Int J Clin Exp Med 2014;7(9):2632-2638
www.ijcem.com /ISSN:1940-5901/IJCEM0001203

Original Article

Effects of xuelian injection on cerebral TNF-α, IL-1β and MMP-9 in rats experienced focal cerebral ischemia/reperfusion

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Received June 24, 2014; Accepted July 9, 2014; Epub September 15, 2014; Published September 30, 2014

Abstract: Xuelian, as the raw material and also one of the representatives in the ethnodrugs (Uygur drugs) in Xinjiang, playing a role in anti-inflammation, clearing and activating channels and collaterals, improving body immunity, promoting blood circulation and enhancing the metabolism of cells. The aims of present study is to explore the protective effects of Xuelian injection on cerebral ischemia-reperfusion injury. Rat model of cerebral ischemia-reperfusion injury was created by the middle cerebral artery embolization (MCAO). The expressions of tumor necrosis factor alpha (TNF-α), interleukins 1β (IL-1β) and matrix metalloproteinase-9 (MMP-9) were investigated with immunohistochemistry and HE staining at 24 h of reperfusion following 2 h ischemia in the basal ganglia. The infarct volumes were recorded to evaluate the protective effects of high-dose, low-dose and middle-dose Xuelian injection on cerebral ischemia-reperfusion injury. The result indicated the administration of Xuelian injection significantly reduced TNF-α, IL-1β and MMP-9 expression, reduced infarct volume in MCAO rats ($P < 0.01$). The present study provides in vivo evidence that high-dose Xuelian injection protects against cerebral ischemia reperfusion injury. The mechanism is related to the decrease of cerebral levels of TNF-α, IL-1β and MMP-9.

Keywords: Cerebral ischemia and reperfusion, xuelian injection, TNF-α, IL-1β, MMP-9, rats

Introduction

Xuelian injection is pure Chinese medicine prescription injection Tianshan snow lotus as raw material Traditional medicine showed Saussurea has bitter taste with lightly sweet, warm-natured, modern pharmacological studies have shown Tianshan snow lotus flower contains lotus alkaloid, polysaccharide and other ingredients, It is the following functions: Snow lotus alkaloid can reduce the permeability of blood vessels, reduce inflammatory exudation, constrict the blood vessels, improve blood circulation, and help to fight inflammation. The studies in the recent years have found that inflammatory reaction is one of important mechanisms in the Ischemia/Reperfusion injury. All of the immersion of inflammatory cells, formation of cerebral edema, the production of various cell factor and adherence factors as well as the matrix metalloprotease, etc. are closely related to the process of cerebral infarct [1]. Upon cerebral ischemia, all of the star-type cells, microglia as well as endothelial cells could produce cellular factors, such as tumor necrosis factor alpha (TNF-α), interleukins-1β (IL-1β) and activated factors of blood platelets, etc. the cellular factors have a chemotaxis on the leukocytes, promoting the leukocytes and endothelial cells present expressions of adherence factors, and the leukocyte worsens the ischemic cerebral injury via its toxic products, macrophage action and immunological reaction [2-4]. Xuelian injection is a kind of Chinese single injection produced by Xinjiang, Xuelian as the raw material and also one of the representatives in the ethno drugs (Uygur drugs) in Xinjiang, playing a role in anti-inflammation, clearing and activating channels and collaterals, improving body immunity, promoting blood circulation and enhancing the

metabolism of cells. The present study touches upon the expressions of TNF-α, IL-1β and matrix metalloproteinase-9 (MMP-9) in the basal ganglia in the brain tissues in the SD rats after cerebral ischemia/reperfusion and also conducts an research on the protection action of Xuelian injection on the cerebral ischemia/reperfusion injury.

Materials and methods

Drugs and reagents

2, 3, 5 triphenyltetrazolium chloride (TTC) and Evans blue (EB), purchased from Sigma company of America; 2% TTC solution and 2% EB solution prepared by 0.2 mol/L phosphate buffer solution (PPS), which are stored in a dark place; rabbit anti-rat TNF-α and MMP-9 polyclonal antibody (from Wuhan Boshid Biological Project Co., Ltd), SP streptavidin-peroxidase detection reagent box (from Beijing Zhongshan Gold Bridge Biological Technology Co., Ltd and DAB color liquid (products of Fluka). And Xuelian injection, purchased from Xinjiang Xiyu Pharmacy Co., Ltd (Batch No. 08120301, specification: 2 ml/pcs).

Animals and grouping for experiment

40 SD rats, unlimited to the male or female, with the weight between 230 ~ 250 g, were provided by Animals Center of Xinjiang Local Disease Research Experiment Institute (License No. XYXK Xin 2003-003). The animals were divided into five groups: sham operation group: (n = 8); ischemia/reperfusion model group (I/R group, n=8); high-dose Xuelian injection group: 73.2 μl/g (n = 8); medium-dose Xuelian injection group: 26.6 μl/g (n = 8); and low-dose Xuelian injection group: 13.3 μl/g (n = 8). We gave Xuelian injection to the front abdominal compartment before blocking the median sacral artery. After two hours of ischemia, we gave another immediate administration before reperfusion. The sham operation group was given constant volume of 0.9% physiological saline at the same time points as ischemia/reperfusion group.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Experimentation and Ethics Committee of Xinjiang Medical university (Permit Number: 27-2956). All surgery was performed under chlorine hydrate, and all efforts were made to minimize suffering.

Preparation of focal ischemia/reperfusion model of rats

We prepared the middle cerebral artery occlusion (MCAO) of the left brain in reference with the method of Longa [2]. The SD rat was anesthetized with 10% choral hydrate and fixed to ly on the testing table. Make regular public hair disinfection, and then cut the fascia lat open to show the left sternocleidomastoid. After that slip it from between sternocleidomastoid and muscle mass on front neck into depth and then to reach the common carotid artery (CCA), and then the left CCA, the internal carotid artery (ICA) and external carotid artery (ECA) will be floating. Make ligation of ECA and CCA with occlusion at the distal end of heart of ICA by artery clamp and cut a slit rapidly down below the fork between ECA and ICA. At this time, move forward a fishing line with an end burned into globular shape slowly towards the direction ICA into the head through the artery slit of CCA. Take the fork of CCA as a mark, push the fishing line for about 18 mm ~ 20 mm until the feeling of slight resistance, that is, arrival of thinner front artery of the brain. And then block all the blood supply of the front cerebral artery , fasten the stump, fix the fishing line and join the subcutaneous organ and the skin, and then the MACO model comes into being. After two hours of ischemia. Take out the fishing line till the fork between ICA and ECA to proceed and accomplish reperfusion. For the sham operation group, the fishing line would be inserted at the fork of the common carotid artery without occlusion of MCA. During the period of operation, the temperature was controlled strictly within 23°C ~ 25°C.

Marks of neural functional behavior defects

After 24 hours of reperfusion, we made marks to the neural function and behavior of the animals according to the Longa method, with the standards stated as below [2]:

0 point, failure to observe the symptoms of neural functional defects; 1 point, the forepaws on the opposite sides in the operation were unable to be unfolded fully (mildly focal of neural functional defects); 2 points, rotation opposite to the operating direction (moderately focal neural functional defects); 3 points, fall down opposite to the direction of operating (heavy neural functional defects); 4 points, unable to move spontaneously and become unconscious. After 2 hours of MCAO, make marks before the reperfusion and the remaking within 2 ~ 3 points suggested the model making is successful.

Measurement of focal size of cerebral infarction [3]

After 24 h of ischemia/reperfusion, kill the rat and then cut its head fast and put the brain tissue into the refrigerator of -20°C to make it frozen. After that, cut the organ into coronary slices with the thickness of 2 mm. Put the slices into 1% TTC phosphate buffer solution and then into constant incubator at 37°C to hatch and dye for 20 min. The normal brain tissue was dyed into red, while the infarction to be white. And then take the brain tissue out and place it into 4% paraformaldehyde to fix it for 2 hours. The area of various sections was then measured by applying the software of Image pro plus 5.0, and the volume of infarction was calculated as: $V = \Sigma (S_1 + S_2) d/2$. In the formula, V refers to the total volume; S_1 and S_2 indicate the area of head and tail of the slices respectively; d refers to the thickness of slices.

Examination of histopathology

10% choral hydrate was used to deeply anesthetize the rats in each group when the experiment ended. And then the chests of animals were opened and 250 ml icy physiological saline was used to perfuse via the heart. Followed that, 4% paraformaldehyde (4°C, pH 7.4) of 250 ml was applied to perfuse. The animals were perfused first fast and then slowly and then were fixed, the head s would be cut and the brains were taken out after perfusion and fixing and the brain tissue was fastened into paraformaldehyde for 6 ~ 8 h. Take a lump of brain of 1 ~ 4 mm from the rear optic chiasma and embed it with a waxy. The slice of it was in coronary shape with a thickness of 4 μm. The regular dye of HE was conducted then and the histopathological changes were observed under the light microscope and pictures would be taken.

Immunohistochemical detection of TNF-α, IL-1β and MMP-9 proteins

The materials from the rats of each group were taken after 24 h of reperfusion at the same time point from the sham operation groups. 10% choral hydrate was used to anesthetize the animals. And then the chest of animals was opened and 250 ml physiological saline was used to perfuse through the heart. Subsequently from that, 4% paraformaldehyde (4°C, pH 7.4) of 250 ml was applied to perfuse. The head was cut and the brain was taken out after perfusion and fixing and the brain tissue was fastened into paraformaldehyde for 6 ~ 8 h. Take a lump of brain of 1 ~ 4 mm from the rear optic chiasma and embed it with a waxy. The slice of it was in coronary shape with a thickness of 4 μm. The specimen was dewaxed later and the antigens were recovered with high pressure and heat. SP method for immunohistochemistry staining was applied to dye over TNF-α, IL-1β and MMP-9. Negative control was used with PBS instead of the primary antibodies, in which the positive results would be that the cytoplasm was dyed into brown in the expression of coloring of TNF-α, IL-1β and MMP-9. Select five different high magnificence of the cortex surrounding the infarction of the slices and calculate the number of positive cells respectively.

Statistical analysis

Statistic analysis was conducted using the statistic software of SPSS16.0. The calculating data is expressed as $\bar{x} \pm s$. The difference between groups were analyzed with one-way anova, the difference has statistically significant when $p < 0.05$.

Ethical consideration

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Experimentation and Ethics Committee of Xinjiang Medical University (Permit Number: 27-2956). All surgery was performed under chlorine hydrate, and all efforts were made to minimize suffering.

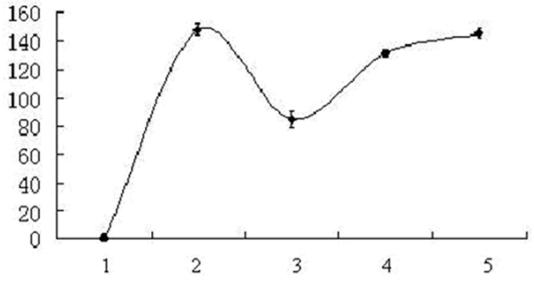

Figure 1. Comparison of cerebral infarct volumes of rats in each group. 1. Sham operation group; 2. I/R group; 3. High-dose group; 4. Medium-dose group; 5. Low-dose group.

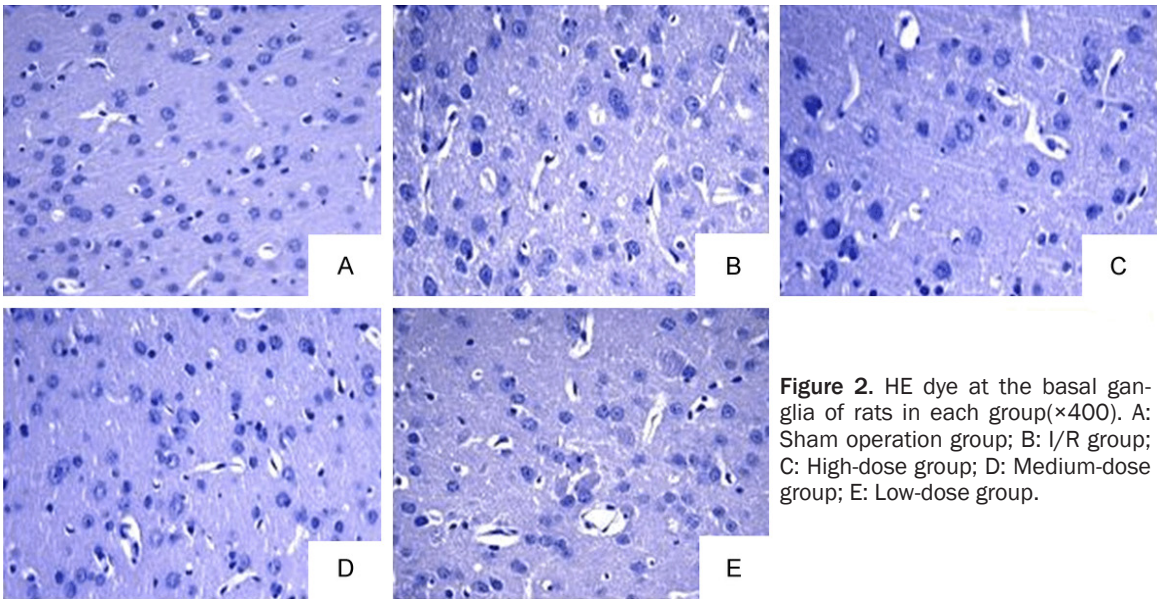

Figure 2. HE dye at the basal ganglia of rats in each group(×400). A: Sham operation group; B: I/R group; C: High-dose group; D: Medium-dose group; E: Low-dose group.

Results

Effects of Xuelian injection on the infarct volumes of SD rats after ischemia 2 h/reperfusion 24 h

The experimental results suggests, through dyeing of TTC, big area of pale infarction occurs to the tissue slice in the I/R group and low-dose group; comparatively small infarction occurs to that in the high-dose group and medium-dose group, while complete dyeing of red comes out in the sham operation. The infarct volumes of the cerebral tissues in the high-dose group and medium-does group shrunk clearly to be (84.75 ± 6.02) mm^3 and (131.25 ± 2.99) mm^3 respectively. Compared with I/R group, the differences for both of them were statistically significant (P = 0.00, P < 0.05); however, the infarct volume in the low-dose group was (145.25 ± 3.59) mm^3, the difference was not statistically significant (P = 0.42, P > 0.05). See **Figure 1**.

Results of histopathology examination

The results of the present study showed that, the neural structures were normal in the basal ganglia region in the sham operation group, the cellular kernels and cytoplasm were dyed clearly and presented chromatography, the distribution of cells possesses specificity and the arrangements were clear and neat. However in the medium-dose group, low-dose group and Ischemia 2 h/Reperfusion 24 h group, the neural cells embodied deep blue, the cytoplasm was red, the arrangement and distribution of cells were irregular with diffusive aggregated distribution and the number of cells was clearly decreasing. The cellular structure was complete. The cellular kernel karyopyknosis displayed in a shape of round, spindle, triangle and polygons. The inflammatory cells immersed inside and the clearance surrounding the neurons and neural colloid cells was widened. The number of neural cells at basal ganglia in the high-dose group decreased, the volume shrunk

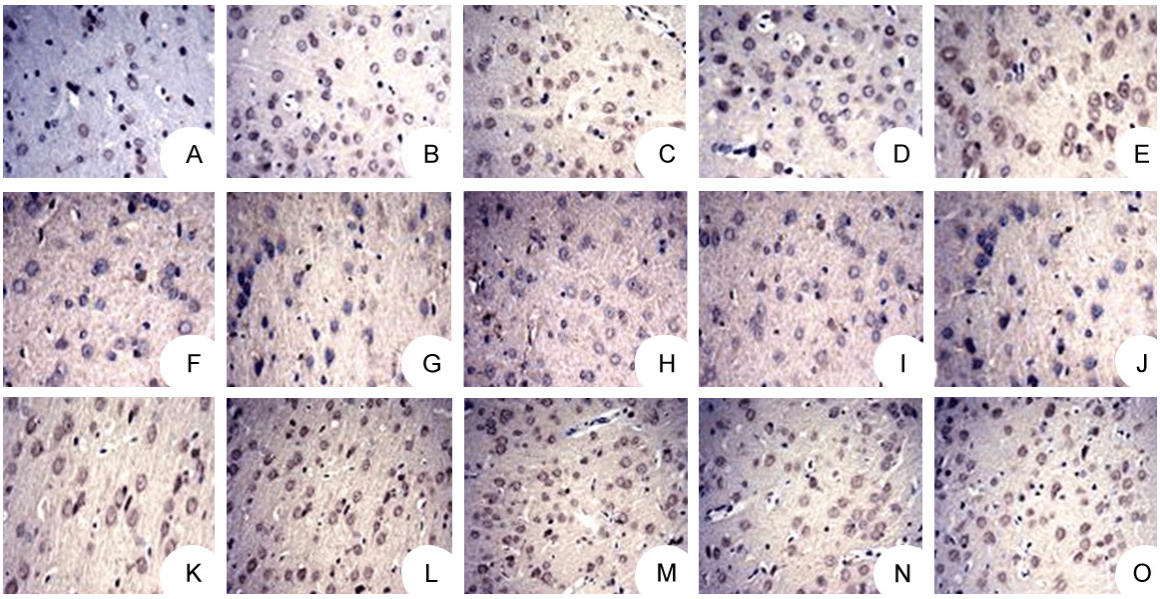

Figure 3. Expressions of TNF-α, IL-1 and MMP-9 at basal ganglia region of brain tissue of SD rats (× 400). TNF-α: A: Sham operation group; B: I/R group; C: High-dose group; D: v; E: High-dose group. IL-1β: F: Sham operation group; G: I/R group; H: High-dose group; I: High-dose group; J: High-dose group. MMP-9: K: Sham operation group; L: I/R group; M: High-dose group; N: High-dose group; O: High-dose group.

Table 1. Expressions of TNF-α, IL-1 and MMP-9 proteins at basal ganglia region of rats in each group (n = 8, pcs/high magnification visual, $\bar{X} \pm s$)

Identification of group	TNF-α	IL-1β	MMP-9
Sham operation group	15.20 ± 4.42$^{\#}$	26.2 ± 2.55	38.20 ± 1.05$^{\#}$
I/R group	46.83 ± 3.52$^{*,\#,\Delta}$	46.6 ± 2.07$^{*,\Delta}$	75.00 ± 1.24$^{*,\Delta}$
High-dose group	25.50 ± 6.34$^{\#}$	31.2 ± 1.92	36.75 ± 2.05$^{\#}$
High-dose group	39.00 ± 5.64$^{\#\Delta}$	54.6 ± 1.17$^{*,\Delta}$	51.00 ± 3.54$^{*,\#,\Delta}$
High-dose group	55.00 ±5.66$^{*,\#,\Delta}$	54.27 ± 2.19$^{*,\Delta}$	68.47 ± 2.14$^{*,\Delta}$

Note: Compared with sham operation group, $^{*}P < 0.05$; Compared with I/R group, $^{\Delta}P < 0.05$; Compared with high-dose group, $^{\Delta}P<0.05$.

and swelling occurred to a few cells and the dye of nucleus was light. Shown as **Figure 2**.

Comparison of expressions of TNF-α, IL-1β and MMP-9 proteins at basal ganglia in the brain tissue of SD rats in each group

At basal ganglia, compared with the sham operation group, the expressions of TNF-α protein in the low-dose group and I/R group increased remarkably and the difference was statistically significant (average P = 0.00, P < 0.01); the expressions of TNF-α proteins in high-dose group and medium-dose group increased but the difference was not statistically significant (P = 0.24 and P = 0.072, P > 0.05). Compared with the sham operation, the expressions of IL-1β proteins in the medium-dose group, the low-dose group and I/R group increased notably, and the difference was statistically significant (average P = 0.00, P < 0.01); but the expression of IL-1β in high-dose group was not seen in clear changes, and the difference was not statistically significant (P = 0.317, P > 0.05). Compared with sham operation group, the expressions of MMP-9 in medium-dose group, low-dose group and I/R group increased remarkably and the difference was statistically significant (average P = 0.00, P < 0.01); but the expression of MMP-9 protein in high-dose group failed to display clear changes and the difference was not statistically significant (P = 0.588, P > 0.05). Some positive cells have the morphological characteristics of the normal neurons, but some other cells display the morphological characteristics after ischemia, such as the reduction of cytoplasm, compact and triangle nucleus or even disappearance of kernels. For the detailed data, see **Figure 3** and **Table 1**.

Discussion

Cerebral Ischemia/Reperfusion is a common clinical histopathological process. To learn about the change law of the infarct volumes after cerebral Ischemia/Reperfusion can provide reference for the treatment of cerebral ischemia. The infarct volume is one of most important objective indexes in evaluating the degree of the local ischemia and ischemic cerebral injury. The present experiment, through the results of the study on the cerebral infarct volumes and histopathology, proves that the changes appeared to the ischemic histopathology and the infarct volumes to different extents of the brain tissue of SD rats in each group, and the high-dose Xuelian injection can effectively protect the cerebral tissue injury after Ischemia/Reperfusion in causing the reduction of number of neural cells at basal ganglia and lessening the volume shrinking and reducing the inflammatory immersion and infarct volumes.

TNF-α is a type of pro-inflammatory factors [7] with multi propertied of promoting inflammation and is also one of the initiation factors of inflammatory reaction. After cerebral ischemia, the expression of TNF-α has a property of neural toxicity and can promote the adherence of leukocytes and endothelial cells [9] and activate macrophage. The neuroglia cells could release the inflammatory media to activate leukocyte and promote the leukocytes move to the cerebral injury place so as to cause the subsequent inflammatory reaction to worsen the cerebral injury. Some people have already the relationship between the expression of TNF-α after temporary focal cerebral ischemia and the blood-brain barrier (BBB) injury: the mice was made MCAO 1 h and then the expression of TNF-α could be seen on the neurons, star cells and ependyma cells at 2 h after reperfusion.6h after reperfusion, BBB began to be injured and at 12 h after reperfusion, the range of injury was clearly increasing. The application of TNF-α antibody can reduce the BBB injury remarkably, which showed that TNF-α is a key inflammatory mediator [10] in the changes of the permeability of BBB in the course of reperfusion. TNF-α could cause the cramp of micro artery, increase of the permeability and release BBB through the direction toxic action on the capillary at early stage. The present study demonstrated that the expression of protein TNF-α increased remarkably, which was consistent with the reports from the most documentation, while the expression of TNF-α in the high-dose group and medium-dose group had no clear increase. The results showed that the medium-dose and high-dose Xuelian injection could stop the starting link of inflammatory cascade reactions through the inhibiting of expression of TNF-α protein. TNF-α can also work with IL-1β to aggravate the range of BBB injury [11]. IL-1β is a key mediator to trigger immune and inflammatory reaction. The temporary cerebral ischemia can induce the expression of IL-1β and IL-1β can cause inflammatory reaction through promoting of adherence of leukocyte with endothelium cells to bring injury to the BBB [12]. The results of the present study demonstrated that the expression of IL-1β protein in the low-dose group and I/R group increased clearly while the expression of IL-1β protein in the high-dose group did not change apparently. The experimental results suggested that the trend of changes in expressions of IL-1β protein and TNF-α protein are consistent, which also conforms to the conclusions in the related documentation reporting that TNF-α can stimulate the synthesis of endothelia cells and express IL-1β. Meantime, the results also proved the high-dose Xuelian injection can further inhibit the expression of IL-1β protein of the cellular factors to participate the cascade reaction along with the suppression of TNF-α.

At the early time of ischemia/reperfusion, the expression of cellular factors and adherence factors followed in a trend to promote the ischemic injury developed into inflammatory injury. Leukocytes gathered, infiltrated and produced a large number of proteolytic enzymes, oxygen free radicals and other effective factors to cause the injury of the endothelial cells and its basement membrane in the cerebral capillary and can induce vasogenic edema and hemorrhage.

Rosenberg, et al. had ever made an experiment injecting the rat brains with gelatinase and found that MMP-9 can destroy the close connection of capillary and basement membrane, so as to cause BBB injury and then bring the tissue injury [13]. Some experiments had ever studied the expression of MMP in the permanent cerebral ischemic animal model and found

that active MMP-9 started to appear 4 h after ischemia and meantime, Evans blue dyeing proved the increase of permeability, indicating MMP-9 plays an active action in the occurrence of vasogenic edema [14].

The results of the experiment suggests, the expressions of MMP-9 protein in the medium-dose group, low-dose group and I/R group increased remarkably but the expression of MMP-9 protein in the high-dose group had no clear changes, which shows that high-dose Xuelian injection plays a comparatively clear in inhibiting of expression of MMP-9 after Ischemia/Reperfusion and then to reduce the permeability of BBB and display a function of protecting nerves.

Conclusion

To summarize, we make an analysis on the results of the experiment: Xuelian injection can effectively inhibit the expression of TNF-α and IL-1β of the brain tissue of rats after ischemia/reperfusion, block the inflammatory cascade reaction and then to further stop the activation of MMP-9, reduce the elevation of expression of MMP-9, bring improvement to the permeability of BBB, lessen cerebral edema and further to reduce the subsequent neural injury. These results provide some certain experimental basis for the clinical application of Xuelian injection on pre-prevention and treatment of ischemic cerebrovascular disease.

Acknowledgements

This project was financially supported by Science and technology supporting Xinjiang Foundation (2013911119) of The Xinjiang Uygur Autonomous Region science and Technology Department.

Disclosure of conflict of interest

The authors declare that they have no competing interests.

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References

[1] Liao SL, Chen WY, Raung SL, Kuo JS, Chen CJ. Association of immune responses and ischemic brain infraction in rat. Neuroreport 2001; 12: 1943-1947.

[2] Jean WC, Spellman SR, Nusshanm ES, Low WC. Reperfusion injury after focal cerebral ischemia: the role of inflammation and the therapeuptic horizon. Neurosurgery 1998; 43: 1382-1397.

[3] Rosenberg GA. Ischemic brain edema. Pro Cardiovasc Dis 1999; 42: 209-216.

[4] Small DL, Morley P, Buchan AM. Biology of ischemic cerebral cell death. Prog Cardiovasc Dis 1999; 42: 185-207.

[5] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20: 84-91.

[6] Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2, 3, 5-Triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 1986; 17: 1304-1308.

[7] Baskaya MK, Doğan A, Rao AM, Dempsey RJ. Neuroprotective effects of citicoline on brain edema and blood-brain barrier breakdown after traumatic brain injury. J Neurosurg 2000; 92: 448-452.

[8] Zaremba J. Contribution of tumor necrosis factor alpha to the pathogenes is of stroke. Folia Morphol (Warsz) 2000; 59: 137-143.

[9] Lavine SD, Hofman FM, Zlokovic BV. Circulating antibody against tumor necrosis factor-alpha protects ra brain from reperfusion injury. J Cereb Blood Flow Metab 1998; 18: 52-58.

[10] Graba TJ. The role of inflammation after acute stroke: utility of pursuing anti-adhesion molecule therapy. Neurology 1998; 5: 62-68.

[11] Gong C, Qin Z, Betz AI, Liu XH, Yang GY. Cellular localization of tumor necrosis factor alpha following focal cerebral ischemia in mice. Brain Res 1998; 801: 1-8.

[12] Arvin B, Neville LF, Barone FC, Feuerstein GZ. The role of inflammation and cytokine in brain injury. Neurosci Biobehav Rev 1996; 20: 445-452.

[13] Rosenberg GA, Dencoff JE, Mcguire PG, Liotta LA, Stetler-Stevenson WG. Injury-induced 92-kilodalton gelatinase and urokinase expression in rat brain. Lab Invest 1994; 71: 417-422.

[14] Gasche Y, Fujimura M, Morita-Fujimura Y, Copin JC, Kawase M, Massengale J, Chan PH. Early appearance of activated matrix metalloproternase after focal cerebral ischemia in mice a possible role in blood-brain barrier dysfunction. J Cereb Blood Flow Metab 1999; 19: 1020-1028.