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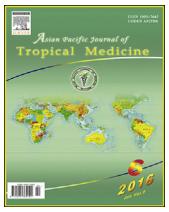

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Expression and significance of angiostatin, vascular endothelial growth factor and matrix metalloproteinase-9 in brain tissue of diabetic rats with ischemia reperfusion

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ABSTRACT

Objective: To discuss the expression and significance of angiostatin, vascular endothelial growth factor and matrix metalloproteinase-9 in the brain tissue of diabetic rats with ischemia reperfusion.

Methods: A total of 60 male Wistar rats were randomly divided into the normal group, sham group, diabetic cerebral infarction group and single cerebral infarction group according to the random number table, with 15 rats in each group. The high sucrose diet and intraperitoneal injection of streptozotocin were performed for the modeling of diabetic rats, while the thread-occlusion method was employed to build the model of cerebral ischemia reperfusion. The immunohistochemical staining was performed to detect the expression of angiostatin, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) in the brain tissue.

Results: The expression of angiostatin after the reperfusion in the brain tissue of rats in the single cerebral infarction group and diabetic cerebral infarction group was increased 6 h after the reperfusion, reached to the peak on 1 d and then decreased gradually. The expression of angiostatin in the diabetic cerebral infarction group 6 h, 1 d, 3 d and 7 d after the reperfusion was significantly higher than that in the single cerebral infarction group ($P < 0.05$). VEGF began to be increased 1 h after the reperfusion in the single cerebral infarction group and diabetic cerebral infarction group, reached to the peak at 6 h and then decreased gradually. The expression of VEGF in the diabetic cerebral infarction group at each time point after the reperfusion was significantly lower than that in the single cerebral infarction group ($P < 0.05$). MMP-9 began to be increased 1 h after the reperfusion in the single cerebral infarction group and diabetic cerebral infarction group, reached to the peak on 1 d and then decreased gradually. The expression of MMP-9 in the diabetic cerebral infarction group at each time point after the reperfusion was significantly higher than that in the single cerebral infarction group ($P < 0.05$).

Conclusions: The high glucose environment in which the diabetic cerebral infarction is occurred is to induce the formation of MMP-9 at first and then activate and increase the expression of angiostatin. Afterwards, the expression of VEGF is inhibited, resulting in the poor angiogenesis after cerebral infarction, which thus makes the injury of brain tissue after cerebral infarction even worse than the non-diabetes mellitus.

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1. Introduction

The impairment of cerebral blood supply can cause the ischemia and hypoxia of brain tissue and then result in the cerebral infarction. The focus of cerebral infarction mainly consists of the ischemic penumbra and central necrotic area. The central area would induce the apoptosis of brain cells because of ischemia, while the collateral circulation in which the penumbra exists could provide the blood for the focus and thus there would still be a great number of alive neuronal cells [1,2]. If repairing the metabolic function of brain after the injury as soon as possible, the function of some neuronal cells can be recovered. Accordingly, the building of effective collateral circulation would be of critical significance for reversing the injury of neuronal cells and improving the neural function [3]. The prognosis of patients with diabetes mellitus and cerebral infarction is even poorer than that of patients with single cerebral infarction. The animal experiment proved that [4] the combined diabetes mellitus could significantly reduce the collateral circulation of cardiac muscular tissue and focus of cerebral infarction and thus aggravated the tissue injury after the cerebral ischemia. There have been many researches that reported the diabetes mellitus could aggravate the injury of cerebral infarction tissue, but no definite conclusion could be drawn. Therefore, in this study, by building the model of diabetic rats with ischemia reperfusion injury and observing the expression of angiostatin, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) in the focus tissues, it was to discuss the possible pathophysiological mechanism of diabetic rats with ischemia reperfusion injury, in order to provide the new thought for the further clinical treatment and pharmaceutical development. The findings were summarized as follows.

2. Materials and methods

2.1. Materials

A total of 60 male Wistar rats were provided by Laboratory Animal Center of School of Medicine, Shandong University, with the weight of (180–230) g and average weight of (206.3 ± 13.8) g; 60 rats were randomly divided into 4 groups according to the random number table: normal group, sham group, diabetic cerebral infarction group and single cerebral infarction group, with 15 rats in each group. Rats in each group were divided into 5 subgroups according to 1 h, 6 h, 1 d, 3 d and 7 d of ischemia reperfusion, with 3 rats in each subgroup. The feeding conditions were as follows: the temperature of feeding room was maintained at (20–22) °C, the room humidity was 50% and rats were given the diet and water freely, with 12 h of lighting and 12 h of darkness by turns. The experiment was performed 1 week after the feeding. The experimental protocol, operation and animal ethics of this study were reviewed and approved by School of Medicine, Shandong University.

2.2. Methods

2.2.1. Modeling of diabetic rats

Rats in the diabetic cerebral infarction group were given the high sucrose diet for 2 months and then the intraperitoneal injection of streptozotocin (STZ, which was purchased from Sigma). The blood glucose and weight were measured 1 month and 2 months after the injection. The modeling standards were as follows: the weight of rats was (290–360) g and the blood glucose level was (16.7–25.6) mmol/L (not fasting).

2.2.2. Modeling of cerebral ischemia reperfusion

The model of cerebral ischemia reperfusion was built using Zea-Longer thread-occlusion method [5] for rats in the diabetic cerebral infarction group and single cerebral infarction group: rats were placed on the operating table at 25 °C in a supine position. After being fixed, they were given the intraperitoneal injection of 35 mg/100 g 10% chloral hydrate. An incision was done in the middle of neck to separate the external carotid artery, internal carotid artery and common carotid artery. The proximal part of external carotid artery and internal carotid artery were ligated. The eye scissors were used to cut an incision at the distal part of common carotid artery (about 5 mm to the bifurcation) and the nylon thread was inserted, with the depth of about 25 mm. The thread occlusion was then fixed. The skin and muscle were sutured and disinfected layer by layer. After 1.5 h of thread occlusion, the thread was pulled out to realize the ischemia reperfusion. The rectal temperature of rats was maintained at (36.5–37.5) °C during the operation and the right middle cerebral artery was chosen as the embolization artery. The nylon thread was inserted in rats of sham group with the depth of about 16 mm and the left operations were the same as above. The modeling standard of ischemia reperfusion was: when the rats were awakened from the anesthesia, the Longa score [6] was employed to evaluate the neural function of rats, where a score of 0 indicated no neurologic deficit, a score of 1 failure to extend left forepaw fully, a score of 2 circling of forepaws when walking, a score of 3 falling to the left when walking ahead and 4 no spontaneous walk and loss of consciousness. The score of 1–3 indicated the successful modeling.

2.2.3. Sampling

The detection was performed at 5 time points of 1 h, 6 h, 1 d, 3 d and 7 d after the ischemia reperfusion. The head of rats was broken and the brain was collected. After being placed in the liquid nitrogen, they were fixed and dehydrated. Afterwards, they were treated with the common paraffin embedding, with the slice thickness of 4 μm. The immunohistochemical staining was employed to detect the expression of angiostatin, VEGF and MMP-9 in the brain tissue. The slices were deparaffinized using the regular method. After adding 3% H_2O_2, it was placed in the microwave for 10 min to repair the antigen. After 15 min of adding the goat serum blocking solution and 3 h of adding the primary antibody, it was washed with 0.1 mol/LPBS for 3 times, with 5 min each time. Afterwards, the secondary antibody was added for 1.5 h and it was washed with 0.1 mol/LPBS for 3 times, with 5 min each time. After the DAB staining, it was restained with hematoxylin. The primary antibody was replaced by PBS in the negative control, with the remained steps same as above. The antibodies of angiostatin, VEGF and MMP-9 were all purchased from Sigma.

2.3. Outcome evaluation

The angiostatin and VEGF with positive staining were mainly in the cell membrane and cytoplasm, while MMP-9 was

mainly in the cytoplasm. The positive expression appeared to be yellow or yellowish brown. Ten fields were randomly selected for the observation under the microscope at ×400. The number of positive cells in each field was counted and the average of 10 fields was calculated.

2.4. Statistical analysis

The data was treated with SPSS19.0. The measurement data was expressed by mean ± sd. The two-way repeated-measures ANOVA was employed for the comparison among different time points, while SNK-q test for the comparison between groups. $P < 0.05$ indicated the significant difference.

3. Results

3.1. Results of focal staining after cerebral infarction

The sections of brain tissue of rats in the diabetic cerebral infarction group and single cerebral infarction group appeared to be pale in the infarction area. The number of neuronal cells was significantly reduced in the central area of infarction, with the understain in the cytoplasm. The area around the infarction had the cellular degeneration, swelling and understained cytoplasm; some small blood vessels had the obvious dilatation and congestion, with the inflammatory infiltration around. The degree of cerebral infarction for rats in the diabetic cerebral infarction group was even worse than that in the single cerebral infarction group.

3.2. Evaluation results of neural function for rats in each group

Rats in the diabetic cerebral infarction group and single cerebral infarction group had the Homer syndromes such as the droopy right eyeball and blepharophimosis when they awakened after the anesthesia, with the bending of left forelimb, falling to the left or circling to the left when walking; the score of neural function was 1–3; rats in the sham group also had the Homer syndromes such as the droopy right eyeball and blepharophimosis, but no other defected neural function; rats in the normal group had the normal neural function and behavior.

3.3. Expression of angiostatin in brain tissue of rats in each group

The expression of angiostatin in the brain tissue of rats in the normal group was limited in the nervous plexus; while the expression of angiostatin in the sham group was significantly higher than that in the normal group ($F = 14.035$, $P < 0.05$). The expression of angiostatin after the reperfusion in the brain tissue of rats in the single cerebral infarction group and diabetic cerebral infarction group was increased 6 h after the reperfusion, reached to the peak on 1 d and then decreased gradually ($F = 7.558$, $P < 0.05$). The expression of angiostatin in the diabetic cerebral infarction group 6 h, 1 d, 3 d and 7 d after the reperfusion was significantly higher than that in the single cerebral infarction group ($F = 4.951$, $P < 0.05$), as shown in Table 1.

3.4. Expression of VEGF in brain tissue of rats in each group

The expression of VEGF for rats in the normal group and sham group was limited in the in the brain tissue, which was mainly in the nervous plexus; while the expression of angiostatin in the sham group was significantly higher than that in the normal group ($F = 29.406$, $P < 0.05$); VEGF began to be increased 1 h after the reperfusion in the single cerebral infarction group and diabetic cerebral infarction group, reached to the peak at 6 h and then decreased gradually ($F = 14.338$, $P = 0.000$). The expression of VEGF in the diabetic cerebral infarction group at each time point after the reperfusion was significantly lower than that in the single cerebral infarction group ($F = 8.095$, $P < 0.05$), as shown in Table 2.

Table 1

Expression of angiostatin in brain tissue of rats in each group (Mean ± SD).

Group	1 h	6 h	1 d	3 d	7 d
Normal group	0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
Sham group	0.91 ± 0.16	0.93 ± 0.17[a]	0.92 ± 0.21[a]	0.94 ± 0.19[a]	0.95 ± 0.20[a]
Single cerebral infarction group	0.94 ± 0.17	1.02 ± 0.18[ab]	1.27 ± 0.19[ab]	1.12 ± 0.16[ab]	1.03 ± 0.19[ab]
Diabetic cerebral infarction group	0.96 ± 0.19	1.14 ± 0.21[abc]	1.38 ± 0.21[abc]	1.18 ± 0.18[abc]	1.17 ± 0.09[abc]

Note: [a] compared with the normal group, $P < 0.05$; [b] compared with the sham group at the same time point, $P < 0.05$; [c] compared with the single cerebral infarction group at the same time point, $P < 0.05$.

Table 2

Expression of VEGF in brain tissue of rats in each group (Mean ± SD).

Group	1 h	6 h	1 d	3 d	7 d
Normal group	0.21 ± 0.07	0.18 ± 0.05	0.19 ± 0.07	0.19 ± 0.06	0.18 ± 0.03
Sham group	1.04 ± 0.09[a]	0.98 ± 0.08[a]	1.03 ± 0.05[a]	1.03 ± 0.07[a]	0.99 ± 0.06[a]
Single cerebral infarction group	3.24 ± 0.13[ab]	15.79 ± 1.05[ab]	14.79 ± 0.79[ab]	9.04 ± 0.21[ab]	6.01 ± 0.17[ab]
Diabetic cerebral infarction group	3.34 ± 0.15[abc]	11.06 ± 0.68[abc]	13.04 ± 0.73[abc]	7.31 ± 0.28[abc]	4.73 ± 0.15[abc]

Note: [a] compared with the normal group, $P < 0.05$; [b] compared with the sham group at the same time point, $P < 0.05$; [c] compared with the single cerebral infarction group at the same time point, $P < 0.05$.

3.5. Expression of MMP-9 in brain tissue of rats in each group

The expression MMP-9 was only found in the hippocampus, vascular endothelium and nervous plexus of brain tissue for rats in the normal group and sham group, with the scattered distribution and limited positive expression; where the expression of MMP-9 in the sham group was significantly higher than that in the normal group (F = 14.035, P < 0.05); MMP-9 began to increase 1 h after the reperfusion in the single cerebral infarction group and diabetic cerebral infarction group, reached to the peak on 1 d and then decreased gradually (F = 7.558, P = 0.000). The expression of MMP-9 in the diabetic cerebral infarction group at each time point after the reperfusion was significantly higher than that in the single cerebral infarction group (F = 4.951, P < 0.05), as shown in Table 3.

Table 3
Expression of MMP-9 in brain tissue of rats in each group (Mean ± SD).

Group	1 h	6 h	1 d	3 d	7 d
Normal group	0.08 ± 0.03	0.09 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.09 ± 0.03
Sham group	0.73 ± 0.12[a]	0.77 ± 0.15[a]	0.69 ± 0.11[a]	0.73 ± 0.11[a]	0.74 ± 0.13[a]
Single cerebral infarction group	2.28 ± 0.08[ab]	8.10 ± 0.49[ab]	36.69 ± 4.95[ab]	20.47 ± 1.59[ab]	13.29 ± 1.32[ab]
Diabetic cerebral infarction group	2.74 ± 0.10[abc]	12.04 ± 0.74[abc]	46.79 ± 5.71[abc]	28.39 ± 2.30[abc]	17.30 ± 1.78[abc]

Note: [a] compared with the normal group, P < 0.05; [b] compared with the sham group at the same time point, P < 0.05; [c] compared with the single cerebral infarction group at the same time point, P < 0.05.

4. Discussion

The cerebral infarction is mainly caused by the necrotic lesion because of the ischemia and hypoxia that is induced by the impairment of cerebral blood supply. For the acute cerebral infarction, its focus mainly consists of the ischemic penumbra and central necrotic area, where the nerve cells has the apoptosis in the central necrotic area and the collateral circulation in which the penumbra exists can provide the blood for the focus and thus there will still be a great number of alive neuronal cells. If the function of blood flow can be recovered, the part of damaged neuronal cells will be reversed, which is also the protection mechanism of ischemia reperfusion. Yang *et al.* [7] ligated the common carotid artery of rats and the results showed that the vessel density around the cerebral infarction was significantly increased after the ischemia. The further study indicated the formation of collateral circulation and new vessels. Such self-protection mechanism might be helpful for the recovery of neural function [8,9]. The angiogenesis of brain tissue is a complicated process. Firstly, the basement membrane began to be decomposed to induce the division of endothelial cells, which would be proliferated into the cytokine. Afterwards, the vascular cavity would be formed and then the new vessels [10,11]. Many regulatory factors are involved in the process of angiogenesis, such as the angiostatin, matrix metalloproteinase-9 and vascular endothelial growth factor. Where, the vascular endothelial growth factor belongs to the positive regulatory factor and the increased expression will be helpful for the angiogenesis; while the increased expression of negative regulatory factor such as the angiostatin will be adverse to the angiogenesis.

Kim *et al.* [12] performed the continuous observation on rat model of cerebral infarction. The results showed that the expression of MMP-9 was continuously increased in the infarction focus within 24 h of infarction, which indicated that the extracellular matrix began to be decomposed to induce the angiogenesis and then provide the blood for the ischemic penumbra to recover part of injured nerve cells. However, the continuously high expression of MMP-9 would inhibit the angiogenesis. The cause of such situation has not been clear yet. But some evidence [13] indicated that the high expression of MMP-9 would inhibit the level of VEGF. Generally, MMP-9 had the negative regulation on VEGF. According to Soejima *et al.* [14], the high glucose environment could activate the activity of MMPs in the artery and stimulate the expression and activation of MMPs to cause the blocked collateral circulation of coronary artery and aggravate the injury of brain tissue in the infarction focus. The results of this study also indicated that the expression of MMP-9 in the single cerebral infarction group and diabetic cerebral infarction group was significantly higher than that in the normal group and sham group, but the expression of MMP-9 in the brain tissue after reperfusion in the diabetic cerebral infarction group was significantly higher than that in the single cerebral infarction group, which further proved the above conclusion. Some clues regarding the relationship between MMP-9 and VEGF might be found from the data: the expression of VEGF and MMP-9 began to be increased 1 h after the reperfusion in the single cerebral infarction group and diabetic cerebral infarction group, where the expression of VEGF reached to the peak at 6 h and the expression of MMP-9 to the peak on 1 d. It indicated that the ischemia stimulation would all induce the increased expression of VEGF and MMP-9. As the time of ischemia prolonged, the changes in VEGF and MMP-9 began to be diversified. The continuously high expression of MMP-9 would have the inhibition against VEGF and thus block the angiogenesis. It meant that 6 h after the ischemia might be the best time for the treatment of cerebral infarction. At this point, the collateral circulation of ischemic penumbra reached to the peak. Afterwards, the angiogenesis was blocked and the collateral circulation of ischemic penumbra was inhibited [15].

There have been limited researches concerning the relationship between the angiostatin and cerebral infarction. The foreign researches [16,17] reported that the degradation products of MMPs, MMP-7 and MMP-9, could dissolve the plasminogen and then activate the angiostatin. It also proved that the angiostatin could inhibit the formation of VEGF and then block the angiogenesis. Takahashi *et al.* [18] reported that the angiostatin could induce the apoptosis of endothelial cells and then confined the angiogenesis in the stage of lumen formation. Besides, the angiostatin could also inhibit ATP synthase and cause the acidosis of cells and then induce the

apoptosis [19–21]. In addition, it could inhibit the activity of P42/P44 MAP kinase, the VEGF signal transduction pathway, and then block the formation process of VEGF. According to the results of this study, the expression of angiostatin after the reperfusion in the brain tissue of rats in the single cerebral infarction group and diabetic cerebral infarction group was increased 6 h after the reperfusion, reached to the peak on 1 d and then decreased gradually. The time of its increase was later than MMP-9, while the peak and decrease time of its expression were the same as MMP-9. Accordingly, it could be presumed that the expression of MMP-9 was increased gradually and it acted on the substrate of plasminogen to activate the angiostatin, which would inhibit the angiogenesis together.

In conclusion, the high glucose environment in which the diabetic cerebral infarction existed would induce the formation of MMP-9 at first, further activate the expression of angiostatin, inhibit the expression of VEGF and then cause the poor angiogenesis after the cerebral infarction and make the tissue injury after cerebral infarction more serious than the diabetes mellitus.

Conflict of interest statement

We declare that we have no conflict of interest.

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