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Effects of salidroside pretreatment on expression of tumor necrosis factor-alpha and permeability of blood brain barrier in rat model of focal cerebralischemia-reperfusion injury

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ABSTRACT

Objective: To observe changes in expression of tumor necrosis factor (TNF)-alpha and permeability of blood brain barrier after salidroside pretreatment in rats with injury induced by focal cerebralischemia-reperfusion. **Methods:** Forty-five male SD rats were randomly divided into three groups (n=15): control group, ischemia-reperfusion (IR) model group, and salidroside pretreatment group. Before the IR model establishment, the rats in the salidroside pretreatment group were intraperitoneally administered with salidroside at a dose of 24 mg/(kg•d) for 7 d. After 30 min post the last administration, the IR model was induced by occlusion of middle cerebral artery with a filament. After 24 h post the operation, the water content and Evens blue content in the ischemia cerebral hemisphere were determined, and the level of TNF-alpha mRNA was detected by the semi-quantitative RT-PCR. **Results:** Compared with the IR model group, the salidroside pretreatment group had significantly lower (P<0.05) water content and Evens blue content in the ischemia cerebral hemisphere and also had significantly lower (P<0.05) level of TNF-alpha in the ischemic cerebral cortex tissue. **Conclusions:** The salidroside pretreatment alleviated the focal cerebralischemia-reperfusion injury in the rat model, possibly by decreasing the permeability of blood brain barrier, attenuating brain edema and reducing TNF-alpha expression.

1. Introduction

In the process of anesthesia and surgery, patients are vulnerable to low blood pressure which results in drop of cerebral perfusion pressure and reduction of cerebral blood flow. Particularly in patients with cerebrovascular stenosis or occlusion, the low blood pressure easily leads to brain tissue hypoxia and often causes reperfusion injury after recovery of cerebral blood flow[1]. Therefore, it is of important clinical significance to protect brain from cerebral ischemia-reperfusion injury. However, the exact mechanisms on cerebral ischemia-reperfusion injury are still unclear, and the treatment outcomes are also not satisfied. Salidroside, the main active ingredient of *Rhodiola rosea*, has many widely accepted functions such as radio-resistance, anti-hypoxia, anti-fatigue and anti-aging[2]. Meanwhile, it can clean excessive blood lipid, prevent formation of atherosclerotic plaques, decrease blood viscosity, speed up erythrocyte flow, improve hemodynamic indicators, resist to myocardial ischemia, and dually regulate nervous system and metabolism. Recent studies have also proved its protective effects on cerebral ischemia-reperfusion injury[3]. The aim of this study was to further explore possible mechanisms by detecting expression of tumor necrosis factor (TNF)-alpha and permeability of blood brain barrier after salidroside pretreatment in rats with focal cerebralischemia-reperfusion injury.

2. Materials and methods

2.1. Rats and grouping

Forty-five young male SD rats of clean grade with mean weight of (272±15) g were bought from Shanghai Slaccas Experimental Animal Co. Ltd (Shanghai, China). They were randomly divided into three groups (n=15): control group, ischemia-reperfusion (IR) model group, and salidroside pretreatment group. Before the IR model establishment, the rats in the salidroside pretreatment group were

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intraperitoneally administered with salidroside at a dose of 24 mg/(kg•d) for 7 d, while each rat of the other two groups were intraperitoneally injected 2 mL normal saline.

2.2. Establishment of animal model

After 30 min post the last injection, the focal cerebralischemia-reperfusion model was established by occluding middle cerebral artery using the line bolt method[4]. All the rats were fasted for 12 h before the operation but had free access to water. After intraperitoneal injection of 10% (v/v) chloral hydrate at a dose of 3.5 mL/kg•BW, neck median of the anesthetized rat was incised. After the right common carotid artery and external and internal carotid arteries were separated and ligated, a round-head nylon line with a diameter of 0.26 mm was put into the common carotid artery though an incision at the bifurcation point. After 18-20 mm long nylon line had entered the common carotid artery, resistance appeared which indicated successful occlusion of middle cerebral artery. Then the nylon line was ligated and fixed, and the fur was sutured. After 60 min occlusion, reperfusion was made by drawing the line outwards. All these steps except the insertion of nylon line were done in the control group. The rats kept autonomous respiration during the operation. The rats with bending arms, left-prone circling behavior and paralysis of left limbs were used in the following trial.

2.3. Sample collection and determination of Evans blue content

The rats were injected 20 g/L Evans blue at a dose of 2.0 mL/kg•BW via femoral vein after 23 h post the operation. One hour later, they were executed and then perfused normal saline via left ventricle until clear liquid flowed out of right auricle. After head was cut, right and left cerebral hemispheres were separated, followed by the removal of arachnoid from cortical surface. Subsequently, each cerebral hemisphere was weighed and immersed into a known volume of formamide solution to measure brain volume. Then dimethylformamide five times as large as the brain volume was added and incubated in a 50℃ water bath for 72 h. Following centrifugation at 1 500 r/min for 10 min, the yielded supernatants were detected at 635 nm using a spectrophotometer. The absorbancy together with the Evans blue standard curve was used to estimate the Evans blue content in brain (μg/mL).

2.4. Determination of water content in brain

After the external water was removed with filter paper, water content in the ischemia side of the brain was determined using the dry-wet mass method. Briefly, the separated right brain tissue was measured with an analytical balance within 5 min. Later, it was kept in a 52℃ medical electric oven for 72 h and the dry weight was determined. The water content was calculated using the following formula:

$$\text{Water content (\%)} = (\text{Wet weight} - \text{Dry weight})/\text{Wet weight} \times 100\% \quad (1)$$

2.5. Detection of TNF-alpha mRNA levels

Total RNA of the ischemic cerebral cortex tissue was extracted using the Trizol reagent. The expression of TNF-alpha gene was observed by RT-PCR using 1 μg total RNA. The reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) gene at the same dose was used as an internal control. The primer sequences are as follows: 5′-CCA ACA AGG AGG AGA AGT-3′ (The first primer for TNF-alpha gene), 5′-GTA TGA AGT GGC AAA TCG-3′ (The second primer for TNF-alpha gene), 5′-ACA GTC AGC CGC ATC TTC TT-3′ (The first primer for GAPDH gene) and 5′-TTG ATT TTG GAG GGA TCT CC-3′ (The second primer for GAPDH gene). The following PCR reaction program was used: initial denaturation at 94℃ for 3 min; denaturation at 94℃ for 45 s; renaturation at 48℃ for 45 s (TNF-alpha gene) or at 55℃ for 45 s (GAPDH gene); extension at 72℃ for 45 s. After 30 cycles, further extension was performed at 72℃ for 5 min. Fifteen microliter of the PCR products was separated electrophoretically on 1.8% agarose gel. Semi-quantitative analysis was conducted using the Gel-pro analyzer. The arbitrary unit (Au) of TNF-alpha gene to that of GAPDH gene was used to indicate the relative expression level of TNF-alpha RNA. Here, the Au was the product of the area of electrophoresis band and fluorescence intensity.

2.6. Statistical analysis

All statistical analyses were done with the SPSS 13.0 statistical software. Quantitative variables are expressed as mean±SD. Two-tailed Student's *t*-test was used to compare continuous data for between-group differences. Statistical significance was set at $P<0.05$.

3. Results

3.1. Effects of salidroside pretreatment on water content and Evens blue content in ischemia cerebral hemisphere

Compared with the control group, the other two groups had significantly higher ($P<0.05$) water content in the ischemia side of the brain and Evens blue content in the ischemia cerebral hemisphere. However, both indicators were significantly lower ($P<0.05$) in the salidroside pretreatment group than in the IR model group. The results of the water content and Evens blue content are represented in Table 1.

3.2. Effects of salidroside pretreatment on expression of TNF-alpha in ischemic cerebral cortex tissue

As evidenced by the RT-PCR results (Figure 1), the focal cerebralischemia-reperfusion increased TNF-alpha mRNA levels, while the salidroside pretreatment remitted such increasing effect. The TNF-alpha mRNA level was significantly lower ($P<0.05$) in the control group than in the other two groups (0.58±0.06 *vs.* 1.07±0.18 for the IR model group; 0.58±0.06 *vs.* 0.81±0.09 for the salidroside pretreatment group), while the salidroside pretreatment group had significantly lower ($P<0.05$) TNF-alpha mRNA level than the IR model group.

Table 1

Water and Evens blue contents in ischemia cerebral hemisphere after salidroside pretreatment (n=15).

Group	Water content (%)	Evens blue content (μg/mL)
Control group	75.21±0.37[a]	14.13±1.08[a]
Ischemia-reperfusion model group	82.47±0.28[bc]	16.82±0.48[bc]
Salidroside pretreatment group	78.11±0.31[bd]	16.23±0.78[bd]

Within columns, different letters indicate that there is a significant difference ($P<0.05$).

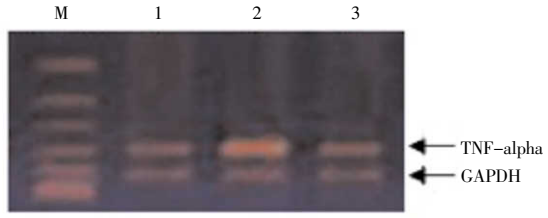

Figure 1. Electrophoresis of RT-PCR products of TNF-alpha gene. Lane M: DNA ladder; Lane 1: Control group; Lane 2: Ischemia-reperfusion model group; 3. Salidroside pretreatment group. The GADPH gene was used as an internal control to indicate the increase in the TNF-alpha mRNA levels.

4. Discussion

Cerebral ischemia-reperfusion injury is related with many factors such as free radical generation, intracellular calcium overload, toxicity of excitatory amino acids, high aggregation of leucocytes, and lack of high-energy phosphate compounds. Acute focal cerebral ischemia results in cell death in central ischemic region characterized by necrocytosis[5]. Some mechanisms have been clearly stated to explain cerebral ischemia-induced injury. Cerebral ischemia lasting for 5-7 min causes exhaustion of cellular energy, blockage of potassium ion channel, decease of membrane potential, and release of glutamic acids from nerve endings. Glutamic acid receptors, including N-methyl-*D*-aspartate and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainite receptors, are also activated, resulting in opening of calcium ion channel on cell membrane and calcium ion overload. The high concentration calcium ions activate nitrous oxide systems, increase nitric oxide and $O^{\bullet}$, induce lipid peroxidation, and thus damage membrane structure and DNA. Additionally, calcium ions also stimulate various kinds of enzyme, aggravate cell injury and energy disorder, cause cascade reactions including cellular edema, membranolysis, release of desmoenzymes and imflamatory madiators, and finally lead to cell necrosis[6].

Internal carotid and vertebral artery, which supply brain tissue with blood, form the circle of Willis (circulus arteriosus) to protect brain from ischemia. Cerebral vascular resistance mainly depends on the contraction and relaxation of brain capillary. Thereafter, cerebral blood flow and cerebral vascular resistance can be used to indicate the status of cerebral aortas and capillaries. Researches about global cerebral ischemia of anesthetized dogs and rats have demonstrated that salidroside remarkably increases cerebral blood flow of the anesthetized dog but has no effect on blood pressure. The results show that salidroside promotes blood circulation without side-effect on operation and anesthesia[7].

Inflammatory reaction plays an important role in the induction of injury by cerebral ischemia-reperfusion, while excessive inflammatory reaction not only affects local blood supply but also directly damage brain tissue structure. In the case of cerebral ischemia, local endothelial cells and leukocytes are activated by a great number of diffusible inflammatory mediators such as TNF-alpha and interleukin 1 which are produced by lesion tissues, so that their infiltration to brain tissue is sped up. These leukocytes release a great number of toxic oxygen free radicals and proteolytic enzymes which will cause focal cerebrovascular trauma, increase vascular permeability and aggravate cerebral edema. Salidroside is reported to reduce TNF-alpha expression levels of rats with cerebral ischemia-reperfusion injury[8], thus protecting brain tissue by relieving cerebral edema and reducing neutrophils infiltration. Our research also supports this result. The decrease in the expression levels of inflammatory factors may be used to interpret the potential protection mechanism of salidroside.

Evans blue, which can bind to plasma proteins, can enter brain tissue when blood brain barrier is destroyed. For this reason, its seepage quantity reflects the permeability of blood brain barrier. Salidroside was found to reduce the Evans blue content in brain tissue[7], which is in line with our result. Therefore, salidroside can alleviate global cerebral ischemia-reperfusion injury by reducing structural and functional changes of blood brain barrier, increasing stability of brain capillary endothelial cells and decreasing vascular permeability.

In conclusion, salidroside pretreatment can efficiently reduce the damage degree of blood brain barrier, attenuate brain edema and decrease TNF-alpha expression level in rats with focal cerebralischemia-reperfusion injury. Considering the little side effects of salidroside, it may be a candidate agent to prevent reperfusion-induced injury for anesthetists. Nevertheless, its specific mechanisms need to be further explored.

Conflict of interest statement

We declare that we have no conflict of interest.

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