Angong Niuhuang Pill Attenuates Blood-Brain Barrier Dysfunction through Inhibiting Lipopolysaccharide Translocation in Intracerebral Hemorrhagic Mice -

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Objective: Intracerebral Hemorrhage (ICH) induces Blood-Brain Barrier (BBB) dysfunction. The purpose of this study was to investigate whether Angong Niuhuang Pill (ANP) attenuates blood-brain barrier dysfunction by inhibiting lipopolysaccharide translocation in ICH mice.

Methods: Male CD-1 mice were randomly divided into sham, ICH, and ANP-treated groups. The mice were subjected to intrastriatal injection of bacterial collagenase or PBS (Sham group). Then they were administrated intragastrically with vehicle (for the sham and ICH group) or ANP (0.1, 0.2, or 0.4 g/kg) respectively.

Results: The results showed that treatment with ANP significantly promoted functional recovery in ICH mice compared with the ICH group (p < 0.05). Brain water content was significantly decreased in the ANP group when compared with that of ICH group (p < 0.05). The intestinal permeability to FITC-dextran in ICH group was higher compared to the Sham group, and the increase of intestinal permeability was abated partially by ANP treatment (p < 0.01). ANP attenuated the pathological changes of the jejunal villus and the ileal villus in ICH mice. In line with above results, ANP inhibited the increase of Lipopolysaccharide (LPS) level in serum (p < 0.05). ANP administration significantly decreased ICH-induced Evans blue extravasation (p < 0.01). In the ICH group, the levels of medullary differentiation factor 88 (MyD88) and matrix metallopeitin-9 (MMP-9) were increased while the expression of claudin-5 were reduced (p < 0.05). However, ANP not only significantly reduced the expression of MyD88 and MMP-9 but also increased the expression of claudin-5 (p < 0.05). ANP treatment had no effect on the Toll-Like Receptor 4 (TLR4) expression.

Conclusion: These findings indicate that ANP attenuates BBB dysfunction by inhibiting lipopolysaccharide translocation in ICH mice.

Keywords: Angong niuhuang pill; Intracerebral hemorrhage; Blood-brain barrier; Lipopolysaccharide

INTRODUCTION

Intracerebral Hemorrhage (ICH) is a stroke subtype resulting from bleeding within the brain parenchyma without trauma. Approximately 10-15% of strokes are ICH and ICH accounts for an even greater percentage of strokes in Asian patients [1]. Brain edema is a critical factor in the subsequent development of neurologic deficits by increasing intracranial pressure or by causing a substantial midline shift. While multiple forms of edema develop after ICH, the primary type is vasogenic cerebral edema resulting from an increase in permeability of Blood-Brain Barrier (BBB) [2].

Thrombin, fibrin and erythrocyte components are involved in BBB disruption and brain injury after ICH [1]. Blood components also induce an inflammatory response in the perihematoma area [3]. There is evidence that these elements of the inflammatory response can alter BBB function in ICH [4]. Significant upregulation of TLR4 expression occurs at approximately 2 to 6 hours after ICH [5-7], accompanied by the appearance of infiltrating neutrophils in the hematoma at 4 h after ICH [8]. TLR4 signaling in the neutrophils further mediate a release of proinflammatory cytokines, which contributes to the detrimental inflammatory response [9,10]. In the Central Nervous System (CNS), microglia and neurons express Toll-Like Receptor 4 (TLR4). TLR4 is stimulated by increases of cytokine and chemokine, which will lead to a sustained CNS inflammation independent of blood-derived leukocytes [11].

Notably, intestinal barrier dysfunction is a common complication after stroke that leads to malabsorption, malnutrition, hypoimmunity, and poor prognosis in patients [12,13]. Increased intestinal permeability caused by stroke lead to the Lipopolysaccharide (LPS) of the intestine translocation into the blood circulation, triggering a systemic inflammatory response and release of various inflammatory mediators [14]. Blood LPS then cross the BBB and exacerbate a neuroinflammation in the brain, in turn worsening the prognosis of patients [15,16]. TLR4 recognizes LPS and produce an inflammatory response [17]. Previous study showed that agent which protects intestinal barrier alleviates ICH-induced neurological impairment [18].

Angong Niuhuang Pill (ANP), a well-known traditional Chinese medicine, has been widely used to treat stroke in China. It contains buffalo horn, calculus bovis, radix curcumae, radix scutellariae, and so on. ANP is considered as an adjunct therapy with conventional medicine for ischemic stroke patients and hemorrhagic stroke patients [19-21]. Recent findings demonstrate that ANP attenuates the infarction sizes, the BBB permeability and neurological deficit in the MCAO rats with 2 h ischemia plus 22 h reperfusion [22]. Meanwhile, clinical studies suggest that ANP ameliorates the neurological impairment in ischemic stroke patients [23,24].

Up to date, almost all the researches hypothesize that ANP can cross the BBB. A good many of studies focus on elucidating the mechanism of action of ANP on the cells in brain. However, we think that not all components of ANP penetrate the BBB. Therefore, whether ANP exhibit a peripheral action and then improve neurological function of ICH mice? This study aims to investigate whether ANP attenuate BBB dysfunction through inhibiting lipopolysaccharide translocation in ICH mice.

MATERIALS AND METHODS

Chemicals and reagents

Angong Niuhuang Pill were purchased from Beijing Tong Ren Tang Chinese Medicine Co. Ltd. (Beijing, China). Ketamine, xylazine, collagenase VII, Fluorescein Isothiocyanate-Dextran (FITC-D), Evans blue, trichloracetic acid were purchased from Sigma-Aldrich (Shanghai, China). LPS ELISA kit were purchased from Cloud-Clone CORP (CCC, USA). Rabbit anti-TLR4, rabbit anti-MyD88, rabbit anti-MMP-9, and rabbit anti-claudin-5 were purchased from Abcam (Cambridge, UK). RIPA lysis buffer, 10% SDS-PAGE, horseradish peroxidase-labeled secondary antibody, ECL kit, β-actin were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Image Quant LAS 4000 were purchased from GE Healthcare Bio-Sciences (Tokyo, Japan).

Drugs

ANP was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na).

Animals

Male CD-1 mice weighing 22 g to 25 g were purchased from Jinan Pengyou Experimental Animal Breeding Co. Ltd. (License number: SCXK (Lu) 20140007). The mice were placed in an appropriate environment at 22-23°C and 60% humidity under a 12 h light/dark
cycle, with freely available water and food. The use of experimental animals and the experimental design complied with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Ethics Committee of Yantai University (approval number, YTU20180124).

**ICH model**

Mice were anesthetized with ketamine-xylazine (80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, intraperitoneal injection). They were fixed in a stereotactic frame after anesthetization, and a hole was drilled into the right side of the head (coordinates 0.2 mm anterior, 3.5 mm ventral, and 2.2 mm lateral to the bregma). Then, 500 nL of phosphate-buffered saline containing 0.075 U collagenase VII was injected using a micro-infusion pump. The needle was then maintained in place for 5 min after injection. The needle was then withdrawn, and the hole was sealed with bone wax and sutured.

**Grouping and treatment**

Mice were randomly assigned to five experimental groups: sham group (Sham), ICH group (ICH), ANP at dose of 0.1 g/kg group (ANP 0.1), ANP at dose of 0.2 g/kg group (ANP 0.2), and ANP at dose of 0.4 g/kg group (ANP 0.4). At 0.5 h after ICH, mice in ANP groups were intragastrically administrated with ANP at dose of 0.1, 0.2, 0.4 g/kg. The mice in the sham and model groups were subjected to 0.5% CMC-Na.

**Evaluation of neurological function**

Garcia test was performed to assess neurological function at 48 h after ICH. Garcia test includes spontaneous activity, side stroking, vibrissae touch, limb symmetry, lateral turning, forelimb walking, and climbing.

**Measurement of brain water content**

After Garcia test, the brains of the anesthetized mice were dissected through the midline. A total of 5 parts from each brain were harvested: the cerebellum, the ipsilateral cortex, the contralateral cortex, the ipsilateral striatum, and the contralateral striatum. The five parts of samples were weighed on an electronic analytical balance (Mettler-Toledo, Shanghai, China). The samples were dried at 100 °C for 24 h before determining the dry weight. Brain water content (%) was calculated as (wet weight - dry weight)/wet weight × 100%.

**Evaluation of FITC-D concentration**

Intestinal mucosal permeability was evaluated by assaying the FITC-D concentration in blood, which reflected the level of intestinal barrier dysfunction. Briefly, food and water were withdrawn for 12 h. Then the mice were gavaged with FITC-D at dose of 300 mg/kg. After 3 h, the serum of animals was collected. FITC-dextran concentrations were determined using a fluorescence spectrophotometer (Molecular Devices, Shanghai, China) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

**Histopathological examination of intestine**

The jejunum and ileum of mice were fixed in 10% formaldehyde and then were sliced into 5μm thick sections with a microtome. Hematoxylin-Eosin staining was performed for the histopathological examination of intestine.

**Evaluation of evans blue concentration**

BBB permeability was assessed with Evans blue method. Evans blue was dissolved in normal saline. The mice were intraperitoneally injected with Evans blue at a volume of 0.01 ml/g. Three hours after, the mice were transcardially perfused with cold PBS. Right cortex and right striatum were harvested and homogenized with 50% trichloroacetic acid. The supernatants were collected and were analyzed at 610 nm by spectrophotometry. Quantitative evaluation of BBB permeability was performed by calculating the amount of Evans Blue deposition per gram tissue.

**Determination of LPS levels in serum**

Serum LPS levels were quantified using the LPS ELISA kit following the manufacturer’s instructions.

**Western blot analysis**

The right cortex and right striatum were lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and centrifuged at 12,000 g for 20 min at 4°C. Total protein (20 μg) was separated on 10% SDS-PAGE. The membranes were incubated overnight at 4°C with the primary antibodies: rabbit anti-TLR4 (1:1000), rabbit anti-MyD88 (1:1000), rabbit anti-MMP-9 (1:1000), and rabbit anti-claudin-5 (1:1000). The membranes were processed with the corresponding horseradish peroxidase-labeled secondary antibody (1:1000). Bands were visualized using the ECL kit and then the bands were quantified using Image Quant LAS 4000. β-actin (1:1000) was served as the loading control.

**Statistical analysis**

All data were analyzed using GraphPad Prism 6.0 and are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Kruskal-Wallis H test or Least Significant Difference (LSD). p < 0.05 were considered significant.

**RESULTS**

**ANP improved neurological function in ICH mice**

Compared to sham group, ICH induced a severe neurobehavioral deficit (p < 0.01). In contrast, treatment with ANP attenuated (p < 0.01) significantly the neurobehavioral function deficit induced by ICH (Figure 1).

**ANP decreased brain edema in ICH mice**

In the ipsilateral basal ganglia, brain water content was significantly increased in the ICH group compared with that of the sham group (p < 0.01). Compared with the ICH group, ANP treatment reduced (p < 0.05) brain water content (Figure 2).

**ANP attenuated intestinal permeability in ICH mice**

Low FITC-D levels was detected in the sham group. ICH caused a significant increase in the level of serum FITC-D compared to the sham group (p < 0.01). However, ANP administration markedly decreased (p < 0.01) FITC-D levels in ICH mice(Figure 3).

**ANP ameliorated the injuries of jejunum and ileum in ICH mice**

In the sham group, the intestinal jejunal and ileal villi were intact and slender, and the epithelial cells were arranged neatly. The jejunal villi and the ileal villi were shed and the epithelial cells were disordered in ICH mice. The pathological changes of the jejunal villi and the ileal villi were attenuated after treatment with ANP (Figure 4 and 5).
ANP decreased LPS levels in ICH mice

ICH led to a significant increase in the level of serum LPS compared to that of the sham group (p < 0.01). Compared with the ICH group, ANP treatment significantly decreased (p < 0.05) the LPS levels in ICH mice (Figure 6).

ANP attenuated BBB permeability in ICH mice

Compared with the sham group, the content of Evans blue in the right hemisphere exhibited a significant increase in the ICH group (p < 0.01). ANP administration significantly decreased (p < 0.01) the Evans blue levels in ICH mice (Figure 7).

ANP has no effect of TLR4 expression in ICH mice

Compared with the sham group, the TLR4 expression in ICH group did not show any change (p > 0.05). Furthermore, treatment with ANP also did not influence (p > 0.05) TLR4 expression in ICH mice (Figure 8).

ANP downregulated MyD88 and MMP-9 expression in ICH mice

MyD88 and MMP-9 expression were significantly increased (p <
0.01) in the ICH group in compared with the sham group. However, treatment with ANP significantly decreased (p < 0.05) MyD88 and MMP-9 expression in ICH mice (Figure 9).

ANP upregulated Claudin-5 expression in ICH mice

The results showed that there was a significant decrease (p < 0.05) in claudin-5 expression in ICH mice. Compared with the ICH group, treatment with ANP significantly increase (p < 0.05) the expression of claudin-5 in ICH mice (Figure 10).

DISCUSSION

In this study, we found that ANP not only improved neurological function of ICH mice but also restored brain water content and Evans blue dyes extravasation. ANP also attenuated injured intestinal mucosa permeability in ICH mice. Furthermore, ANP treatment decreased LPS levels, the expression of MyD88, MMP-9, and enhanced claudin-5 expression.

ICH caused neurological function dysfunction [25] - disruption of BBB integrity and subsequent increased permeability are the major aspects of brain injury after ICH. BBB injury results in the formation of brain edema and swelling, the increased intracranial pressure, and
the shift of the midline, which aggravates the outcomes of ICH [3].

This study suggests that ANP treatment improves neurological function and decreases BBB permeability, then results in a significant reduction of brain edema.

ICH can cause intestinal mucosal barrier damage, and LPS translocates into the blood circulation. These findings indicate that treatment with ANP attenuates the intestine injury and the intestinal mucosa permeability and then decreases LPS levels in blood.

TLR4 plays a key role for innate immune responses and LPS can binds to TLR4 triggering the TLR4/MyD88/MMP-9 signaling pathway. In our study, ANP did not affect the TLR4 levels in ICH mice. MyD88, a critical adapter protein for TLR4, leads to the activation of downstream NF-κB and the subsequent production of pro-inflammatory cytokines [26,27]. Previous studies have shown that NF-κB triggers the production of MMP-9 [28]. MMP-9 is implicated as a pathological mediator during the acute phase of stroke. Putative mechanisms include the proteolysis of neurovascular substrates such as tight junction proteins essential for BBB integrity and components of the extracellular matrix integral to cerebrovascular and neuronal viability [29]. BBB integrity is compromised during CNS infection, increasing permeability and strongly contributing to secondary edema, which directly influences patient prognosis. MMP-9 plays an important role in regulating TJ protein expression [30,31]. Claudin-5 is the essential integral proteins in tight junction, and its levels have been considered as indicators of normal and dysfunctional states of the BBB. Our study showed that ANP regulates the TLR4/MyD88/ MMP-9 signaling pathway and then enhances claudin-5 levels.

There is a limitation in the present study. This experiment did not detect inflammatory factors in the brain. Therefore, whether ANP play a role in regulating the central inflammation in ICH mice should be elucidated in the further study.

In summary, ANP improves the neurological function of ICH mice. ANP attenuates the blood-brain barrier dysfunction by inhibiting lipopolysaccharide translocation in ICH mice. This study suggests ANP attenuate blood-brain barrier dysfunction by inhibiting lipopolysaccharide translocation in ICH mice. Further, ANP regulates the TLR4/MyD88/ MMP-9 signaling pathway and enhances the expression of claudin-5.

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