

Effect of Berberine on Angiogenesis and HIF-1 α / VEGF Signal Transduction Pathway in Rats with Cerebral Ischemia - Reperfusion Injury

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ABSTRACT

Objective: To investigate the effect of berberine on angiogenesis and signal transduction pathway of hypoxia-inducible growth factor-1 α (HIF-1 α) / vascular endothelial growth factor (VEGF) in rats with cerebral ischemia-reperfusion injury.

Study Design: An experimental study.

Place and Duration of Study: Luoyang Central Hospital Affiliated with Zhengzhou University, China, from 2016 to 2017.

Methodology: Forty-five rats were randomly divided into control group, model group and berberine group, 15 rats in each group. The model of rat with cerebral ischemia-reperfusion injury was duplicated by suture method. Neurological score was evaluated before, after operation, and 7 days after administration. Microvessel density (MVD) of cerebral ischemia-reperfusion cortex was detected by immunohistochemistry. HIF-1 α mRNA and VEGF mRNA of cerebral ischemia-reperfusion cortex were detected by RT-PCR. HIF-1 α , and VEGF protein expression of cerebral ischemia-reperfusion cortex was detected by Western Blotting.

Results: After operation and 7 days of administration, the neurological scores of the control group, model group, and berberine group were different (all $p < 0.001$). After 7 days of administration, neurological score of berberine group was lower than that of model group ($p < 0.001$), MVD, HIF-1 α mRNA and VEGF mRNA expression. HIF-1 α and VEGF protein expression levels were lower in model group than berberine group (all $p < 0.001$).

Conclusion: Berberine can promote angiogenesis in the rat with cerebral ischemia-reperfusion injury. Its mechanism may be activation of HIF-1 α / VEGF signal transduction pathway.

Key Words: Berberine, Cerebral ischemia, Reperfusion injury, Angiogenesis, Vascular endothelial growth factor, Hypoxia-inducible growth factor-1 α .

INTRODUCTION

Cerebral ischemia-reperfusion injury means after restoration of blood supply to the ischemic brain tissue, neurological damage and morphological changes in the brain tissue is more serious than before the restoration of blood supply.¹ However, if the blood supply to ischemic brain tissue is restored within the shortest possible time, neurological function of ischemic brain tissue can be maximally restored.² Blood vessels constitute systemic transport pathway of blood. Angiogenesis is the most effective way to restore blood supply of ischemic brain tissue.³ Therefore, one effective method for clinical treatment of cerebral ischemia is to promote angiogenesis.

Berberine (BBR) is a Chinese medicine monomer with clear structure and very safe clinical use. Its pharmacological action is reliable as demonstrated in significant anti-heart failure, inhibition of vascular smooth muscle

proliferation, anti-tumor, anti-platelet, anti-inflammatory and anti-atherosclerosis effects.^{4,5} In recent years, some scholars have done a lot of experimental studies on protective effect of BBR on heart and cerebral vessels.⁶ However, the brain protection mechanism of BBR has not yet been fully elucidated and there is little literature reporting the effect of berberine on cerebral ischemia-reperfusion injury from angiogenesis. Vascular endothelial cell growth factor (VEGF) is the most active factor yet found in promoting angiogenesis, which can promote the division, proliferation and migration of vascular endothelial cells and increase vascular permeability.⁷ VEGF is target gene of hypoxia-inducible growth factor-1 α (HIF-1 α).⁸ Under ischemic and hypoxic conditions, HIF-1 α can activate the transcription of VEGF gene and increase the level of VEGF.

The objective of this study was to investigate the effects of berberine on angiogenesis and HIF-1 α / VEGF signal transduction pathways during cerebral ischemia-reperfusion injury by duplicating model of rat with cerebral ischemia-reperfusion injury.

METHODOLOGY

This study was conducted at Luoyang Central Hospital Affiliated with Zhengzhou University, China, from 2016 to 2017. Forty-five healthy SPF male and female SD rats

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were selected as experimental subjects weighing 200-210 (204.5 ±2.6) grams. One week after laboratory adaptive feeding, 45 rats were randomly divided into control group, model group, and berberine group, 15 rats in each group.

Model of rat with cerebral ischemia-reperfusion injury was duplicated by suture method. The main steps were as follows: The rats in the model group and berberine group were anesthetised by intraperitoneal injection of 10% chloral hydrate (350 mg / kg), fixed in supine position with neck skin preserved. The skin was cut from the middle part of the neck to separate muscle, fascia tissue. The right internal carotid and *arteria carotis communis* were separated, with two arteriole clamp clamping the distal end of *arteria carotis communis* and internal carotid trunk. The proximal end of internal carotid was ligatured with disinfected line. A small incision was cut in the ligatured internal carotid. Meanwhile, one line was placed under internal carotid for standby use. The suture was inserted into the incision, the loose knot tighten, and the arteriole clamp in the internal carotid removed. The suture was inserted for 18 mm along the internal carotid or stopped when there was resistance. The insertion position was kept motionless for 45 minutes, the suture was withdrawn, the arteriole clamp removed for 45 minutes reperfusion, and then 45 minutes similar blockage was carried. After repetition for 3 times, suture wounds were closed and disinfected. Twenty-four hours after operation, the rats in both groups showed left forelimb adduction, inability to extend left forepaw at tail suspension, leftward circling when walking, and thus modeling could be determined as successful. Control group rats did not receive any treatment. Berberine group was given berberine 2 mg / (kg · d) after successful model replication, which was diluted to 4 mL and injected intraperitoneally once daily for 7 days. The control group and model group were injected intraperitoneally with equivalent amount of 0.9% sodium chloride injection.

Neurological score was given to 3 groups of rats in Longal scoring standard after operation and 7 days after continuous administration. Longal scoring standard had five components: 1. 0 point: animals showed no neurological deficit symptoms; 2. 1 point: forepaw could not fully stretch; 3. 2 points: walk circularly outward; 4. 3 points: offside tilt in walking; 5. 4 points: loss of consciousness, inability to crawl. The higher the score, the worse the damage is.

Immunohistochemical detection of cerebral ischemia-reperfusion cortex microvessel density (MVD) was determined. Five rats in each group were anesthetised by intraperitoneal injection of 10% chloral hydrate (350 mg/kg) 1 hour after the last intraperitoneal injection, brain tissue was removed, fixed in 10% formaldehyde, embedded in paraffin, sectioned, dewaxed, hotfixed and sealed in H₂O₂. After addition of CD34 (1: 800), VEGF

(1: 800) and HIF-1 α (1: 800) anti- antibodies, it was incubated overnight at 4°C. The second antibody was incubated at room temperature for 30 minutes, developed and redyed, followed by restoration of blue, dehydration, mounting and filming. The dominating cortex MVD of right anterior and middle cerebral artery was counted by WEIDNER method.⁹

RT-PCR detection of VEGF mRNA was carried out and HIF-1 α mRNA expression levels were also evaluated. Five rats in each group were anesthetised intraperitoneally with 10% chloral hydrate (350 mg/kg) 1 hour after the last intraperitoneal injection to extract the total RNA in the brain tissue with cerebral ischemia-reperfusion injury. The extracted total RNA was reversely transcribed into complementary cDNA for amplification with cDNA as a template. One reaction cycle was as follows: denaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, gradient annealing at 56-60°C for 30 seconds and extension at 72°C for 40 seconds. Following the reaction conditions described above for one reaction cycle, the reaction was continued for 35 cycles. The product obtained by the reaction was separated by electrophoresis in an agarose gel, the absorbance of the separated gel bands was scanned and the relative contents of the respective mRNAs were compared with reference to absorbance values. Upstream primer of HIF-1 α during amplification: 5'-AACAAACAGAATCTGTCCTCAAACC-3', downstream primer: 5'-CAGGTAATGGAGACATTGCCAG-3', fragment length 296bp; upstream primer of VEGF: 5'-GGCCTCTGAAACCATGAACT-3', downstream primer: 5'-ATGCTGCAGGAAGCTCATCT-3', fragment length 385bp; upstream primer of β -actin-1: 5'-AGTGTGACGTGGACATCCGCAAAG-3', downstream primer: 5'-ACTCACATCTGCTGGAAGGTGGAC-3': fragment length 390bp; upstream primer of β -actin-2: 5'-TCAG-GTCATCACTATCGGCAAT-3', downstream primer: 5'-AAAGAAAGGGTGTAAAACGCA-3', fragment length 431 bp.

Western Blotting detection of VEGF and HIF-1 α protein expression levels: Five rats in each group were anesthetised by intraperitoneal injection of 10% chloral hydrate (350 mg / kg) 1 hour after the last intraperitoneal injection. Brain tissue was removed and homogenised. Total protein in brain tissue was extracted and 40 μ g protein was added to loading buffer for boiling denaturation. The denatured protein sample was added into SDS-PAGE gel, followed by electrophoresis (spacer gel 100V, separation gel 120V) and membrane transfer (200 mA, 80 min) to the cellulose acetate membrane. After 2 hours blockage, VEGF and HIF-1 α primary antibody was incubated overnight at 4°C, secondary antibody was incubated at room temperature for 60 minutes, coated with ECL luminescent reagent. The film was exposed in the darkroom, and then soaked in the film developer and fixing solution. The absorbance of the

bands was scanned and the relative amounts of each interest protein were compared by absorbance values.

All statistical analyses were performed on SPSS version 23.0 statistical software. Measurement data was indicated by mean \pm SD. Multiple sample averages were compared using single factor analysis of variance. P-value of <0.05 was considered significant.

RESULTS

After operation and seven days of administration, the neurological scores of the control group, model group, and berberine group were different (all $p < 0.001$). After seven days of administration, neurological scores of the model group and berberine group were higher than those of the control group (all $p < 0.001$), and neurological score of the model group was higher than that of the berberine group ($p < 0.001$, Table I).

After seven days of administration, the expression of MVD, VEGF mRNA and HIF-1 α mRNA in cerebral ischemia-reperfusion cortex of control group, model group, and berberine group were different (all $p < 0.001$); and MVD of cerebral ischemia-reperfusion cortex in the berberine group and model group was higher than that in the control group (all $p < 0.001$). MVD of cerebral ischemia-reperfusion cortex in berberine group was higher than the model group ($p < 0.001$). The expression levels of VEGF mRNA and HIF-1 α mRNA in cerebral ischemia-reperfusion cortex of berberine group and model group were higher than those in control group (all $p < 0.001$). The expression levels of VEGF mRNA and HIF-1 α mRNA in cerebral ischemia-reperfusion cortex of berberine group were higher than that in model group (all $p < 0.001$, Figure 1, Table II).

After seven days of administration, the expression levels of VEGF and HIF-1 α protein in cerebral ischemia-reperfusion cortex in control group, model group and berberine group were different (all $p < 0.001$); and expression levels of VEGF and HIF-1 α protein in cerebral ischemia-reperfusion cortex of berberine group and model group were higher than those of control group (all $p < 0.001$). The expression levels of VEGF and HIF-1 α protein in cerebral ischemia-reperfusion cortex

of berberine group were higher than those of model group (all $p < 0.001$, Figure 2).

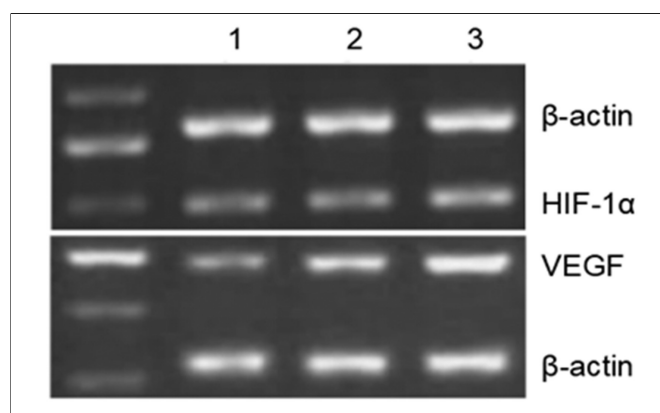


Figure 1: 1 RT-PCR detection results of HIF-1 α mRNA and VEGF mRNA. 1: Control group; 2: Model group; 3: Berberine group.

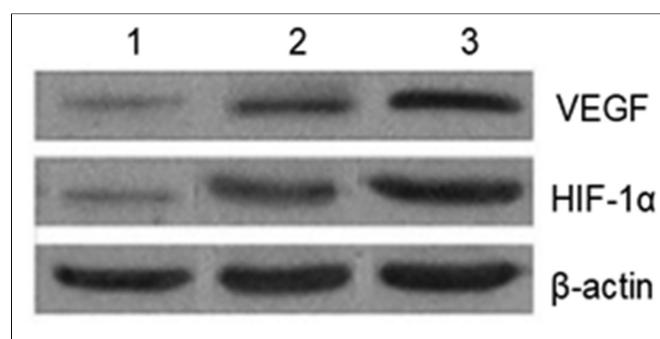


Figure 2: Western blotting detection results of HIF-1 α and VEGF protein expression. 1: Control group; 2: Model group; 3: Berberine group.

DISCUSSION

After cerebral ischemia, nerve cells in brain tissue become denatured, necrotic and apoptotic due to ischemia and hypoxia, resulting in severe damage to nerve function.¹⁰ If brain tissue blood supply restored in cerebral ischemia part, damaged brain function can be largely restored. Angiogenesis at the cerebral ischemic site can largely restore the blood supply to the cerebral ischemic site and improve ischemia and hypoxia at the cerebral ischemic site.¹¹ The cerebral ischemic site of

Table I: Comparison of neurological scores of rats in each group (score).

Groups	The number of mice	After operation		After 7 days of administration	
		Mean \pm SD	p-value	Mean \pm SD	p-value
Control group	15	0.00 \pm 0.00	<0.001	0.00 \pm 0.00	<0.001
Model group	15	2.14 \pm 0.28		2.98 \pm 0.65	
Berberine group	15	2.16 \pm 0.19		1.24 \pm 0.46	

Table II: Comparison of MVD, VEGF mRNA and HIF-1 α mRNA expression levels in each group..

Groups	The number of mice	MVD		VEGF mRNA		HIF-1 α mRNA	
		Mean \pm SD	p-value	Mean \pm SD	p-value	Mean \pm SD	p-value
Control group	5	7.16 \pm 0.65	<0.001	0.287 \pm 0.050	<0.001	0.185 \pm 0.040	<0.001
Model group	5	11.61 \pm 1.38		0.396 \pm 0.070		0.302 \pm 0.060	
Berberine group	5	16.05 \pm 1.72		0.601 \pm 0.080		0.447 \pm 0.050	

the brain will show angiogenesis, but angiogenesis ability of the cerebral ischemic site is too small to restore blood supply to the cerebral ischemic site.^{12,13} In the clinics, angiogenesis at cerebral ischemic sites can be stimulated by external factors, so as to effectively restore the blood supply to the cerebral ischemic site. Therefore, promotion of angiogenesis in cerebral ischemic site is a hot research topic in recent years in the field of cerebral ischemia treatment.^{14,15}

The process of angiogenesis is mainly composed of proliferation, migration, adhesion, and cavity-like structure formation of vascular endothelial cells.¹⁶ The above processes of the vascular endothelial cells in the body are done under the action of various active factors. VEGF is one of the strongest angiogenic promoters yet found. After their combination, VEGF and VEGF receptors stimulate the differentiation and maturation of vascular endothelial precursors and promote the migration and adhesion of vascular endothelial cells.¹⁷ The expression of VEGF gene in the body is related to the change of the microenvironment in which the expression of VEGF gene is increased when ischemia and hypoxia appear in the microenvironment.¹⁸ HIF-1 α is a key core transcription factor that regulates the body's oxygen homeostasis and is present in a variety of cells in the body. HIF-1 α , an active nuclear protein transcribed under hypoxic condition of the body, is closely related to inflammation, vasorelaxation, angiogenesis, and hematopoiesis.¹⁹ The promoter region of VEGF gene has a binding site for binding of HIF-1 α . HIF-1 α binds to the promoter of VEGF gene under the condition of ischemia and hypoxia and promotes the expression of VEGF gene.

Berberine, also known as berberinum, is a main active ingredient of the traditional Chinese medicine coptis and has long been shown to have anti-bacterial and anti-inflammatory effects for the treatment of gastrointestinal diseases such as diarrhoea.²⁰ In recent years, more studies have shown berberine effects in neuroprotection, anti-tumor, anti-atherosclerosis, lower blood glucose and lipid metabolism regulation for treatment of a variety of diseases.²¹ Moreover, berberine can penetrate the blood-brain barrier and act directly on nerve cells, and thus has drawn more and more attentions in protection of cerebral ischemia.²²

The results showed that after administration, neurological score of rats with cerebral ischemia-reperfusion injury in berberine group was lower than that of the model group, indicating that berberine can effectively restore neurological function of rats with cerebral ischemia-reperfusion injury. After administration, MVD in cerebral ischemia-reperfusion cortex of berberine group rats was higher than that of model group, indicating that berberine can promote angiogenesis; After administration, expression levels of VEGF mRNA and HIF-1 α mRNA,

VEGF and HIF-1 α protein in cerebral ischemia-reperfusion cortex of berberine group were higher than those in model group, indicating that berberine can up-regulate HIF-1 α , promote VEGF expression, and then play a protective role in cerebral ischemia-reperfusion injury.

CONCLUSION

Based on the role of VEGF and HIF-1 α in promoting angiogenesis and the important regulatory effect of HIF-1 α on VEGF expression, we speculated that berberine could enhance VEGF expression, angiogenesis and nerve cell recovery of rat with cerebral ischemia-reperfusion injury by up-regulating HIF-1 α .

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