Pakistan J. Zool., vol. 54(5), pp 2253-2258, 2022 DOI: https://dx.doi.org/10.17582/journal.pjz/20201028131014

Effect of Intravenous Thrombolysis on the Early Treatment Time Window and Blood-Brain Barrier Marker Protein Expression

Zhongxi Zhang[1], Ping Zou[2*], Jingfang Zhang[1], Yujuan Zeng[1] and Yujie Tang[1]

[1]Department of Internal Medicine, Clinical College, Changsha Medical University, Changsha, 410219, China
[2]Department of Traditional Chinese Medicine, The First Affiliated Hospital of Changsha Medical University, Changsha, 410219, China

ABSTRACT

The objective of this study was to explore the effects of intravenous thrombolysis on the early treatment time window and blood-brain barrier marker protein expression in rats with acute basilar artery occlusion. Sprague Dawley rats (n=180) were randomly divided into 6 groups, 30 in each group, which were sham operation group, model group, and intravenous thrombolysis for 0, 2, 4, and 6h groups. The rats were scored by Zea-Longa method. The effects of intravenous thrombolysis on brain injury and blood-brain barrier were detected by TTC staining. The expressions of ICAM-1 and MMP9 mRNA were detected by RT-PCR. The expression levels of ICAM-1 protein and MMP9 protein were detected by immunohistochemistry and Western blot. We found that compared with the model group, intravenous thrombolysis could significantly improve the behavioral disorder of rats, and significantly reduce the infarct size of rat brain tissue, and the expression levels of ICAM-1 protein and MMP9 protein. We conclude that intravenous thrombolysis could significantly improve the therapeutic effect of acute basilar artery occlusion in rats within 2-4 h, and the treatment effect was the best at 4h.

Article Information
Received 28 October 2020
Revised 15 November 2020
Accepted 04 December 2020
Available online 01 December 2021 (early access)
Published 10 June 2022

Authors' Contribution
ZXZ and PZ grouped the rats. JFZ and YJZ conducted the experiments. YJT tested the protein expression data. All authors conducted the experiments, analysed the results and wrote the manuscript.

Key words
Intravenous thrombolysis, Basilar artery occlusion, Blood-brain barrier, ICAM-1, MMP9

INTRODUCTION

Acute basilar artery occlusion (BAO) is an ischemic stroke disease, which can lead to quadriplegia, bulbar palsy, coma, etc. In case of poor treatment, the mortality rate can reach 90% (Khilchuk *et al*., 2018). With the rapid development of medical technology, all kinds of diagnosis and treatment technologies have developed rapidly, but the disability and mortality rate are still as high as 80%. Therefore, BAO is one of the major diseases that endanger human health in the world. Studies have shown that the main cause of BAO is local thrombus or plaque formed by atherosclerosis, which leads to arterial occlusion (Huo *et al*., 2016). At present, the main thrombolytic therapy methods are arterial thrombolysis, intravenous thrombolysis and combined intravenous and arterial thrombolysis, among which intravenous thrombolysis is the more effective. Nevertheless, it is found that it has little effect on patients with severe occlusion of the main middle cerebral artery and internal carotid artery, which is mainly manifested by low vascular recanalization rate. The NINDS experiment shows that when the time window of thrombolytic therapy is within 3h, the effect of thrombolytic therapy with rt-PA at the dose of 0.9mg/kg is the best (Gerber *et al*., 2017). It has been found that brain injury can reach more than 70% after cerebral ischemia and reperfusion (Hu *et al*., 2017). It is known that IP ischemic penumbra is an early symptom of ischemic brain tissue, which can be used as the main marker for early diagnosis of the body (Huan *et al*., 2018). Treatment time window refers to a certain time range after the onset of cerebral ischemia, during which the severity of cerebral ischemia injury can be reduced and the recovery of organism function can be promoted after diagnosis and treatment (Dorado *et al*., 2017). At present, however, the specific action mechanism of intravenous thrombosis in treating acute BAO is still unclear. Therefore, this article mainly studied the early therapeutic time window and blood-brain barrier marker protein expression changes in treatment of acute basilar artery occlusion rates by invasive thrombosis.

MATERIALS AND METHODS

Experimental animal

Sprague Dawley healthy rats (n=180) weighing 220-

* Corresponding author: zouping654321@126.com
0030-9923/2022/0005-2253 $ 9.00/0

280g, were purchased from Animal Experimental Center of Nanjing Health Department. Rats were exposed to light cycle from 6: 00 to 18: 00 every day for 12 h. Their indoor temperature was set at 24±2°C and relative humidity was set at 58±1%. During the experiment, rats in each group were fed *ad libium*. Rats were randomly divided into 6 groups, each of 30: Sham operation group, model group, intravenous thrombolysis for 0, 2, 4, and 6h groups.

Establishment of acute BAO rats model

According to Busch's method, after anesthetizing rats, the external carotid artery was ligated, and the ligature was inserted into the bifurcation of internal and external carotid arteries. After fixation for 4 h, the common artery was clamped, and PBS was used to promote embolus pushing; while for the sham operation group, the same amount of PBS was pushed in for 30 seconds.

Method for intravenous thrombolysis

After 0, 2, 4 and 6 h of the emboli were pushed in, by inserting and fixing the puncture needle, 5000U/kg urokinase was injected from the external carotid artery for about 20 min.

Neurobehavioral score

After successful modeling, rats were scored with reference to Zea-Longa method (Talia *et al*., 2017), with: 0 score for normal walking; 1 point for the rat's right forelimb is bent and cannot be straightened normally; 2 points for the rat offsets to the left when walking; 3 points for the rat dumps to the left; 4 points for the rat is unable to walk, and is unconscious. The data were recorded and analyzed according to the above indicators.

Determination of cerebral infarct size

After successful modeling of rats, 12% chloral hydrate was injected into rats from abdominal cavity, and rats were quickly decapitated when they were in clouding of consciousness. The rat brain tissue was quickly frozen at -80°C, sliced, stained in 1% TTC, at 37°C for 30 min. After that, the tissue was taken out and the cerebral infarct size was calculated.

Test of blood-brain permeability of rats

A certain amount of samples was weighed, and put into formamide solution (1mL/100mg). After 24 h of water bath at 60 °C, the samples were centrifuged for 3min at 600r/min, and the supernatant was taken for centrifugation twice. 0.2mL supernatant was sucked and added to the 96-well plate, and its absorbance value was detected at 630nm, in which the blank solution was formamide, and the Evans blue content (μg/mg) was calculated from the standard curve.

Immunohistochemistry

Six rats in each group were injected with 12% chloral hydrate from abdominal cavity. When they were in clouding of consciousness, they were decapitated and their brains were taken out quickly. The primary antibodies were ICAM-1 monoclonal antibody (1: 1200) and MMP9 monoclonal antibody (1: 1200), incubated at 4°C overnight. After that, they were incubated for 30min according to the instructions of second antibodies, and finally developed with DAB. Picture processing of samples was carried out with high power microscope, and images were analyzed with Image pro-plus.

RT-PCR

12% chloral hydrate was injected into rats from abdominal cavity. When the rats were in clouding of consciousness, they were decapitated quickly and put into liquid nitrogen for freeze-drying. A small amount was ground into powder. After RNA extraction and reverse transcription according to the instructions of RNA extraction and cDNA synthesis, the mRNA expression of ICAM-1 and MMP9 was detected by RT-PCR. The primer sequences are shown in Table I.

Table I. Design results of ICAM-1 and MMP9 primers.

Gene	Primer sequence (5′→ 3′)	Product length
GAPDH	F:GCAGTGGCAAAGTGGAGATTC	147
	R:CGCAGGATACTTTGCTGACTGC	
ICAM-1	FCGATTGACCTCAGCGCTGTGCT	163
	R:GTCAAGTGACAAGCCTGTACGT	
MMP9	F:CAGGTCTCACAGCGCATCCTCGG	143
	R:GTGGCCCATACTTTAGGCCGATC	
	R:ACGGCTTATTGCAGCGTTACGGCC	

Western blot

Six rats in each group were injected with 12% chloral hydrate from abdominal cavity. When the rats were in clouding of consciousness, they were decapitated and their brains were taken out quickly. 150mg of brain tissue was weighed from each sample. After protein extraction and concentration determination, it was denatured by boiling water bath for 5min. Three types of proteins were separated by 5×SDS-Page electrophoresis, and then the transfer membrane experiment was carried out by Western blot. The transfer membrane was sealed by 5% skimmed milk powder for one hour, and then incubated overnight

with ICAM-1 antibodyand MMP9 antibody. After that, it was washed with TBST, incubated at room temperature after adding secondary antibody, and then imaged by chemiluminescence after 2h. Its gray value was analyzed by LABWORK software, and GAPDH was used as internal reference to calculate the ratio carefully.

Statistical analysis

All the data in this study were processed by SPSS20.0 statistical analysis software (IBM Company, USA); the measurement data were expressed by mean±standard deviation ($\bar{x}$±s). The comparison between groups was made by one-way analysis of variance or repeated measures analysis of variance. The pairwise comparison between groups was made by LSD-t test; the counting data were expressed by percentage (%), and the comparison between groups was analyzed by $\chi 2$; $P<0.05$ indicated statistically significant difference.

RESULTS

Table II shows results according to Zea-Longa evaluation standard. The score of normal groups is significantly lower than that of model group, proving that rat BAO model was successfully established. The results showed that the behavior of rats began to recover after operation carried out 2h after arterial occlusion. The therapeutic effect was more obvious after 4 h, but gradually decreased after 6 h. Therefore, it can be judged that basilar artery thrombosis carried out 2-4 h after BAO can improve rat's behavior.

It can be seen from Table II that the EB content gradually decreased within 0-4h after intravenous thrombolysis treatment and increased at 6h. It can be seen that intravenous thrombolysis could reduce the damage of blood-brain barrier, and the EB content was significantly decreased after treatment.

Table II shows that the expression of ICAM-1 and MMP9 decreased gradually within 0-4h after intravenous thrombolysis treatment, and the decrease was more significant than that of model group at 4h. At 6h, the expression of ICAM-1 and MMP9 gradually increased, indicating that the expression of ICAM-1 and MMP9 could be decreased within 4h after intravenous thrombolysis.

Besides that mRNA and protein (Fig. 1) expression of ICAM-1 and MMP9 decreased gradually within 0-4 h after intravenous thrombolysis, and the decrease was more significant than that of model group at 4h. At 6h, the mRNA expression of ICAM-1 and MMP9 gradually increased. Therefore, the mRNA expression of ICAM-1 and MMP9 could be decreased within 4h after intravenous thrombolysis.

After successful modeling of rats, 12% chloral hydrate was injected into rats from abdominal cavity. When the rats were in clouding of consciousness, they were quickly decapitated for TTC staining. The results are shown in Figure 1. It can be seen from Figure 2 that the white area of brain tissue in rats of model groupis significant. That is, the cerebral infarct size is larger, which proves that the model is successfully established. Intravenous thrombolysis was performed 0, 2, 4, 6h and 6 h after artery occlusion, and it was found that the cerebral infarct size was gradually reduced. The therapeutic effect at 6 h after operation was lower than that at 4 h, but it was still effectively comparable with model group.

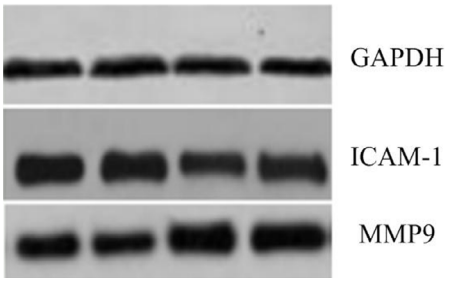

Fig. 1. Results of expression level of ICAM-1 protein and MMP9 protein

Table II. Effect of intravenous thrombolysis on rat behavior, EB content and expression of ICAM-1 and MM9 in rat brain.

Group		Sham operation	Model group	0h	2h	4h	6h	F	P
Score		1.40±0.21	1.70±0.72	1.65±0.23	1.60±0.72	1.46±0.31	1.63±0.56	13.247	0.001
EB connotation		4.16±0.16	10.38±1.32	6.32±0.74	5.26±1.13	4.21±0.75	5.89±1.26	26.127	0.001
Immunohisto-chemistry	ICAM-1	0.21±0.01	0.67±0.09	0.63±0.14	0.56±0.34	0.38±0.01	0.45±0.34	14.125	0.001
	MMP9	0.15±0.02	1.36±0.07	1.0±0.01	0.87±0.03	0.34±0.01	0.64±0.02	11.134	0.001
mRNA expres-sion levels	ICAM-1	0.52±0.01	1.00±0.07	0.84±0.03	0.76±0.13	0.51±0.14	0.59±0.01	13.124	0.001
	MMP9	0.67±0.05	1.00±0.03	0.87±0.01	0.79±0.03	0.46±0.04	0.62±0.07	19.132	0.001

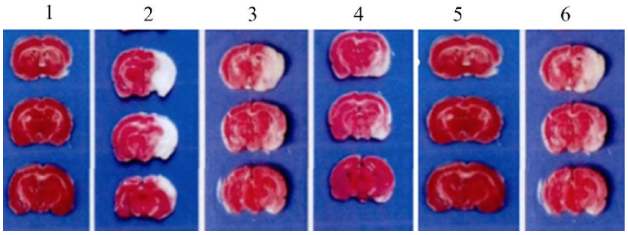

Fig. 2. Effect of intravenous thrombolysis on TTC staining; 1 is sham operation group; 2 is model group; 3, 4, 5, and 6 are respectively intravenous thrombolysis for 0, 2, 4, and 6h groups

DISCUSSION

In this study, we treated rats with acute artery occlusion by intravenous thrombolysis. Compared with model group, intravenous thrombolysis could significantly improve the behavioral disorder of rats and reduce the infarct size of rat brain tissue. Compared with model group, immunoblotting, Rt-PCR and Western blot showed that intravenous thrombolysis could reduce the expression level of ICAM-1 and MMP9. If effective therapeutic drugs are given early, it can improve IP, raise rCBF to the threshold of protein synthesis inhibition and electrical activity termination, and then reduce the infarct size. As cerebral ischemia can cause local expression of some cytokines, such as IL-1β, TNF-α and ICAM-1, endothelial cells and leukocytes promote the up-regulation of the expression of various leukocyte adhesion factors, which eventually leads to the adhesion of these leukocytes on the vascular wall. This results in the destruction and injury of vascular endothelium in ischemic sites, causing reperfusion injury and intracranial hemorrhage (Dorado *et al*., 2017).

ICAM-1 is an adhesion molecule in immunoglobulin family (Berrouschot *et al*., 2016). In animal experiments, when the body was in cerebral ischemia, it was found that ICAM-1 began to express two h after ischemia and one h after reperfusion. Meanwhile, it was reported that leukocyte deposition and up-regulation of ICAM-1 expression after cerebral infarction showed a significant correlation with hemorrhagic transformation after intravenous thrombolysis (Mak *et al*., 2016). Therefore, ICAM-1 expression and leukocyte infiltration may be related to the time window of thrombolytic therapy for acute BAO. MMPs is a group of proteases used to degrade extracellular matrix proteins, generally existing in the form of zymogen. Although it has generally has extremely low expression level, its expression will increase significantly in some physiological diseases, and it will then participate in tissue repair. When gene knockout or exogenous inhibition is given, it can alleviate reperfusion and ischemia injury, and alleviate the secondary vascular brain edema during cerebral hemorrhage (Wajima *et al*., 2017). It is found that the expression of MMPs increased after ischemia-reperfusion, and the permeability of BBB and MMP-9 is most closely damaged (Peña *et al*., 2017; Talia *et al*., 2017).

In recent years, the incidence of acute artery occlusion has gradually increased, and its mortality remains high. It has become one of the major diseases endangering human health (Shukla *et al*., 2017). Acute arteryocclusion can lead to quadriplegia, bulbar palsy, coma, etc. In case of poor treatment, the mortality rate can reach 90% (Huang *et al*., 2017). With the rapid development of medical technology, all kinds of diagnosis and treatment technologies have developed rapidly, but the disability and mortality rate are still as high as 80% (Wang *et al*., 2018). Therefore, the therapeutic study of acute artery occlusion has become the main focus of cardiovascular disease research. Blood-brain barrier plays an important role in the study of ischemic diseases. It has been found that there are many mechanisms to destroy blood-brain barrier, and many experimental and clinical studies have shown that blood-brain barrier injury is closely related to acute artery occlusion (Sun *et al*., 2017). MMPs is a group of proteases used to degrade extracellular matrix proteins, generally existing in the form of zymogen. Although it has generally has extremely low expression level, its expression will increase significantly in some physiological diseases, and it will then participates in tissue repair. When gene knockout or exogenous inhibition is given, it can alleviate reperfusion and ischemia injury, and alleviate the secondary vascular brain edema during cerebral hemorrhage (Lindberg, 2011). It is found that the expression of MMPs increased after ischemia-reperfusion, and the permeability of blood-brain barrier and MMP9 was most closely damaged. It is also found that ulinastatin can reduce rats blood-brain barrier injury and improve rats brain injury by reducing the activity of MMP9, so as to protect brain tissue (20). In recent years, studies have shown that the cause of brain injury is related to inflammatory reaction. ICAM-1 is an adhesion molecule in immunoglobulin family that mediates the adhesion between endothelial cells and leukocytes and promotes the penetration of leukocytes in blood vessels. When the body is in cerebral ischemia, animal experiments show that ICAM-1 can begin to express after reperfusion for 1h, 2h after ischemia. It is found that leech injection can reduce the expression of ICAM-1 protein, improve the focal ischemia-reperfusion effect of rats, and resist the inflammatory effect caused by cerebral ischemia (Jia *et al*., 2020). At the same time, it has been reported that leukocyte deposition and ICAM-1 expression up-regulation after

cerebral infarction are significantly correlated with hemorrhage transformation after intravenous thrombosis. Therefore, ICAM-1 expression and leukocyte infiltration may be related to the time window of thrombolytic therapy for acute BAO (Chao *et al*., 2020).

To sum up, intravenous thrombolysis could have therapeutic effect on rats with acute BAO within 2-4 h, and the therapeutic effect was the best at 4h. At the same time, it could significantly reduce the expression level of ICAM-1 protein and MMP9 protein.

ACKNOWLEDGEMENTS

The research project has been supported by Changsha Medical University Clinical Medicine College Students Science and Technology Innovation and Entrepreneurship Training Base, Xiangke University (2019) No. 8, project number: 2019RS5005 and Research and Practice on the Construction of Simulated Hospitals under the Internet + Background, Xiang Jiaotong (2019) No. 291.

Statement of conflicts of interests

The authors have declared no conflict of interest.

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