Jianshen Lishui Decoction Suppresses the Expression of MMP-9 in Rats with Cerebral Hemorrhage

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Abstract: To clarify the effect of Jianshen Lishui decoction on the expression of matrix metalloproteinase-9 (MMP-9) in rats with cerebral hemorrhage.

1. Introduction

Method: The experimental rats were divided into a Blank group, a Sham Operation group, a Model group, and a Jianshen Lishui group. The Blank group was not given any treatment, the Model group received collagenase to induce the hemorrhage rat model, the Sham Operation group received saline instead of collagenase to induce the hemorrhage rat model, and the Jianshen Lishui group received collagenase to induce the hemorrhage rat model and then administered gastric irrigation with the Jianshen Lishui decoction. At the corresponding time points (6 h, 24 h, 3 d, and 7 d), neurological deficit and changes in cerebral water content were assessed in each group. The pathological changes in the brain tissue were assessed using hematoxylin staining and the expression level of MMP-9 was assessed using a Western blot method in each group at each time point.

Results: In the Blank group and Sham Operation group, there were no neurological defects or changes in brain water content. In the other groups, neurological function deficits and the brain water content increased gradually from 6 h. In the Model group and Jianshen Lishui group, the neurological function deficits and the increase in brain water content reached a peak at 3 ($P<0.05$). In the Jianshen Lishui group at 6 h, 24 h, 3 d, and 7 d, the neurological function deficits and the increase in brain water content were lower than that in the Model group ($P<0.05$). 2. In the Blank group and Sham Operation group, there was no rise in MMP-9, and in the other groups, MMP-9 started to increase gradually from 6 h, reaching a peak at 24 h ($P<0.05$) in the Jianshen Lishui group and at 3 d in the Model group ($P<0.05$). In the Jianshen Lishui group at 24 h, 3 d, and 7 d, MMP-9 was significantly lower than that in the Model group ($P<0.05$). 3. In the Model group and the Jianshen Lishui group at 6 h, the brain tissue surrounding the hematoma presented with hyperemia and swelling, and the cells became loose. At 24 h, liquefaction appeared around the bleeding position, cell necrosis was observed, tissue edema was getting worse, and the perivascular space widened, which was most visible at 3 d in the Model group and along with gliocyte proliferation. At 7 d, the edema around the bleeding position gradually reduced, along with gliocyte proliferation, and cell necrosis was relieved. While at 6 h, 24 h, 3 d, and 7 d, the bleeding range was smaller and tissue edema was less in the Jianshen Lishui group compared to the Model group.

2. Materials and Methods

2.1. Animals and experimental materials

2.1.1. Experimental animals

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One hundred and twenty specific-pathogen-free (SPF) 20-month-old female rats were purchased from Changsha Tianqin Biotechnology Co., Ltd., Permit no. SCXK Xiang 2019-001).

2.1.2. Experimental reagents and drugs

The following were purchased: MMP-9 antibody (Abcam, Cambridge, MA; LOT: GR324586-6), Western blotting primary anti-diluent (Biosharp, Hefei, China; LOT: 70015829), collagenase (Sigma-USA, Burbank, CA; LOT: 50190619), internal reference Anti (Biosharp, LOT: 69070015), ECL chemiluminescence kit (Biosharp, LOT: 69070015, BL523A), Sds-page protein loading buffer 5X (Biosharp, LOT: 69078121C), Jianshen Lishui decoction fried-free particles (3 g sanqi powder, 15 g danshen, 15 g poria cocos, 15 g polyporus umbellatus, 10 g alisma, 10 g atractylodes, and 6 g cassia twigs) (Jiangyin Tianjiang Pharmaceutical Co., Ltd., Jiangyin, China).

2.2. Research methods

2.2.1. Cerebral hemorrhage rat model

The rats fasted for 4 h and were denied water for 2 h before surgery. After intraperitoneal anesthesia with 3.5% chloral hydrate (10 ml/kg), according to the Gary method, rats were fixed on the stereoscopic location apparatus so that their anterior fontanelle and the posterior fontanelle were on the same plane[10]. A small hole of about 1-mm diameter was drilled 1 mm behind the anterior fontanelle and 3 mm to the right of anterior fontanelle and a needle was inserted 6 mm with the microsyringe of the stereotaxic instrument which was the position of the right caudate nucleus. Then, collagenase VII (0.5 U) was injected into the rats in less than 10 mins and the needle was held there for 5 mins after the injection, then withdrawn slowly. The successful establishment of the cerebral hemorrhage model was assessed according to the study by Matsushital[11], and involved an ethology assessment of the awakened rats which was scored as follows: 0 points: no symptoms of nerve injury; 1 point: inability to completely extend the contralateral forepaw; 2 points: turn circle to the paralyzed side; 3 points: fall to the opposite; 4 points: inability to walk spontaneously, loss of consciousness. Animals with a functional score of ≥2 points were selected for the experiment and those with a functional score of <2 points were excluded. Then, animals we added to each group to achieve the total number required.

To establish a sham operation model, the same method was used; however, collagenase was replaced by normal saline.

2.2.2. Grouping and modeling

One hundred and twenty adult male SD rats aged 20–22 months with weight of 300 ± 35 g were selected. To group the rates, SPSS 18.0 (IBM Corp., Armonk, NY) was used to generate random sequences twice. For the first sequence, rats were randomly divided into a Blank group (n=20, without any treatment), a Sham Operation group (n=20, injection of equal dose of normal saline), and the Model group (n=80, injection of collagenase to prepare the cerebral hemorrhage model). After the cerebral hemorrhage model was established, the rats were then randomly divided into two groups: A: Model group and B: Jianshen Lishui group, each group with 20 animals.

2.2.3. Intervention methods

After modeling, the Blank group was not given any treatment, the Sham Operation group was given gastric irrigation with normal saline, and the Model group was given normal saline by gastric irrigation. The Jianshen Lishui group was given gastric irrigation of Jianshen Lishui decoction with a density of 380 mg/kg dispensed, twice a day for 7 d.

2.2.4. Sampling and processing

At the corresponding time points of 6 h, 24 h, 3 d, and 7 d, rats were narcotized with 10% chloral hydrate. After fixing the rat and opening its chest, a blunt perfusion needle was inserted into the aorta along the apex of the heart, and the needle was fixed with hemostatic forceps. Normal saline was flushed through the blunt-tipped perfusion needle after the auricle was split and until the fluid was bloodless. Then, the cardiac fixation solution was changed to 4% paraformaldehyde, and during the fixation, the speed of paraformaldehyde solution perfusion was altered from fast to slow until the rat became stiff as a plate. The cranial cavity was opened, the brain was extracted rapidly, and the specimens were prepared for preservation according to the requirements of Western blotting detection.

2.3. Observation outcomes

2.3.1. Neurological deficit scale

Rats were evaluated for neurological deficits at 6 h, 24 h, 3 d, and 7 d after modeling. The reference scoring criteria were as follows: 0 point, normal nerve function; 1 point, mild nerve function defect (left forelimb flexed while tail lifted); 2 points, moderate neurological impairment (turning circle to the left when walking); 3 points, moderate neurological impairment (tilting to the left); 4 points, no spontaneous walking and decreased consciousness; and 5 points, death related to bleeding.

2.3.2. Determination of brain water content

Anesthetized rats in different groups and at different time points, The heads of the rats were cut off, the brain was extracted quickly, a 4-mm piece of brain tissue from the bleeding side was cut with a blade centered on the tip of a needle, and then the wet weight was weighed immediately.

2.3.3. Detection of MMP-9 expression with Western blot

Imprinted buffer liquid (0.5 mL) was placed on extracted coronal brain slices (about 3-mm thick), taken from 4-mm away from the anterior cerebral pole and isolated as left and right cortex as well as left and right basal ganglia, and ultrasonic homogenization was performed. Then the liquid was placed into a 1.5-ml centrifuge tube, an electric tissue grinding rod was used to grind the tissue, protein lysate was added, and after 5 minutes of pyrolysis, the solution was centrifuged for 10 minutes at 4℃ and 1000 rpm, and then the precipitation organization was removed and the supernatant was returned. The protein concentration of each sample was measured and electrophoresis separation was performed using gel with a concentration of 15%, and 50 μg of the protein sample was added to each lane. After the bromophenol blue reached the bottom of the separation adhesive, the membrane was transferred, the PVDF membrane was placed in a 5% skim milk blocking solution at room temperature for 1 h, primary antibody was added, and the solution was incubated at 4℃ overnight. The next day, the PVDF membrane was washed with WB washing solution thrice, then secondary antibodies were added, and the solution was incubated at room temperature for 1 h. Then, 3 mL of chemiluminescence solution (ECL) was added, and then photosensitive film development was performed. The gray value of the protein signal was measured using the Image J image analysis system. β-actin, as the internal reference protein, was used to uniformly process the relative expression of the protein.

2.3.4. Pathological detection with HE coloration

Paraffin sections were prepared from samples fixed with neutral formaldehyde, sections with a general thickness of 3-5 μm were created, and the process continued as follows: 1. The samples were dewaxed twice with xylene, each for 10 mins; 2. The xylene was removed using anhydrous ethanol in two stages, each for 5 mins; 3. Samples were rinsed with 95% and 80% ethanol, each for

10 mins, rinsed with tap water for l min (the tissue was not flushed with running water directly), and then rinsed with distilled water for 1 min; 4. Samples were stained with hematoxylin stain for 4 mins and rinsed with tap water for 2 mins; 5. Samples were differentiated with 1% hydrochloric acid alcohol for 20 s (under microscope) and rinsed with tap water for 2 min; 6. Samples were placed in 1% dilute ammonia return blue for 30 s (under mirror control), rinsed for 2 min with tap water, and 1 min with distilled water; 7. Samples were dyed with eosin for 90s; 8. Samples were dehydrated with 80% ethanol for 10 s, 95% ethanol for 10 s, and anhydrous ethanol for 5 min; 9. Samples were placed in anhydrous ethanol for 10 min; 10. Samples were placed in xylene for 10 mins; 11. Samples were mounting with neutral or Canadian gum, and the histopathological changes were observed under the microscope.

2.4. Statistical method

Statistical software SPSS 18.0 (IBM Corp.) was used for statistical analysis and measurement of data with normal distribution, a t-test was used for comparisons between two groups, and an analysis of variance was used for comparison between three groups. A non-parametric method was used to compare non-normally distributed data, and a difference of $P<0.05$ was considered statistically significant.

3. Results

3.1. Comparison of neurological function in each group

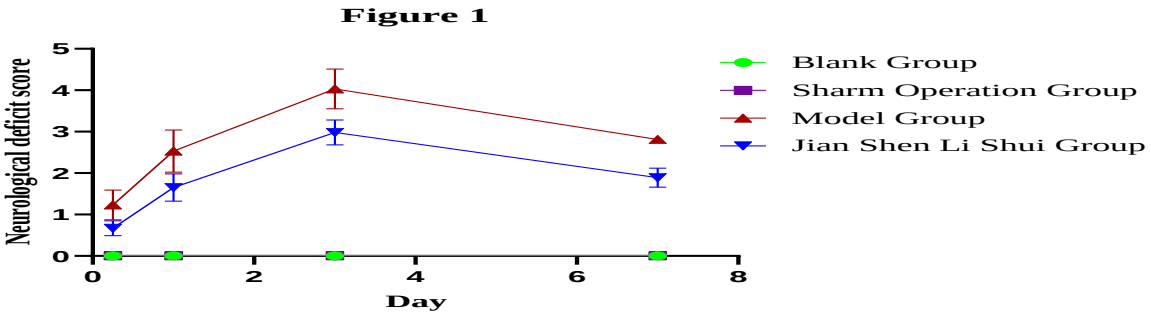

Figure 1 Comparison of neurological function defect scores at various time points of rats in each group（$\bar{x} \pm s$, n=10）

Note: No neurological deficits were found in the Blank group and the Sham Operation group, whilst neurological deficits did appear gradually in the other groups from 6 h. Neurological deficits peaked in the Model group and the Jianshen Lishui group at 3 d ($P<0.05$). Neurological deficits at 6 h, 24 h, 3 d, and 7 d in the Jianshen Lishui group were lower than those in the Model group ($P<0.05$).

3.2. Comparison of brain water content in each group

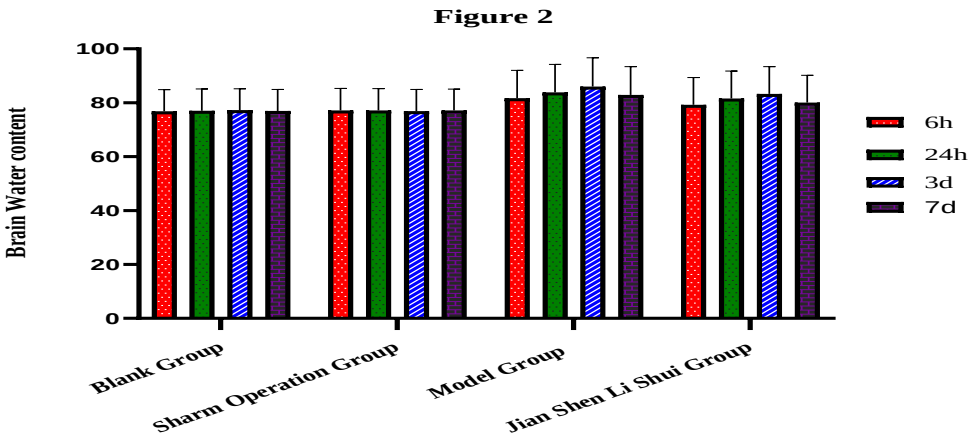

Figure 2 Comparison of brain water content of rats in each group (±, n=10, %)

Note: There was no change in the brain water content in the Blank group and Sham Operation group, while the water content of brain tissue in other groups increased gradually from 6 h. The water content of brain tissue in the Model group and the Jianshen Lishui group reached its peak at 3 d ($P<0.05$). The brain water content in the Jianshen Lishui group at 6 h, 24 h, 3 d, and 7 d was lower than that in the Model group ($P<0.05$).

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3.3. Comparison of MMP-9 expression levels in each group

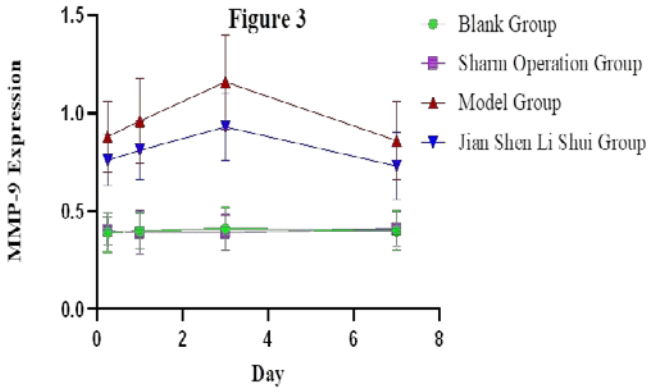
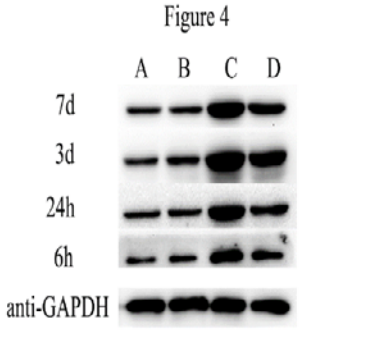


Figure 3 MMP-9 expression of rats at each time point (±, n=10, %)



A:Blank Group B:Sharm Operation Group

C:Model Group D:Jian Shen Li Shui Group

Figure 4 MMP-9 expression of rats at each time point (±, n=10, %)

Note: MMP-9 did not increase in the Blank group and the Sham Operation group, while MMP-9 increased gradually in the other groups from 6 h. At 3 d, the expression of MMP-9 in the Model group and the Jianshen Lishui group reached its peak. MMP-9 expression in the Jianshen Lishui group was lower than that of the Model group at 6 h, 24 h, 3 d, and 7 d ($P<0.05$). Details are shown in Tables 3 and 4.

3.4. Cerebral hemorrhage in each group observed by HE staining

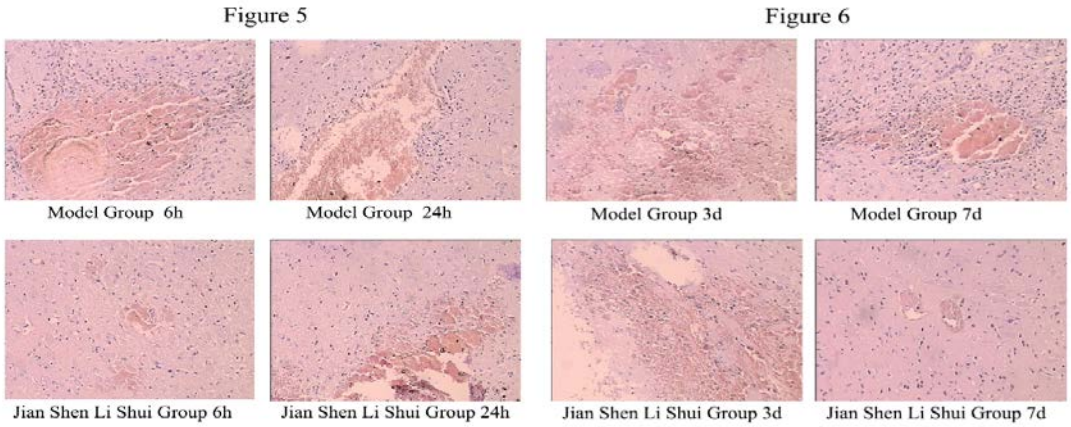


Figure5.6 Cerebral hemorrhage in each group observed by HE staining

Note: In the Model group and the Jianshen Lishui group at 6 h, there was congestion and swelling of the cerebral tissue around the hematoma and the cells became loose. At 24 h, the bleeding site was liquefied, the cells were necrotic, the tissue edema was aggravated, and the perivascular space was widened. The above manifestations were most obvious at 3 d in the Model group, accompanied by glial cell proliferation. At 7 d, the tissue edema around the hemorrhagic lesion was gradually reduced, glial cell proliferation remained, and cell necrosis was alleviated. However, at 6 h, 24 h, 3 d, and 7 d, the range of hemorrhage and tissue edema in the Jianshen Lishui group was smaller than that in the Model group.

4. Discussion

Cerebral hemorrhage is often accompanied by primary hematoma, its prognosis following rehabilitation treatment is often related to the size and location of the hematoma. Furthermore, cerebral hemorrhage is often accompanied by cerebral edema[12]. Cerebral hemorrhage belongs to the category of “stroke” in the field of Traditional Chinese medicine. Two studies published in Frontiers in Pharmacology and Chinese Journal of Integral Medicine, respectively[13,14], showed that traditional Chinese medicine can treat cerebral hemorrhage in a multi-target and multi-channel manner to improve the symptoms of cerebral hemorrhage.

Based on Chinese medicine theory, animal experiments and clinical research conclusions, Professor Liu Tai, a famous Chinese medicine doctor in Guangxi, suggests that the key mechanism of intracerebral hemorrhage is “blood accumulation in the brain,” makes the brain lose the functions of consciousness and movement control, which causes hemiplegia, aphasia, , and even coma due to cerebral apoplexy. The Jianshen Lishui decoction is a classical prescription that includes wuling powder, sanchi, and danshen. Alisma, the sovereign drug of the prescription, with sweet and mild flavor, acts on the kidney and bladder to remove dampness and promote diuresis. Tuckahoe and grifola, as the minister drugs, enhance the efficacy of alisma. Atractylodes, as the adjuvant drugs, improves the efficacy by invigorating the spleen and promoting diuresis. Sanqi unblocks the meridians, and stops bleeding without leaving blood stasis. Danshen nourishes and activates the

blood, improves cerebral circulation, and has the effect of warming yang and qi, promoting diuresis and dissipating stasis. This prescription is suitable for the treatment of cerebral hemorrhage with cerebral edema.

The results of preliminary animal experiments suggest that Jianshen Lishui decoction, in the treatment of cerebral hemorrhage rats, shows efficacy in down-regulating the expression of caveolin-1, protecting the integrity of the BBB, and protecting the nerve function of cerebral hemorrhage rats[8]. In addition, a study published in Oncotarget[6] suggested the positive correlation between caveolin-1 expression and MMP-9 activity and expression. MMP-9 is an important participant in cerebral hemorrhage and cerebral edema, which is closely related to the development of cerebrovascular diseases. Traditional Chinese medicine not only alleviates cerebral hemorrhage and cerebral edema[15], but also reduces the damage to the BBB in animal models[16]. High MMP-9 expression level was detected during these injuries, and the up-regulation of MMP-9 levels are related to the loss of nervous system function, indicating MMP-9 has a harmful effect in terms of brain injury. Traditional Chinese medicine may have strong anti-inflammatory and anti-apoptotic effects on the loss of nerve function by inhibiting the expression of MMP-9[17].

Therefore, this study mainly investigated the factors of cerebral hemorrhage and cerebral edema in rats in terms of the expression level of the relevant protein MMP-9, to observe the protective effect of Jianshen Lishui decoction on brain tissue and brain function in rats with cerebral hemorrhage and cerebral edema. The results showed that in the Blank group and the Sham Operation group, there were no neurological deficits or brain edema, and the relevant protein MMP-9 was up-regulated. From 6 h, the neurological deficit was progressively aggravated in the other groups, accompanied by an increase in brain water content, and MMP-9 was also gradually up-regulated. Compared with the Blank group and the Jianshen Lishui group at 3 d, the Model group and the Jianshen Lishui group reached its peak in nerve function loss, brain water content, and expression of MMP-9, while the above observation indicators of the Jianshen Lishui group were all lower than that of the Model group at 6 h, 24 h, 3 d, and 7 d. The Jianshen Lishui decoction has obvious effects on early acute cerebral hemorrhage with edema, and is beneficial to the overall condition and recovery of neurological deficits in experimental rats.

In conclusion, the preliminarily findings of this study were that the Jianshen Lishui decoction could restore the neurological function of cerebral hemorrhage and improve cerebral edema in a timely manner by down-regulating MMP-9 proteins. However, the detailed molecular mechanisms of the interaction between the Jianshen Lishui decoction and MMP-9, and whether other molecules also indirectly participate in the regulation of MMP-9, remain unclear, and further studies are needed in the future. Recent studies have found that cerebral hemorrhage may be related to susceptibility genes[18], and this provides us with new ideas for future research aimed at guiding the clinical prevention and treatment of cerebral hemorrhage.

5. Conclusion

Jianshen Lishui decoction suppresses the expression of MMP-9 in rats with cerebral hemorrhage, thereby relieving the deficits of neurological function and reducing brain water content. Our findings may provide new ideas for future research aimed at guiding the clinical prevention and treatment of cerebral hemorrhage.

Patients with cerebral hemorrhage have abnormal blood-brain barrier (BBB) function and internal environment disorders with various substances in the blood invading the brain tissue, resulting in edema around the hematoma which increased sustainably within 3 d to 14 d, providing the important pathological basis for disability and death caused by cerebral hemorrhage[1-3]. Therefore, it is particularly important to protect BBB function in the treatment of cerebral hemorrhage. Matrix metalloproteinase-9 (MMP-9) which is found in microglia, endothelial cells, astrocytes, T cells, and neutrophils, causes cerebral hemorrhage and cerebral edema by destroying the basal membrane of cerebral blood vessels and the BBB, causing an inflammatory response in the brain tissue.

After cerebral hemorrhage, MMP-9 is involved in the process of BBB injury and promotes the

formation of vasogenic cerebral edema. Therefore, suppressing the expression of MMP-9 could reduce the degree of BBB injury and brain edema[4,5]. In rats with cerebral hemorrhage, the expression of Caveolin-1 is positively correlated with the expression of MMP-9[6]. Previous studies showed that Jianshen Lishui decoction can reduce the expression of Caveolin-1 in rats with experimental cerebral hemorrhage[7,8], and can relieve the neurological damage and neuronal cell apoptosis in rats with cerebral hemorrhage[9]To study this further, this study aims to use a collagenase-induced cerebral hemorrhage rat model to observe the expression levels of MMP-9 and the influence of Jianshen Lishui decoction on the expression of MMP-9 with a view to elucidating the molecular mechanism of Jianshen Lishui decoction in the treatment of cerebral hemorrhage. This study aims to provide the evidence for the efficacy of traditional Chinese medicine for the treatment of cerebral hemorrhage.

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