC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGTCCA-3'
AQP4	Forward	5'-AGTTTAGCTGATGTCGTCGTGTAGC-3'
	Reverse	5'-ACGTGTCGTAGCTAGCTGACTGAAC-3'

my in patients with carotid atherosclerosis [6], but the specific mechanism remains unknown.

Aquaporin-4 (AQP4), a membrane-bound protein that regulates water permeability, is a member of the aquaporin family of water channel proteins that is expressed in the end-feet of astrocytes in the central nervous system (CNS) [7]. Recently, AQP4 has been shown to function, not only as a water channel protein, but also as an adhesion molecule that is involved in cell migration and neuroexcitation, for synaptic plasticity [8]. AQP4 plays an important role in neuroimmunologic functions in injured mouse brain [9]. It is also reported that curcumin attenuates chronic intermittent hypoxia-induced brain injuries by inhibiting AQP4 and the p38 MAPK pathway [10].

In the present study, a rabbit model of carotid artery stenosis was established to induce IBI. Subsequently, the effects of percutaneous transluminal coronary angioplasty (PTCA) on neuron apoptosis and AQP4 expression were studied, providing a reference for elucidating the molecular mechanism of clinical interventional therapy (IT).

Materials and methods

Animal grouping and modeling

30 New Zealand White male rabbits were randomly divided into three groups: Sham group, IBI group (n=10), and IBI+IT group (n=10). Rabbits in IBI group and IBI+IT group were fasted for 12 hours before surgery, but water was given. Rabbits were anesthetized through marginal ear vein injection and under a middle anterior neck incision. The bilateral carotid common arteries (BCCA) were separated. At the proximal end of the common carotid artery, 1 cm away from the bifurcation of the internal and external carotid arteries, the bilateral common carotid arteries and a needle with a diameter of 0.6 mm were tightened together with a No. 0 thread. A slipknot was tied and the line head was left, then the needle was carefully pulled out and the incision was sutured. Rabbit bilateral carotid arteries in the sham group were separated, but not ligated. 12 hours after the surgery, rabbits in IBI+IT group received therapy through PTCA by pumping up the balloon to widen the catheter. One week later, the rabbit brain tissues from each group were collected for subsequent molecular biology experiments. All animal-specific procedures were approved by the Animal Ethics Committee.

Detection of proinflammatory cytokine levels by RT-PCR

Total RNA was extracted from fresh frozen rabbit brain tissue using the TRIzol ™ kit. The RNA concentration and purity of samples from each group was then detected by a spectrophotometer. Reverse transcription was performed after the concentration and purity of the samples were measured. 2 μg of RNA samples were reversely transcribed into cDNA. RT-PCR was performed with cDNA from each sample. The RT-PCR system was as follows: 10 × Buffer 2.5 μL; cDNA 1 μL; forward primer (20 μmol/L) 0.5 μL; reverse primer (20 μmol/L) 0.5 μL; LightCycler® 480 SYBR Green I Master (2 ×): 10 μL; ddH_2O: 5.5 μL. The RT-PCR amplification system was the same for all samples. The primers of each gene are listed in **Table 1**.

Observation of neuronal organelles by TEM

The brain tissue was first cut into pieces with a volume of 1 mm × 1 mm × 1 mm, and quickly fixed with a fixing solution for 2 h. Tissue was post-fixed with 1% osmic acid in 0.1 M phosphate buffer at room temperature (20°C) for 2 h. Phosphate buffer (pH 7.4) was used to rinse the specimens 3 times, each time for 15 minutes. The specimens were dehydrated and infiltrated again, followed by embedding and sectioning. Double staining for transmission electron microscopy was performed with uranium and lead (stained with 2% uranium acetate saturated alcohol solution and lead citrate solution for 15 min, respectively). The sections were dried at room temperature overnight. The images were observed and collected by transmission electron microscopy.

Western blot

The fresh frozen brain tissue from each group was taken out from the -80°C freezer. After preliminary cutting with scissors, the tissues were fully ground with a grinder. The samples were lysed by ultrasound and centrifuged (centrifuge, Eppendorf, Germany), then the supernatant was aspirated and aliquoted into EP tubes. After the concentration of protein was measured by BCA method (BCA Protein Assay Kit, thermofisher, USA) and ultraviolet spectrophotometer method, all protein samples were volume to equal concentrations. The samples were aliquoted and stored at -80°C. Total proteins were separated by SDS-PAGE. At the end of the electrophoresis, proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA), then the membrane was incubated with rabbit primary antibody (1:1000, cell signaling technology, USA) at 4°C overnight. Subsequently, the membrane was incubate with Goat anti-rabbit secondary antibody (1:2000, Sigma, USA) for 1 h in the dark, and then scanned as well as quantified using an Odyssey Scanner.

Nissl staining

The rabbit hippocampus of each group was immersed in a sucrose solution (30%), and frozen sections (30 μm) were prepared. The prepared brain section was attached to a glass slide (pretreated with gelatin) and dried. Subsequently, the sections of each group were placed in cresyl violet, stained at 37°C for 0.5 h, and then separated as well as dehydrated by gradient ethanol, and finally treated with xylene and then mounted. The sections were then observed under a light microscope and photographed.

TUNEL staining of brain tissues

The sliced brain tissues were dried in a 60°C oven for 30 minutes, and then dewaxed with xylene (5 min × 3 times), orderly dehydrated with 100% ethanol, 95% ethanol and 70% ethanol, each repeated for three times. The sections were then incubated with protein kinase K for another 0.5 h. After rinsing with PBS, the terminal deoxyribonucleotide transferase TdT and luciferase-labeled dUTP were added, and the reaction was performed at 37°C for 1 hour. Sections were then incubated with horseradish peroxidase-specific antibodies for another 1 h (37°C) in the incubator. Finally, DAB was used as a substrate, and the reaction was performed at room temperature for 10 min. After the nucleus was stained by hematoxylin, the picture was taken under a light microscope and counted.

Detection of oxidative stress marker

100 μL of 10% brain tissue homogenate was taken. According to the kit instructions, the protein in each sample were quantified by Coomassie brilliant blue staining, meanwhile the levels of SOD, MDA, and GSH-Px were measured.

Statistical analysis

All data were analyzed with SPSS 22.0 software, and data are expressed as the mean ± SD. *T*-test was performed to determine statistically significant differences. A value of $P<0.05$ was considered significant.

Results

Effects of interventional therapy on AQP4 gene expression in rabbit brain tissues

As shown in **Figure 1**, results of RT-PCR and western blot indicates that in the IBI group, expressions of AQP4 mRNA and protein of rabbit brain tissues were significantly decreased ($P<0.05$), while IT significantly up-regulated AQP4 gene expression.

Effects of IT on distribution of hippocampal neurons in rabbits with IBI

As shown in **Figure 2**, the results of Nissl staining suggested that there was significant proliferation of glial cells and loss of Nissl bodies in the hippocampus of rabbits with IBI. After IT, the number of glial cells in the hippocampus was significantly reduced, while the number of Nissl bodies increased significantly ($P<0.05$).

Scores of neuronal function in rabbits from each group

One week after surgery, symptoms of neurologic deficits appeared in all surgical groups except the Sham group, including reduced activity, debility, inability to walk straight, tipping to one side, or rotating. Therefore, mNSS score was

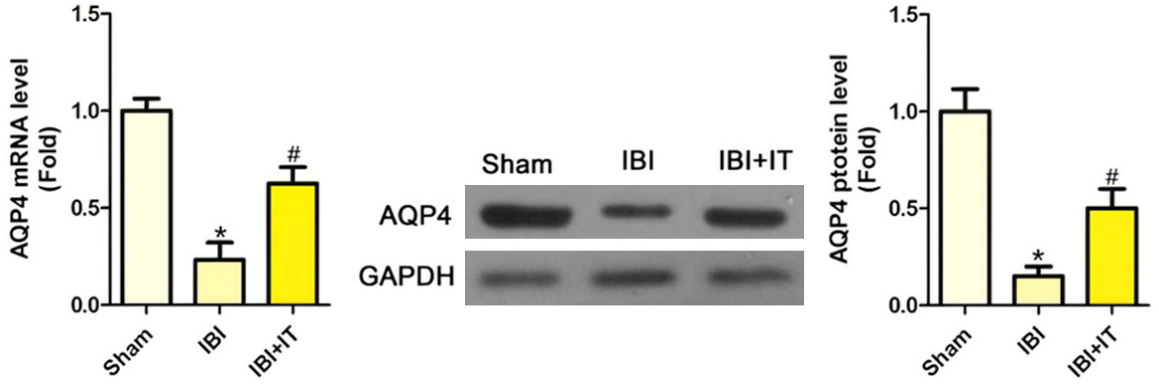

Figure 1. Effects of interventional therapy on AQP4 gene expression in rabbit brain tissues. Sham group, IBI group, and IBI+IT group. *compared with control group. #P<0.05 compared with IBI group.

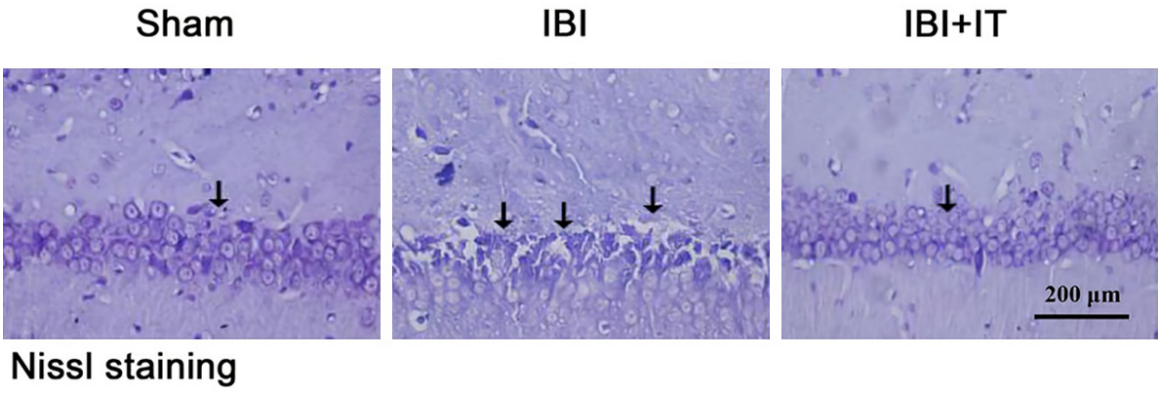

Figure 2. Effects of IT on distribution of hippocampal neurons in rabbits with IBI, Sham group, IBI group, and IBI+IT group.

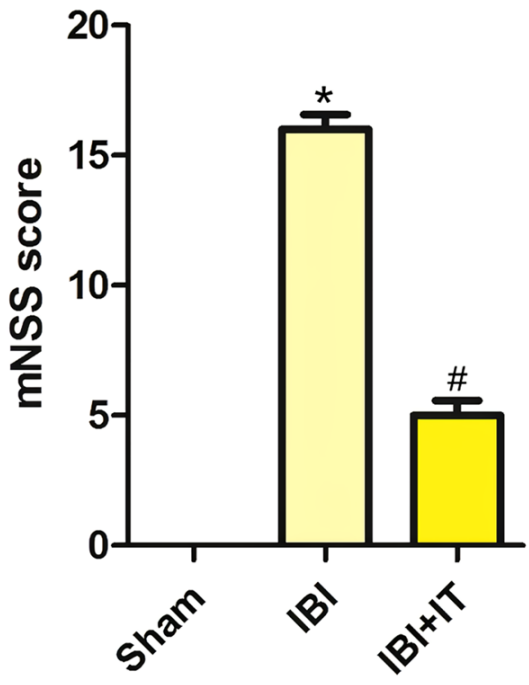

Figure 3. Scores of neuronal function in rabbits of each group. Sham group, IBI group and IBI+IT group, *compared with control group. #P<0.05 compared with IBI group.

used to evaluate the neural function of rabbits in each group. As shown in **Figure 3**, the score of IBI group was significantly higher than that of the Sham group ($P<0.05$). One week after IT treatment, the mNSS score of rabbits in the IBI group was significantly reduced ($P<0.05$), indicating that IT improved the IBI-induced neurologic dysfunction in rabbits.

Results of TUNEL staining in rabbit brain tissues of each group

As shown in **Figure 4**, the results of TUNEL staining showed that the number of TUNEL-positive cells in the rabbit brain tissues of IBI+IT group was significantly lower than that in the IBI group ($P<0.05$), indicating that IT could

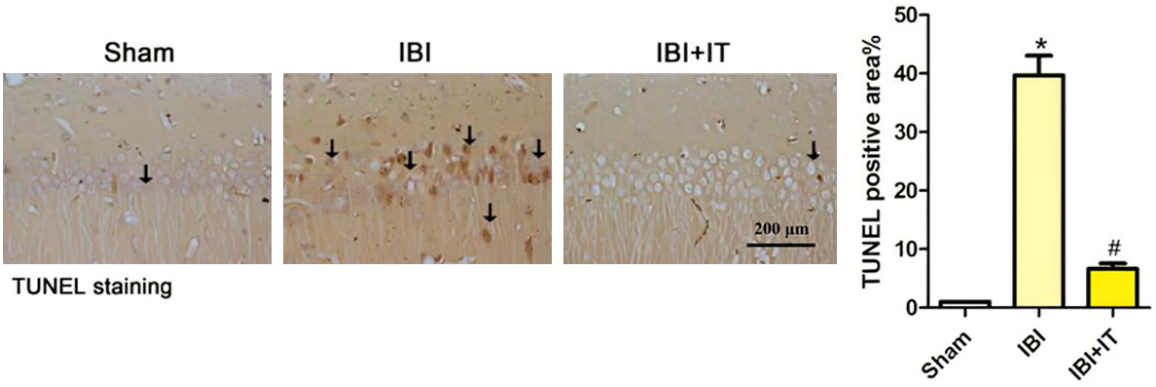

Figure 4. Results of TUNEL staining in rabbit brain tissues of each group. Sham group, IBI group and IBI+IT group, *compared with control group. #P<0.05 compared with IBI group.

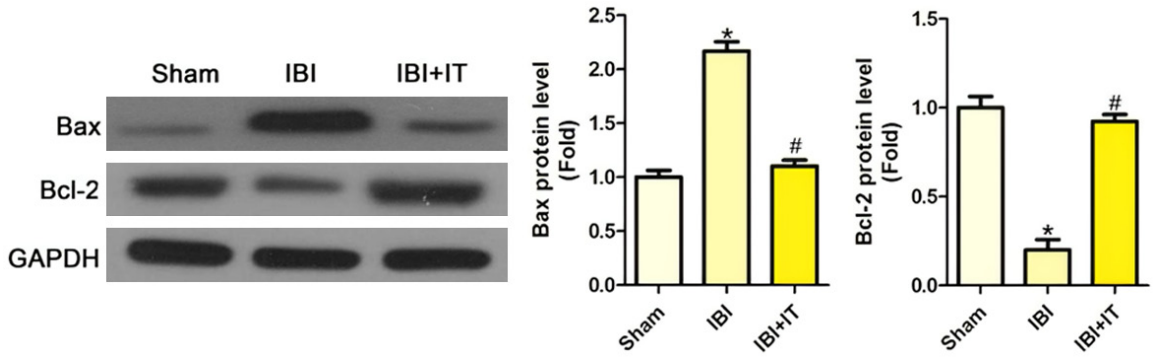

Figure 5. Expressions of apoptosis-related genes in rabbit brain tissues from each group. Sham group, IBI group and IBI+IT group, *compared with control group. #P<0.05 compared with IBI group.

inhibit the apoptosis of cerebral neurons caused by carotid stenosis.

Expressions of apoptosis-related genes in rabbit brain tissues of each group

As shown in **Figure 5**, western blot results (**Figure 5**) showed that the protein expression of the pro-apoptotic gene Bax was significantly up-regulated in the brain tissues of rabbit with IBI, while the expression of the anti-apoptotic protein Bcl-2 protein was significantly suppressed ($P<0.05$). In contrast, after IT, the expression of Bax protein in the brain tissue of IBI rabbits was significantly reduced, and the expression of the anti-apoptotic protein Bcl-2 was significantly increased ($P<0.05$).

Comparison of oxidative stress levels in brain tissue of rabbit from each group

Furthermore, the levels of antioxidant enzymes SOD, oxidases MDA, and GSH-Px in the rabbit brain tissues from three groups were measured. The results suggested that IT significantly inhibited the increase of oxidative stress levels in brain neurons caused by carotid stenosis ($P<0.05$) (**Figure 6**).

Effects of IT on brain neuronal organelles in rabbits with IBI

Finally, the morphologic integrity of the organelles in rabbit brain neurons from three groups was observed by TEM. The results indicated that compared to the control group, the brain neuronal organelles in rabbits from the IBI group showed fragmentation and swelling (**Figure 7A** and **7B**). However, IT significantly alleviated the neuronal organelle damage induced by IBI (**Figure 7B** and **7C**).

Discussion

Ischemic stroke is defined as the sudden reduction or complete interruption of local arteri-

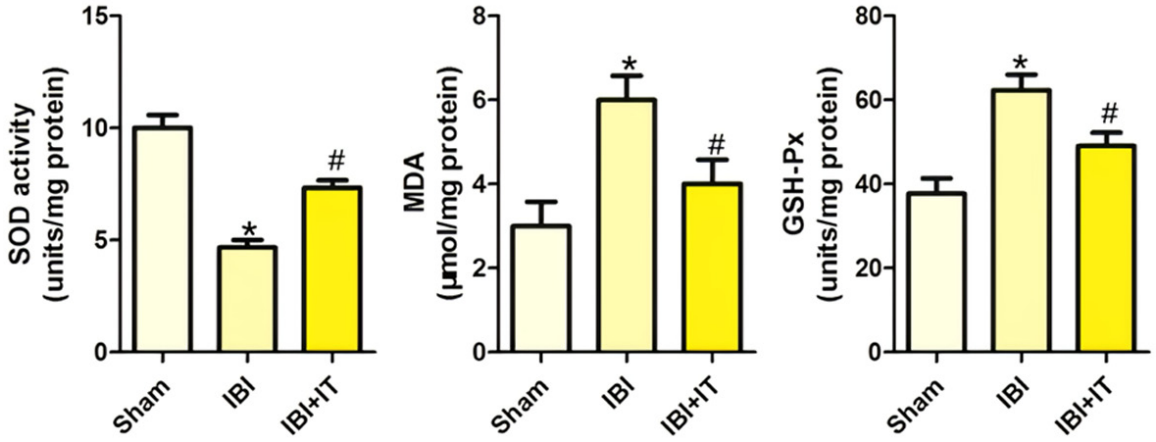

Figure 6. Comparison of oxidative stress levels in brain tissue of rabbit from each group. Sham group, IBI group and IBI+IT group, *compared with control group. #P<0.05 compared with IBI group.

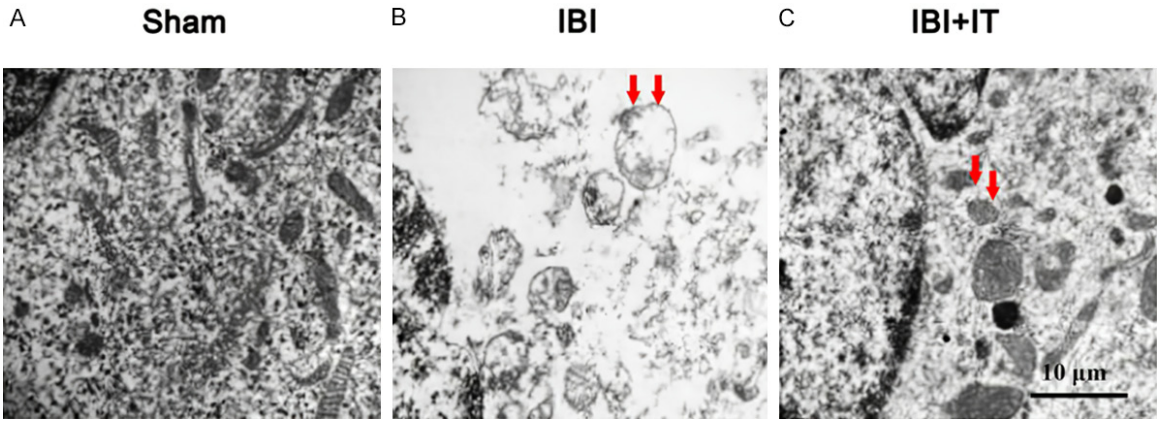

Figure 7. Effects of IT on brain neuronal organelles in rabbits with IBI. Sham group, IBI group and IBI+IT group, *compared with control group. #P<0.05 compared with IBI group.

al blood flow in brain tissues, which results in a sharp decrease in the levels of oxygen, blood and sugar, leading to the collapse of the brain tissue and the rapid occurrence of cerebral dysfunction [11]. It is worth noting that ischemic stroke is also one of the common and serious complications during surgery. For patients who have cardio-cerebral vascular disease, or carotid artery clamping during surgery, or hypotension induced by anesthesia could induce ischemic stroke or even death [12]. In particular, when carotid artery of the patient is narrowed or occluded, the narrowed artery showed a reduction in blood flow. Once hypotension induced by systemic blood volume decline or other factors happens, it can easily cause inadequate perfusion of brain tissue, and thus cause ischemic and hypoxic damage to neurons and lend to nerve function loss, and even result in death [13, 14].

Carotid artery stenosis is one of the most critical risk factors for IBI. The carotid vascular cavity becomes smaller and the elasticity of the blood vessel is lost. The blood flow velocity and blood flow volume in the carotid arteries are significantly reduced, which can subsequently reduce the blood supply to the brain tissue [15]. At present, the methods of establishing carotid stenosis at the animal level include balloon injury method, silicone rubber o-ring method, vascular ligation method, and air modeling method. Although the above methods are currently used to establish models of carotid stenosis, the balloon injury method, silicone rubber ring method, and air modeling method have the characteristics of long modeling time and

difficulty in controlling the degree of stenosis. In contrast, the operations of vascular ligation method are simple and easy, the degree of stenosis is easy to control, and the vascular elasticity loss in the stenosed section is stable [16-18]. Therefore, in the present study, we chose the bilateral carotid artery ligation method to induce a rabbit model of carotid stenosis.

Since Michael De Bakey completed the first carotid endarterectomy in 1953, carotid endarterectomy has gradually become the gold standard for the treatment of carotid stenosis [19]. However, in recent years, with the rapid development of interventional technology and the continuous improvement of operator's skills, direct carotid artery placement has been increasingly used in the treatment of ischemic stroke. Which of the two is better has becoming a hot topic for modern scholars and clinicians. In terms of surgery, traditional carotid endarterectomy has the following disadvantages compared to interventional therapy: 1) General anesthesia used for carotid endarterectomy requires higher general conditions for patients, while patients with carotid stenosis are mostly elderly with underlying diseases such as hypertension and hyperlipidemia. In contrast, interventional therapy often uses local anesthesia, which greatly reduces the risk of anesthesia. 2) Carotid endarterectomy tends to cause tearing of blood vessels, and fragments falling into intracranial blood vessels can cause corresponding intracranial damage. 3) The operation time of carotid endarterectomy is significantly longer than interventional therapy. 4) The risk of stenosis after carotid endarterectomy is higher than interventional surgery [20-22]. Therefore, in cases of clinical blood disorders, some doctors are more inclined to use interventional methods to treat carotid stenosis, but few basic studies have explored the molecular mechanism of protection in the process of interventional surgery. In this study, we found that interventional surgery could significantly up-regulate the expression of the neuroprotective protein AQP4 in brain tissue, and at the same time inhibited the neuronal apoptosis and oxidative stress levels in brain tissues of rabbits with IBI, thus reducing neuronal organelle damage, and ultimately better improving the neural function of rabbits. Previous studies have reported that AQP4 is a potential drug target in neurological disorders, which is consistent with our results [23]. However, the signal pathways involved in neuro-protective effects of interventional therapy require further studies.

In summary, the present research first demonstrated that interventional therapy up-regulated the expression of AQP4 gene, inhibited neuronal cell apoptosis, reduced ischemic brain damage caused by carotid stenosis, and therefore exerted neuro-protective effects.

Acknowledgements

Nantong "13th five-year" science, education and health engineering innovation team project. The study had prior formal approval of hospital's Ethical Review Committee of The Sixth People's Hospital of Nantong. Animal experiments were approved by The Sixth People's Hospital of Nantong (Nantong, China).

Disclosure of conflict of interest

None.

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