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Original Article

Apoptotic cell characteristics of rat brain microvascular endothelia induced by different degrees of hypoperfusion

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Abstract: Hypoperfusion is one of the common causes of ischemic stroke. In this study, decreased blood perfusion and neurological damage were confirmed in ischemic rats. Further, the effect of different perfusion was researched in vivo. We found that hypoperfusion promoted the apoptosis of rats brain microvascular endothelial cells, and the more serious of hypoperfusion, the more obvious of apoptosis. At the same time, this process was related to Tie-2 receptor on cell membranes and Caspase-3 apoptotic pathways. Hemodynamics was one factors affecting the cerebral infarction.

Keywords: Cerebral hypoperfusion, apoptosis, rat brain microvascular endothelial cells, Tie-2

Introduction

As an important organ of the body, brain has almost no energy reserves, and completely depends on the blood circulation [1], Stability of blood flow is essential for cerebral oxygen and glucose supply. Because of vascular obstruction by thrombus or embolus in stroke, brain hypoperfusion with the decrease of cerebral flow velocity and the increase of blood viscosity is obvious [2], resulting in the metabolic or nutritional deficiency for permanent neurological tissues deficits [3, 4].

Hypoperfusion is one of the commonest causes of ischemic stroke [3, 5]. The blood residual supply determines the volume of cerebral infarction and the prognosis of ischemia [6]. According to the relevant reports, cerebral infarction with complete blood occlusion was heavier than that with partially preserved tissue perfusion, and this phenomenon was associated with the neuroprotection of blood perfusion [7]. Hypoperfusion caused brain ischemia and hypoxia, and subsequently a series of brain injuries including early neuronal death [8], glial or astrocytic activation [8, 9], synaptic changes [10], oxidative stress [9, 11], blood-brain barrier damage [12].

Middle cerebral artery occlusion (MCAO) is now the most common used animal model for ischemic stroke. Our previous studies [13, 14] found that the artery was incomplete obstruction in MCAO model: blood flow was significantly decreased in the beginning of ischemia and at a low stable level during a follow-up period [14]. For no good methods to control and detect the perfusion volume in body, hypoperfusion has not been quantified in vivo and that's why the study of hemodynamics is mainly focused on vitro studies [15, 16]. Laminar shear stress (LS) is the transverse pressure on vessels produced by blood flow. As a mechanical parameters which is proportional to the perfusion [17, 18], LS is often used to represent the intensity of flow.

Brain microvascular endothelial cells (BMECs), as the inner layer of the blood vessels, are the great majority of victims in the hypoperfusion injury [14]. It had been proved that LS force of blood flow in the venous system was 1-6 dynes/cm^2, and 10-70 dynes/cm^2 in the arterial sys-

tem [18]. LS force of microvascular blood flow must be smaller than that of the venous system, which is less than 1 dynes/cm^2 under normal condition. Equally, LS is much lower than 1 dynes/cm^2 for ischemic stroke with hypoperfusion. In this study, we used perfusion of 0.1 dynes/cm^2, 0.3 dynes/cm^2, 0.5 dynes/cm^2 and 0.7 dynes/cm^2 in grades for 6 hours to study the apoptotic effects of the rats BMECs (rBMECs) under different degrees of hypoperfusion. We hoped to be able to enrich the mechanism of brain hypoperfusion injury from the perspective of hemodynamics.

Materials and methods

Establishment of middle cerebral artery occlusion (MCAO) model in rats

Adult male Sprague-Dawley rats (250-280 g, about 10 weeks old, Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China) were our experiment models. They were randomly divided into one of two groups: control group (n = 10), ischemic group (n = 10). All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH, USA) guidelines. Right MCAO was adopted to simulate ischemia in rats according to the method of Longa et al [19]. After the rats were anesthetized, an intraluminal nylon monofilament entered the middle cerebral artery following the external carotid artery and internal carotid artery. The middle cerebral artery was stuffed and cerebral infarction was induced simultaneously.

Detection of cerebral blood flow during MCAO

Laser Doppler flowmetry probe (PriFlux System 5000, PerimedAB company) was focused on the surface of the motor cortex of the rats [20]. The cerebral blood flow was measured with this equipment before and after MCAO. Firstly, the normal blood flow levels were observed before operation. Then, MCAO was given and the nylon monofilament was inserted into the middle cerebral artery, the blood flow value was recorded again.

Determination of hemiplegia in rats

The severity of hemiplegia induced by MCAO was assessed by Rogers method [21]. This scale divided the score into 0-7 points: 0 point stands for no hemiplegia. The higher the score, the more severe the hemiplegia. Each rat was evaluated before and after MCAO.

Observation of infarction volume with 2, 3, 5-triphenyltetrazolium chloride (TTC)

Rat brain was stained with 2% TTC perfusion [22] after hemiplegia assessment. 2 mm thick coronal slices (6 slices each brain) were cut and stained: normal area without infarction is bright red, while the infarct area is white.

Culture of rBMECs

rBMECs were cultured referring to the reports [23, 24]. The brain of newborn SD rats (3-5 days) was cut from the skull, sheared to small tissues and filtered with mesh screen. Cells were separated from these tissues with collagenase digestion and then cultured in high glucose DMEM medium of 20% FBS. All experiments were performed in a sterile environment.

Immunocytochemistry for von Willebrand factor (vWF) [24]

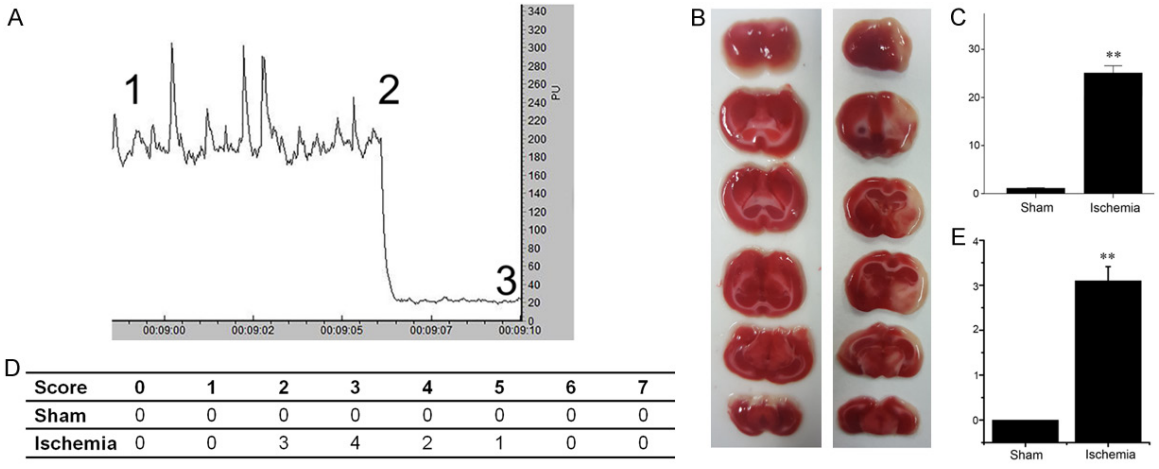
rBMECs processed with 4% paraformaldehyde, 3% H_2O_2, 0.2% Triton X-100, and 5% BSA, successively. Then, cells were incubated with vWF antibody and biotinylated secondary antibody. After Strept-Avidin-Biotin Complex and DAB staining, hematoxylin stained nuclei, vWF were visualized by microscopy. Negative controls were performed by omitting the first antibody.

OGD and LS intervention

OGD model was used for cell ischemia in vitro [25]. rBMECs were cultured in the parallel-plate flow chamber in a sterile environment [26]. The sugar-free culture DMEM medium liquid with 5% CO_2 and 95% N2 mixed gas was selected to provide cells LS in OGD environment for 6 hours. In addition to motionless cells culture medium group (no perfusion), the other groups were medium hypoperfusion with shear stress of 0.1 dynes/cm^2, 0.3 dynes/cm^2, 0.5 dynes/cm^2, 0.7 dynes/cm^2. The computer of parallel-plate flow chamber provided medium dynamic control and regulation.

Annexin V-FITC/PI apoptosis analysis [27]

The cells were resuspended and stained with Annexin V-FITC for 15 min without light at room



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Score	0	1	2	3	4	5	6	7
Sham	0	0	0	0	0	0	0	0
Ischemia	0	0	3	4	2	1	0	0

Figure 1. Cerebral hypoperfusion injury. A: Cerebral blood flow during MCAO in rats by laser doppler flowmetry. The normal blood flow displayed from the 1 point to the 2 point before MCAO. The blood flow fallen sharply from the 2 point to the 3 point when MCAO modeling. B, C: The brain pathology after MCAO. Infarct volume between the sham group and the ischemia group showed. Red was the normal tissue, white was infarcted tissue (**P<0.01). D, E: Neurobehavioral scores described. Rats with different scores were counted in groups. The higher the score, the worse the neural function deficient status (**P<0.01).

temperature following the instructions of the product. The cells were washed 3 times with PBS and stained with PI, and were then detected immediately with flow cytometry.

Hoechst 33258 apoptosis analysis [28]

Cultured cells taken out from parallel-plate flow chamber were stained with Hoechst 33258 without light at 4°C for 30 min, and were observed with the fluorescence microscope.

Western blot

Total protein was extracted with RIPA, underwent protein electrophoresis and transferred to PVDF membrane. The membranes were incubated in primary antibody overnight at 4°C, washed 3 times for 10 min and incubated in second antibody on an orbital incubator for 2 hours. They were photographed with ECL imaging agent in Bio-Rad system.

Statistical analysis

Data was analyzed with a SPSS 22.0 statistical package. ANOVA method was adopted to determine distinguished differences among groups in Annexin V-FITC/PI apoptosis analysis, Hoechst 33258 apoptosis analysis and Western Blot. P<0.05 was considered statistically significant. P<0.01 was proved to be obvious difference.

Results

Changes of cerebral blood flow during MCAO in rats

Laser Doppler flowmetry was used to record the blood flow before and after MCAO. When the rat's middle cerebral artery was blocked, the blood flow of cerebral cortex fallen sharply at the original level of about 10%-20% (**Figure 1A**). This part of the experiment certificated the decreased blood flow during ischemic stroke.

Pathological and behavioral effects of MCAO in rats

Cerebral hypoperfusion can cause severe brain injury, mainly including two aspects: anatomical and behavioral aspects. The pathological infarction volume was detected by TTC staining method [22]. As indicated in **Figure 1B** and **1D**, The rats in the sham control group exhibited all normal areas. In contrast, there was a significant infarct volume in ischemia group. At the same time, Rogers scores was used to evaluate the behavioral lack [21]. Results showed that the score of the rats was 0 who had no action defects before MCAO, But there was a significant hemiplegia after MCAO and the score was statistical increased (P<0.01). This part of the experiment showed that cerebral hypoperfusion resulted in cerebral infarction.

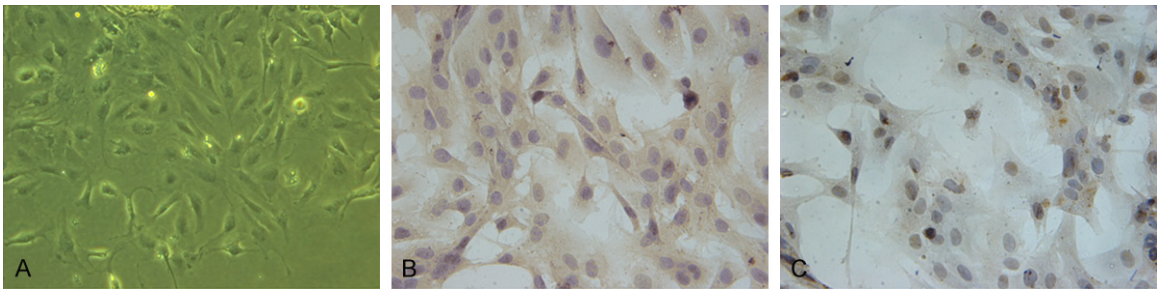

Figure 2. Culture and identification of rBMECs. Cells morphology under inverted microscope in (A) (200×). Cells were positively immunostained of vWF (B) than the control group (C) (400×).

rBMECs characterization

The cultured cells were identified as endothelial cell through morphological observation and immunostaining. rBMECs under microscope were polygonal or fusiform shape which the characteristics are consistent with previous reports (**Figure 2A**). vWF is one of the most reliable endothelial expressed protein marker [24], rBMECs were positively immunostained (**Figure 2B**) than the control group (**Figure 2C**).

Effects of cerebral hypoperfusion on rBMECs apoptosis

OGD model was adopted for cells ischemia in vitro. For rBMECs, exposure to OGD condition for 6h was appropriate to show significant apoptosis (the relative apoptotic rate was 25%-30%), and the expression of self-regulated proteins was provoked strongly. Different degrees of hypoperfusion about 0.1 dynes/cm^2, 0.3 dynes/cm^2, 0.5 dynes/cm^2, 0.7 dynes/cm^2 (the amplitude fluctuation was not more than 0.05 dynes/cm^2) were used during OGD intervention for a total of 6 hours. Apoptosis was detected by AnnexinV/7-AAD flow cytometry and Hoechst 33342 staining. As shown in **Figures 3** and **4** compared with control group (shear stress = 0 dynes/cm^2), the intervention of LS reduced cells apoptosis. The apoptosis rate was gradually decreased correspondingly with the LS gradient of 0.1, 0.3, 0.5, 0.7 dynes/cm^2. It was proved that the hemodynamics played a role against cerebral hypoperfusion injury. The greater the shear stress, the less the apoptosis rate.

Effects of cerebral hypoperfusion on cell protein expression

Expression of protein Tie-2 and caspase-3 were detected while measured the cells apoptotic rates. As shown in **Figure 5**, Tie-2 increased and Caspase-3 decreased accompanied with the increase of LS. Tie-2 in cells membrane could be influenced by the hemodynamics and affected Caspase-3 expression in apoptotic pathway. We speculated that the up-regulation of Tie-2 induced some mechanisms, and ultimately interfered Caspase-3 for anti-apoptosis.

Discussion

Activity of the brain needs adequate cerebral perfusion to maintain the supply of oxygen and nutrition [1]. Hypoperfusion is a risk factor for cerebral infarction [3, 4]. Through this study, we found that hypoperfusion promoted the apoptosis of rBMECs, and the lower the perfusion, the greater the number of apoptotic cells. At the same time, this process might be related to Tie-2 receptor on cell membranes and Caspase-3 apoptotic pathways.

Cerebral hypoperfusion results in cerebral infarction [5, 6]. Mild hypoperfusion, such as transient ischemic attack (TIA) or syncope episodes [29, 30], induced by intracranial vascular atherosclerosis for slight or even reversible blood flow decreases, was almost no clinical symptoms. While severe hypoperfusion, such as typical cerebral parenchyma infarction or watershed infarction, induced by different degrees of vascular occlusion due to thrombus, will cause blood flow reduction or interruption and severe clinical neurological symptoms [31]. Hypoperfusion causes brain damage, not only in oxygen and nutrients lack, but also in microemboli formation with the reduction of cerebral blood flow [32, 33]. Found in studies, it is easy to form microemboli under the condition of low perfusion, hemodynamic changes or vulnerable lesions in the blood vessel [32, 34].

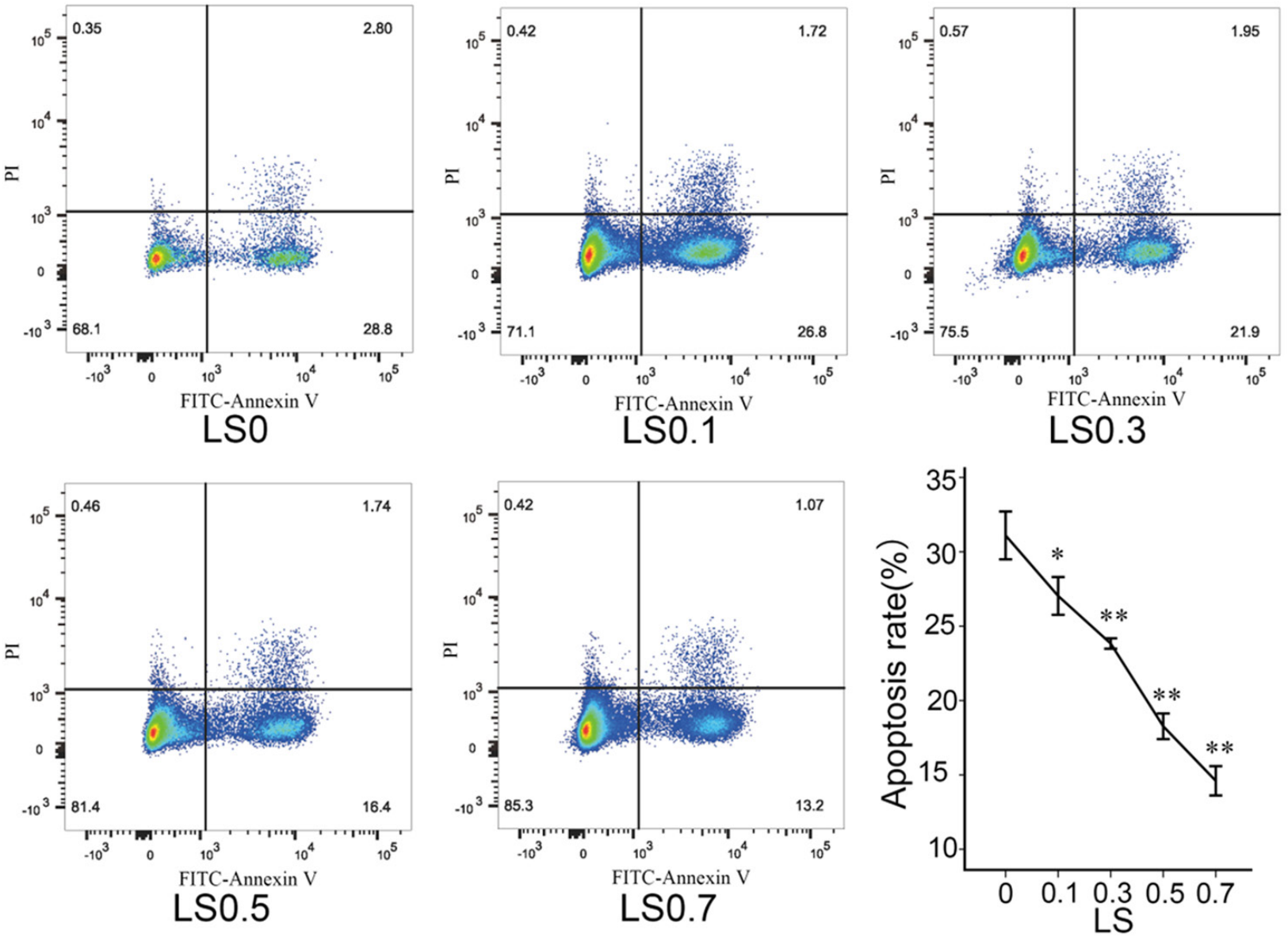

Figure 3. Effects of cerebral hypoperfusion on rBMECs apoptosis by Annexin V-FITC/PI flow cytometry. The value of the two quadrants on the right represented the percentage of apoptosis. The slower the fluid rate, the lower the shear stress force, the more apoptosis and the most obvious apoptosis was in static state of flow (*P<0.05, **P<0.01).

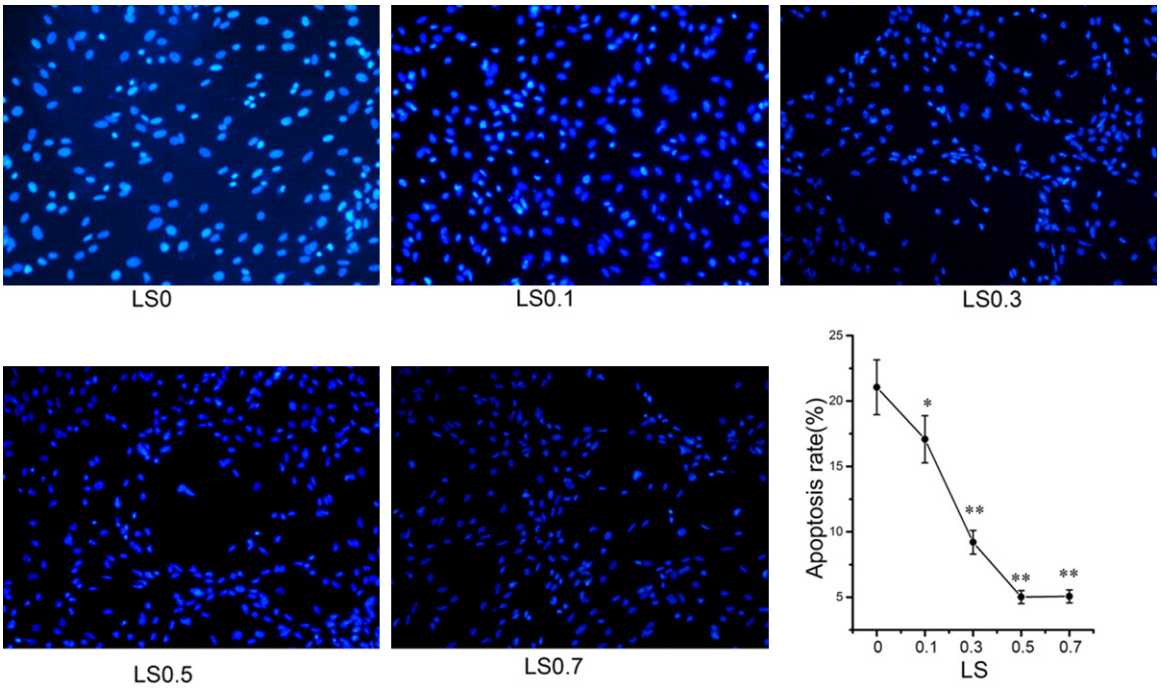

Figure 4. Effects of cerebral hypoperfusion on rBMECs apoptosis by Hoechst 33258 staining. This method was for the nucleus staining, the apoptotic cells were light dyeing and normal cells were dark dyeing. The results were consistent with the previous Annexin V-FITC/PI flow cytometry (*P<0.05, **P<0.01).

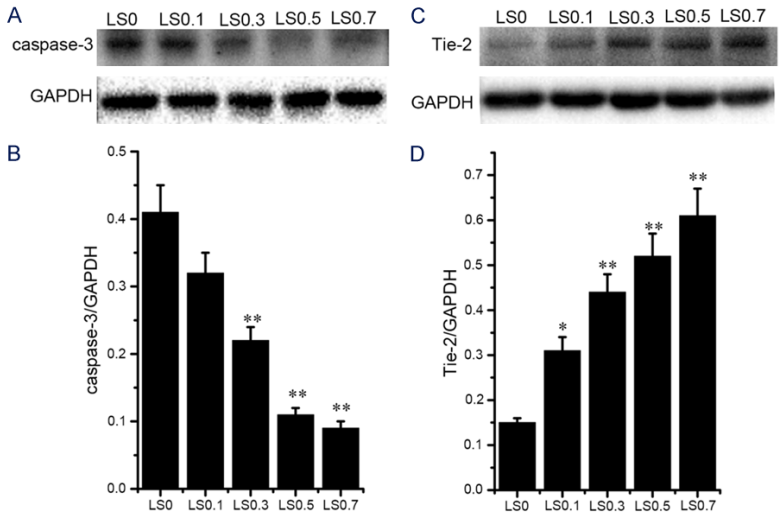

Figure 5. Effects of cerebral hypoperfusion on cell protein expression of Tie-2 and caspase-3. Compared with the protein expression in LS0 group, with the increase of LS, the expression of caspase-3 protein decreased, and the expression of Tie-2 protein increased gradually (*P<0.05, **P<0.01).

Increasing the blood volume could attenuate the formation or enhance the scavenging ability of microemboli [32]. Thus, hypoperfusion can considered to be an important risk factor for cerebral infarction.

BMECs, a major component of the inner lining of the vessels, contact with blood flow directly and be sensitive to the effects of perfusion [35]. LS force is produced by the blood flow and is one of the main components of the mechanical flow force [17, 18]. The dynamic transmission mechanism of hemodynamics has been extensively studied. These studies indicated that receptors on endothelial membrane could directly feel the changes of LS, and through the changes of the membrane structure, the mechanical signal would be transformed into biological signals to cause a series of biochemical changes in endothelial cells [18, 36, 37]. In this study, the smaller the LS force was, the less the residual blood was, and the more obvious the apoptosis of endothelial cells was. To reduce the obstruction of the vascular cavity and increase the

blood flow as much as possible was one measure to reduce brain damage.

Tie-2, a tyrosine kinase receptor, mainly expresses in the vascular endothelial cells and has extensive homology with immune globulin and epithelial growth factor (EGF) receptor [38]. Tie-2 can support vascular integrity, promote angiogenesis and maintain the stability of vascular structure [39]. Caspases is a group of structurally related cysteine proteases in the cytosol. Through cascade activation of different subtypes of Caspases, the degradation of DNA is initiated [40] for the completion of apoptosis. This process is involved in the execution of apoptosis, in which caspase-3 is considered to be one of the key enzymes [41]. There were reports that one of the most important ways for endothelial protection by Tie-2 receptors was caspase-3 pathway [40]. Therefore, we believed that down-regulation of Tie-2 receptor and up-regulation of Caspase-3 expression was related to apoptosis induced by hypoperfusion in this study.

Due to the technical limitations, it is not possible to give accurate and precise control of cerebral perfusion in vivo and that's why we had to do this experiment in vitro. However, we still proved the rudimentary effect of blood perfusion in this experiment.

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Disclosure of conflict of interest

None.

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