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Neuroprotective effects of Hemocoagulase Agkistrodon on experimental traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is the major cause of disability and mortality among young people and is associated with neurodegenerative diseases. However, the available clinical options have limited effectiveness. Here, we investigated the neuroprotective effect of Hemocoagulase Agkistrodon (HCA), a thrombin-like enzyme (TLE) isolated and purified from snake venom. Rats subjected to experimental TBI were administered a single dose of HCA or vehicle 10 min after injury. Neurological function was assessed with modified neurological severity score (mNSS). Brain edema were evaluated by measuring brain water content. Levels of hemoglobin and inflammatory cytokines were detected by Enzyme-linked immunosorbent assay (ELISA). In addition, assays including Evans blue extravasation, Western blot analysis and immunofluorescence staining were utilized to determined blood-brain barrier (BBB) integrity. Our results showed that HCA treatment ameliorated neurological deficits ($p < 0.01$), alleviated brain edema ($p < 0.01$) and hemorrhage ($p < 0.01$), decreased the production of the proinflammatory cytokines IL-1β ($p < 0.01$), TNF-α ($p < 0.01$) and IL-6 ($p < 0.05$), and increased the anti-inflammatory cytokine IL-10 at the contusion site ($p < 0.01$). Moreover, HCA administration reduced BBB disruption by regulating expression of tight junction proteins, including ZO-1, occludin and claudin-5 ($ps < 0.01$). Together, our results demonstrate that HCA might have therapeutic efficacy in acute TBI, suggesting a potential clinical application for mitigating the neuropathological damage associated with TBI.

1. Introduction

Traumatic brain injury (TBI) is a result of external mechanical destruction of the brain parenchyma and is the leading cause of mortality and disability among young people worldwide (Faul and Coronado, 2015). The pathological process of TBI is progressive, consisting of primary injury along with sequential secondary damage. Primary injury is characterized by tissue destruction, intracranial hemorrhage and axonal shearing, which result in acute neurological dysfunction, whereas secondary damage following TBI occurs within hours to days and is associated with brain edema, progressive hemorrhagic injury, inflammation and blood-brain barrier (BBB) disruption, triggering the development of neurodegeneration, including chronic traumatic encephalopathy (CTE), Alzheimer's disease and Parkinson's disease (DeKosky and Asken, 2017; Gardner et al., 2018; Julien et al., 2017). The lifelong physical, cognitive, behavioral, and emotional impairments caused by TBI pose a serious threat to global public health.

TBI includes numerous types of insults to the brain, with one of the most severe being progressive hemorrhagic injury, which is attributed to the continuous bleeding of microvessels ruptured by initial mechanical impact (Fair et al., 2019). In addition, during the secondary injury phase, a series of toxic substances released from damaged tissue also induce vascular structural failure, which then coalesces to exacerbate hemorrhagic progression (Kurland et al., 2012). The extravasated blood from the frustrated tissue compresses adjacent healthy tissue, resulting in expansion or progression of the contusion. Moreover, breakdown products of the extravasated blood and the infiltration of blood-derived cells are toxic to the central nervous system (CNS), initiating inflammatory and immune responses that ultimately lead to neurodegeneration.

Snake venom is a natural biological resource consisting of various active proteins and peptides with potential therapeutic value for clinical

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treatments, including blood coagulation (Matsui et al., 2000). In this study, among the ingredients affecting the coagulation process, we focused on the snake venom thrombin-like enzymes (TLEs). Unlike traditional hemostatic drugs, TLEs belong to a class of serine proteases, which act directly on fibrinogen to polymerize fibrin monomers without activating the clotting cascade (Ullah et al., 2018), thus reducing the risk of thrombosis and disseminated intravascular coagulation (DIC) (Mohamed Abd El-Aziz et al., 2019; Seon et al., 2017). Currently, TLEs have been widely used to prevent and stop surgical bleeding (Waheed et al., 2017). More importantly, additional studies have shown that Batroxobin, the snake venom TLE purified from *Bothrops atrox moojeni*, has been used to treat ischemic cerebral infarction (Gusev et al., 2006), implicating the potential application of snake venom TLEs in the treatment of neurological diseases.

Hemocoagulase Agkistrodon (HCA) is a new TLE isolated and purified from *Agkistrodon acutus* venom. In 2009, Hemocoagulase Agkistrodon for Injection was approved to clinically prevent clotting in superficial wound bleeding during surgical procedures in China. A recent Phase III clinical trial demonstrated that HCA have good hemostatic and coagulative functions without affecting hepatic and renal functions (Wei et al., 2010). Considering the progressive hemorrhagic injury is the key step in TBI pathophysiological cascade, and HCA possesses high hemostatic efficiency and favorable safety profile, we deduced that HCA might be beneficial to TBI therapy. In addition, a number of previous studies have shown that snake venom TLEs displayed neuroprotective effects in several CNS diseases (Fan et al., 2013; Li et al., 2016). Therefore, in the present study, we tried to evaluate the neuroprotection of HCA and possible mechanisms in experimental TBI rat model.

2. Materials and methods

2.1. Animals

All animal care and experimental procedures complied with the guidelines of the Animal Management Rule of the Ministry of Health, People's Republic of China (document no. 55, 2001) and were approved by the Laboratory Animal Ethics Committee of the Chinese Academy of Medical Science, Beijing, China. A sum of 356 adult male Sprague-Dawley rats (220−240 g) were obtained from Vital River Laboratories Technology Co., Ltd (Beijing, China. Animals were kept in a humidity-(45–50 %) and temperature-controlled (21−25℃) room on a 12-h light/dark cycle with ad libitum access to a standard rodent diet and water.

2.2. Surgical procedures

Isoflurane-anesthetized rats were intubated and placed in a stereotactic frame. A craniectomy with a 5 mm diameter was performed 1 mm posterior from the bregma and 2 mm lateral to the sagittal suture in the right hemisphere, with a portable drill to remove the bone flap. A modified Feeney's weight-drop injury (WDI) model was used to induce TBI by advancing a 5-mm impacting rod into the exposed parietal cortex and then dropping a 40 g weight from 25 cm height (Ma et al., 2019). HCA or vehicle (Beijing, Konruns Pharmaceutical Co., Ltd.) was administered intravenously 10 min following injury with 0.1 mL/100 g injection volume. After surgery, the animals were housed under the same conditions as described above.

2.3. HCA injection preparation

The excipients in commercial HCA is Dextran 20. For animal study, the commercial HCA was dissolved with water for injection and the vehicle is 0.8 % Dextran 20. The HCA solution for 0.4 U/kg group was diluted 1:2 (V/V) with 0.8 % Dextran 20 to obtain solution for 0.2 U/kg group.

2.4. Group and drugs administration

Rats were randomly divided into four groups: (1) sham group-rats underwent surgical procedures without impact treated with vehicle; (2) model group-rats underwent surgical procedures treated with vehicle; (3) 0.2 U/kg group-rats underwent surgical procedures treated with 0.2 U/kg HCA; and (4) 0.4 U/kg group-rats underwent surgical procedures treated with 0.4 U/kg HCA.

2.5. Neurological function assessments

Neurological outcomes were assessed using the modified neurological severity score (mNSS) as previously described, a multifunctional evaluation scale consisting of motor, sensory, balance and reflex tests (Chen et al., 2008). Neurological function is graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18). A higher score was associated with more severe neurological injury.

2.6. Evans blue extravasation

Evans blue dye extravasation was used to evaluate BBB integrity as previously described (Zhu et al., 2017). At 24 h and 72 h post-TBI, 3 mL/kg Evans blue dye (4% in phosphate-buffered saline (PBS), E2129, Sigma-Aldrich, St. Louis, MO, USA) was injected intravenously and allowed to circulate for 2 h. The rats anesthetized with isoflurane were transcardially perfused with sterile saline. Brains were quickly harvested, imaged and weighed. Then, the Evans blue dye was extracted in 1 mL formamide for 48 h incubation at 45℃. The concentration of Evans blue dye was measured with spectrophotometry at 620 nm and quantified using a standard curve.

2.7. Brain water content

Brain edema was evaluated by measuring the water content in brain tissue according to the wet-dry weight method. Brains were immediately removed, and the injured hemispheres without the brain stem and cerebellum were weighed rapidly to determine the wet weight. After being dried in an oven at 100℃ for 48 h, the brain tissues were weighed again to determine the dry weight. The brain water content was calculated as follows: brain water content (%) = (wet weight − dry weight)/wet weight × 100 % (Huang et al., 2015).

2.8. Western blot analysis

Rats anesthetized with isoflurane were intracardially perfused with 0.1 M PBS. The whole brain was removed and the ipsilateral brain tissues were homogenized in RIPA lysis buffer (50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, and 10 mM EDTA) supplemented with complete EDTA-free protease inhibitor mixtures (4693116001, Roche, Indianapolis, IN, USA). Protein samples were separated on SDS-polyacrylamide gels and transferred electrophoretically to PVDF membranes (ISEQ00010, Millipore Sigma). The membranes were incubated overnight at 4 °C with antibodies against ZO-1 (40–2200), occludin (33–1500) and claudin-5 (35–2500) from Thermo Fisher Scientific and GAPDH (ab181603) from Abcam. Following incubation with anti-mouse or anti-rabbit IgG-HRP (Thermo Fisher Scientific) secondary antibodies for 1 h at room temperature, signals were detected using ImageQuant LAS 500 (GE Healthcare) (Tang et al., 2020).

2.9. Brain tissue preparation and immunofluorescence staining

Rats anesthetized with isoflurane were intracardially perfused with 0.1 M PBS, followed by 4 % paraformaldehyde (PFA) in 0.1 M PBS. The whole brain was removed, further fixed in 4 % PFA at 4 °C overnight and embedded in paraffin. Coronal sections (5 μm thick) were mounted on

slides for subsequent immunofluorescence staining as previously described (Chen et al., 2018).

Briefly, the brain tissue sections were deparaffinized and rehydrated with graded ethanol washes. Antigen retrieval was conducted by boiling the sections in Tris-EDTA buffer (pH 9.0) for 90 s. The sections were then incubated with 10 % normal goat serum at room temperature for 30 min, followed by immunostaining with antibodies against ZO-1 (1:100, 40–2200, Thermo Fisher, Carlsbad, CA), occludin (1:200, ab216327, Abcam, Cambridge, USA) or claudin-5 (1:100, 35–2500, Thermo Fisher) overnight at 4 °C. After being washed three times with PBS at 5-min intervals, the sections were then incubated with Alexa 488-anti-rabbit or Alexa 488-anti-mouse secondary antibodies (1:200, Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature before being washed and mounted. Images were captured by confocal microscopy using a Leica TCS SP8 (Leica Microsystems, Buffalo Grove, IL, USA). Three brain sections per rat, each separated by 1 mm were subjected to imaged, with three separated images taken per section from a region in proximity to injury site.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Rats anesthetized with isoflurane were intracardially perfused with 0.1 M PBS. The whole brain was removed and the ipsilateral brain tissues were homogenized in ice-cold PBS (pH 7.4) containing protease inhibitor mixtures, followed by centrifugation at 12,000 *g* for 30 min at 4 °C. The supernatant was collected and used to assess the levels of inflammatory cytokines and hemoglobin with commercial ELISA kits according to the manufacturer's instructions.

For IL-1β, TNF-α, IL-6 and IL-10 measurements, protein levels were determined by an ELISA protocol standardized by Peprotech (NJ, USA). The wells of a polystyrene 96-well plate were coated with antibody (1 μg/mL) overnight at room temperature. After the wells were blocked with 1% BSA in PBS, the sample or standard was added to the antibody-coated wells. Then, the biotinylated detection antibody (0.5 μg/mL), avidin-HRP and ABST substrate were added sequentially. Cytokine concentration was measured with an ELISA plate reader at a wavelength of 405 nm with the wavelength correction set at 620 nm and quantified using a standard curve.

The hemoglobin concentration was assayed with an ELISA kit (ab157733, Abcam), and the sample or standard was added to the anti-hemoglobin antibody-precoated wells. After the removal of unbound proteins by washing, anti-hemoglobin antibodies conjugated with HRP were added, which formed complexes with the previously bound hemoglobin. Following the addition of TMB, the quantity of hemoglobin was measured at 450 nm and calculated from the standard curve.

2.11. Statistical analysis

GraphPad Prism 7.00 software was used to conduct the analyses. All data were presented as the mean ± SEM. The differences between two groups were determined using 2-tailed t-tests. The differences among three or more groups were determined using one-way ANOVA followed by Bonferroni's multiple comparisons test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HCA treatment improves neurological performance in TBI rats

To investigate whether HCA exerted neuroprotective effects on brain injury, HCA (Fig. 1A) was administered intravenously 10 min after TBI model establishment. The mNSS assay was applied to evaluate neurological function. At 24 and 72 h post-TBI, the neurological scores in the model group were significantly higher than those in the sham-operated group (ps < 0.01, Fig. 1B and C), indicating that the Feeney's weight-drop model in the present study resulted in neurological deficits. In contrast, TBI rats treated with 0.2 U/kg and 0.4 U/kg HCA exhibited less mNSS scores than the corresponding model group (ps < 0.01, Fig. 1B and C). Moreover, the 0.4 U/kg treatment exhibited a more marked decrease in mNSS score than the 0.2 U/kg treatment at 24 h post-TBI ($p < 0.05$, Fig. 1B). The above results demonstrate that treatment with HCA in the acute phase rescues TBI injury-induced neurological impairments.

A

Amimo sequence of α subunit

DCSSGWSSYEGHCYKVFKQSKTWADAESFCTKQVNGGHLVSLESS 45
GEADFVGQLLAQKLKSAKLHVWLGLRAQNKEKQCSLQWSDGSSLS 90
YENWLEEESKKCLGVHLETGFHKWENFYCEQQDPFVCEA 129

Amimo sequence of β subunit

DCPSDWSSYEGHCYKPFNEPKNWADAENFCTKQHTGGHLVSFQST 45
EEADFVVKLAFQTFDYGLFWFGLSKLWNQCNWQWSNAAMLKYTDW 90
AEESYCVYFKSTNNKWRSLTCRMLANFVCEFQA 123

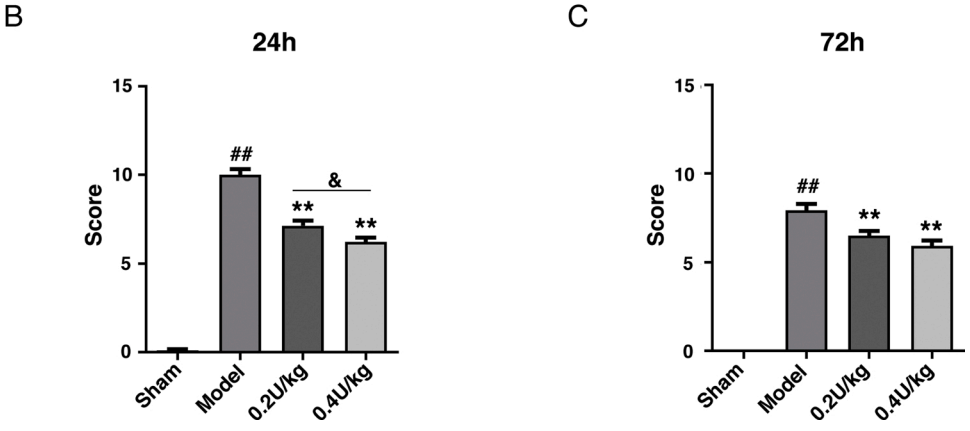

Fig. 1. Effects of HCA treatment on neurological outcomes post-TBI.
(A) Amino acid sequences of HCA. **(B)** mNSS of rats from indicated treatment groups at 24 h post-TBI (n = 26 − 28 per group). **(C)** mNSS of rats from indicated treatment groups at 72 h post-TBI (n = 26 − 28 per group). Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs model group; $^{\&}P < 0.05$ vs 0.2 U/kg group.

3.2. HCA attenuates brain edema in rats with experimental TBI

Next, we examined the brain edema, which is an important indicator in evaluating the severity of TBI. As shown in Fig. 2A, in the injured hemisphere, brain edema as determined by the brain water content at 24 h post-TBI was significantly increased ($p < 0.01$), whereas treatment with HCA markedly decreased the water content compared with that of the model group at the dose of 0.2 U/kg ($p < 0.05$) and 0.4 U/kg ($p < 0.01$) respectively, suggesting that HCA has a therapeutic effect on the attenuation of brain edema. At 72 h post-TBI, although both 0.2 U/kg and 0.4 U/kg HCA administration reduced the water content, and there was no significant difference between the HCA treatment groups and the model group (Fig. 2B). Further analysis revealed a significant reduction in brain water content in the model group at 72 h compared with 24 h post-TBI ($p < 0.05$, Fig. 2C), indicating that there is a gradual recovery of brain edema over time due to the self-healing ability of rats.

3.3. HCA decreases hemoglobin in injured tissue of TBI rats

As shown in Fig. 3, basal protein levels of hemoglobin were low in the ipsilateral hemispheric brain of sham-injured animals. Vehicle-treated injured rats had 18-fold higher and 13-fold higher hemoglobin levels than the corresponding sham controls, at 24 h and 72 h post-TBI (ps < 0.01), respectively, indicating that the TBI model in this study exhibits intracranial hemorrhage. At 24 h after TBI, administration of 0.2 U/kg and 0.4 U/kg HCA significantly reduced the hemoglobin level, reaching 55 % and 47 %, respectively, of the concentrations in brains of the vehicle-treated injured rats (ps < 0.01, Fig. 3A). At 72 h after TBI, only 0.4 U/kg HCA significantly reduced the hemoglobin level to 67 % compared with that of the model group ($p < 0.01$, Fig. 3B). The above results demonstrate that HCA can reduce intracranial hemorrhage after TBI in rats.

3.4. HCA regulates the secretion of inflammatory cytokines in TBI rats

To evaluate the effects of HCA on inflammatory response in TBI rats, we measured the levels of the cytokines IL-1β, TNF-α, IL-6, and IL-10 following TBI. At 24 h post-TBI, 2.3-fold elevation of IL-1β ($p < 0.01$, Fig. 4A), 2.5-fold elevation of TNF-α ($p < 0.01$, Fig. 4 B) and 1.4-fold elevation of IL-6 ($p < 0.01$, Fig. 4C) were observed in vehicle-treated injured rats as compared with sham controls, indicating that there is an inflammatory response triggered in TBI rats. The increase in proinflammatory cytokines was significantly attenuated by HCA treatment. 0.2 U/kg HCA and 0.4 U/kg HCA respectively reduced IL-1β by 28 % and 34 % (ps < 0.01, Fig. 4 A), and reduced TNF-α by 34 % and 39 % (ps < 0.01, Fig. 4B). In addition, 0.4 U/kg HCA marked decreased IL-6 level by 19 % compared with model group ($p < 0.05$, Fig. 4C). At the meantime, 0.4 U/kg HCA significantly increased (by 54 %) the expression of the anti-inflammatory factor IL-10 ($p < 0.01$, Fig. 4D).

At 72 h following injury, the expression levels of inflammatory factors in the ipsilateral hemispheres of vehicle-treated, injured rats remained high; IL-1β by 2.1-fold ($p < 0.01$, Fig. 4A), TNF-α by 2.2-fold ($p < 0.01$, Fig. 4B) and IL-6 by 1.3-fold ($p < 0.05$, Fig. 4C), compared with sham values. HCA treatments significantly reduced the levels of all three cytokines. On average, IL-1β and TNF-α in 0.2 U/kg HCA-treated rats were 24 % ($p < 0.01$) and 32 % ($p < 0.05$), respectively; IL-1β and TNF-α in 0.4 U/kg HCA-treated rats were 32 % and 39 % (ps < 0.01), respectively; IL-6 in 0.4 U/kg HCA-treated rats was 24 % ($p < 0.05$); of the concentrations indicated in model group (Fig. 4A-C). For IL-10, 0.4 U/kg HCA significantly increased the expression of the anti-inflammatory factor IL-10 by 2-fold ($p < 0.01$, Fig. 4D).

In addition, we also measured the levels of IL-1β and TNF-α, two important pro-inflammatory factors in serum. As shown in supplementary Fig. 1, there were 2-fold and 3.5-fold increase of IL-1β ($p < 0.01$) and TNF-α ($p < 0.01$) in model group at 24 h post-TBI, as compared with the control group. 0.4 U/kg HCA markedly decreased the peripheral

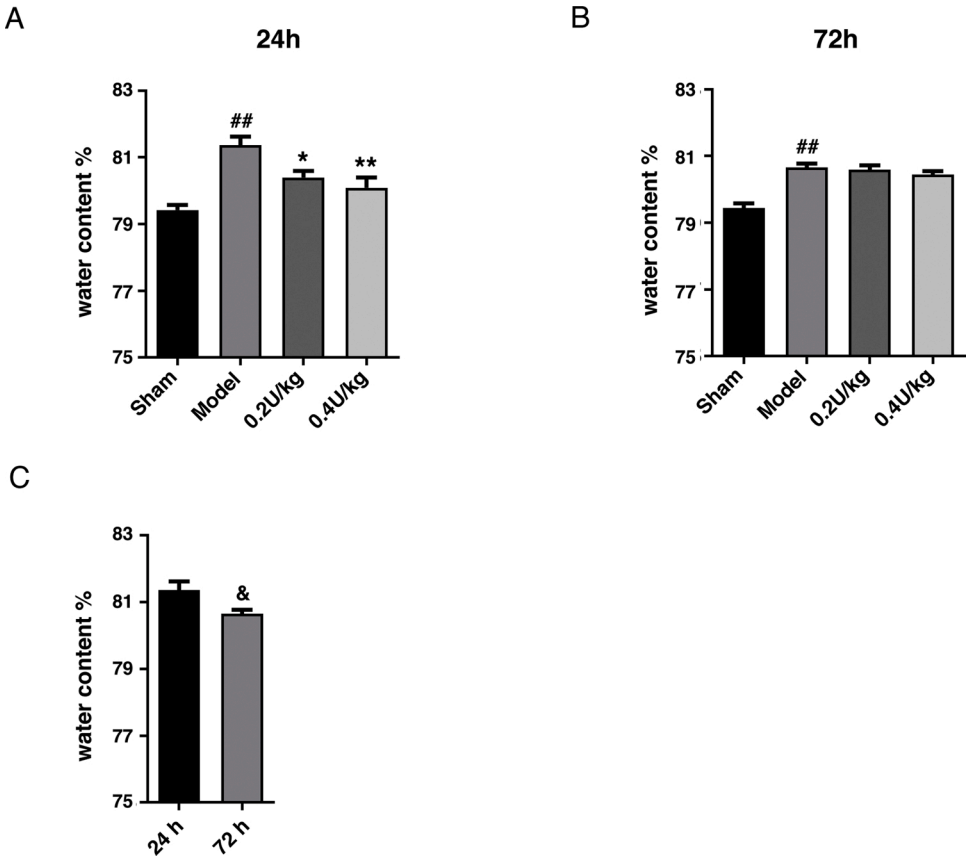

Fig. 2. Effects of HCA treatment on brain edema post-TBI.
(A) Brain water content of rats from indicated treatment groups at 24 h post-TBI ($n = 10 - 11$ per group). **(B)** Brain water content of rats from indicated treatment groups at 72 h post-TBI ($n = 13 - 14$ per group). **(C)** Comparison the brain water content of rats from model groups at 24 and 72 h post-TBI. Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs model group; $^{\&}P < 0.05$ vs model group at 24 h post-TBI.

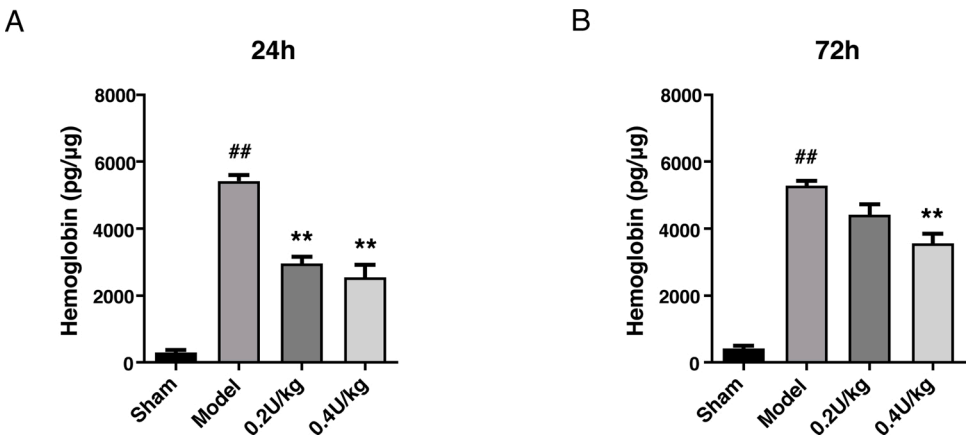

Fig. 3. Effects of HCA treatment on hemoglobin post-TBI.
(A) Hemoglobin measured in the ipsilateral hemispheres from indicated treatment groups at 24 h post-TBI (n = 7 per group). **(B)** Hemoglobin measured in the ipsilateral hemispheres from indicated treatment groups at 72 h post-TBI (n = 8 per group). Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs model group.

cytokines, reaching 47 % ($p < 0.01$, Fig. S1A) and 51 % ($p < 0.05$, Fig. S1B), respectively. Moreover, 1.4-fold and 2.8-fold elevation of IL-1β ($p < 0.05$) and TNF-α ($p < 0.05$) were observed in model group at 72 h following TBI, and this effect could significantly be reduced to 71 % ($p < 0.01$, Fig. S1A) and 43 % ($p < 0.05$, Fig. S1B) after the treatment of 0.4 U/kg HCA, respectively.

Taken together, these results indicate that HCA regulates regulated inflammatory cytokine release induced by TBI.

3.5. HCA treatment attenuates TBI-induced BBB disruption in rats

We next examined the effect of HCA on BBB integrity postinjury. The BBB leakage was determined by Evans blue exudation. As shown graphically in Fig. 5A and C, and quantitatively in Fig. 5B and D, Evans blue levels were low in the ipsilateral hemispheric brain of sham-injured animals. TBI induced significant increase of Evans blue extravasation at 24 h and 72 h post-TBI (ps < 0.01), respectively, indicating that TBI disrupted BBB integrity in this study. At 24 h after TBI, the profound increase in Evans blue leakage were attenuated by HCA treatment; as there were a 26 % reduction in 0.2 U/kg group ($p < 0.05$, Fig. 5B) and a 51 % reduction in 0.4 U/kg group ($p < 0.01$, Fig. 5B) relative to the injured rats treated with vehicle. At 72 h post-TBI, Evans blue extravasation in the ipsilateral hemisphere was significantly declined by 48 % in 0.4 U/kg HCA-treated rats compared with that in the vehicle-treated, injured rats ($p < 0.01$ Fig. 5D). The 0.2 U/kg group tended to have less Evans blue leakage than the model group, but did not reach to significance (Fig. 5D).

Next, we analyzed the expression levels of ZO-1, occludin and claudin-5 in the tissue surrounding the contusion core. Western blot analysis revealed that TBI model in our study markedly decreased the expressions of all three tight junction proteins at 24 h and 72 h post-injury (ps < 0.01, Fig. 6). However, the administration of 0.4 U/kg HCA significantly increased the levels of the three junctional proteins compared with those in the vehicle-treated, injured rats (ps < 0.01, Fig. 6), indicating that HCA can alleviate the TBI-induced loss of tight junction proteins.

Consistent with the Western blot results, immunolabeling analysis further confirmed the effects of HCA on the BBB. We analyzed the distributions of tight junction proteins ZO-1, occludin and claudin-5 in proximity to the brain injury site. The results revealed there were continuous staining of all three tight junctions in the sham groups, whereas a loss of staining was observed in the corresponding region in the model groups (Fig. 7A and C). Notably, 0.4 U/kg HCA obviously rescued the loss of all tight junction proteins, whereas 0.2 U/kg HCA only slightly increased the expression of these proteins. Quantitative analysis also showed significant increase of mean fluorescence intensity in ZO-1, occludin and claudin-5 in 0.4 U/kg groups at two time points (ps < 0.01, Fig. 7B and D).

4. Discussion

At present, the acute therapeutic strategies for treating TBI are still limited to surgical debridement, removal of hematomas, and control of intracranial pressure. There are few treatments for secondary injury and associated neurological sequelae. Therefore, safe and effective pharmacological therapeutics are urgently needed for TBI treatment. This study showed for the first time that the administration of HCA following TBI improved neurobehavioral function, attenuated brain edema and intracranial hemorrhage, and regulated inflammatory cytokine release and BBB integrity. Collectively, our results demonstrate that HCA treatment is effective in alleviating the severity of TBI rats, both clinically and neuropathologically.

The progressive hemorrhagic injury is the insult induced by both primary and secondary damage in TBI (Fair et al., 2019), and coagulopathy increases the chances of poor outcomes in TBI patients. HCA, a new family member of TLEs, has been shown to be a potent clinical drug for hemorrhagic diseases (Wei et al., 2010). Mechanistic studies revealed that HCA was unable to activate FXIII, FX and FII, thus avoiding coagulation pathway activation, which is a risk factor for thrombus formation (Maas and Renne, 2018). Moreover, HCA neither activates plasminogen nor reduces normal plasma fibrinogen levels even at the highest clinical dosage, eliminating the risk of activating the fibrinolytic system (Li et al., 2018). Based on previous reports that TLEs have clinical benefits in preventing surgical bleeding, as well as in treating ischemic cerebral infarction (Gardiner and Andrews, 2008; Kunalan et al., 2018), we attempt to explore the potential therapeutic effects of HCA on clinical symptoms in TBI. Firstly, we showed that postinjury HCA treatment attenuated TBI-induced neurological function deficits, as indicated by decreased mNSS. Secondly, we evaluated brain edema which is a lethal pathological condition caused by abnormal intracranial fluid accumulation in the acute phase of TBI. The formation of brain edema continuously compresses the brain tissue, and the subsequent decrease in perfusion and oxygenation leads to additional ischemic damage (Jha et al., 2019). In this study, HCA treatment obviously alleviated the brain water content at 24 h post-TBI. TBI ruptures blood vessels and induces intracerebral hemorrhage, leading to the formation of intracranial hematoma, a common and serious secondary lesion in craniocerebral injury (Hochstadter et al., 2014). Hemolysis within the hematoma causes neuronal death, neuroinflammation and neurological deficits. It has been reported that hemoglobin released from lysed red blood cells (RBCs) is involved in brain damage (Bulters et al., 2018). Therefore, we also examined the hemoglobulin to assess intracranial hemorrhage and found that hemoglobulin in the ipsilateral hemispheres was significantly reduced after HCA treatment. Taken together, our study provides evidence that postinjury HCA treatment can attenuate TBI-induced clinical symptoms, including neurological function deficits, cerebral edema and intracranial hemorrhage.

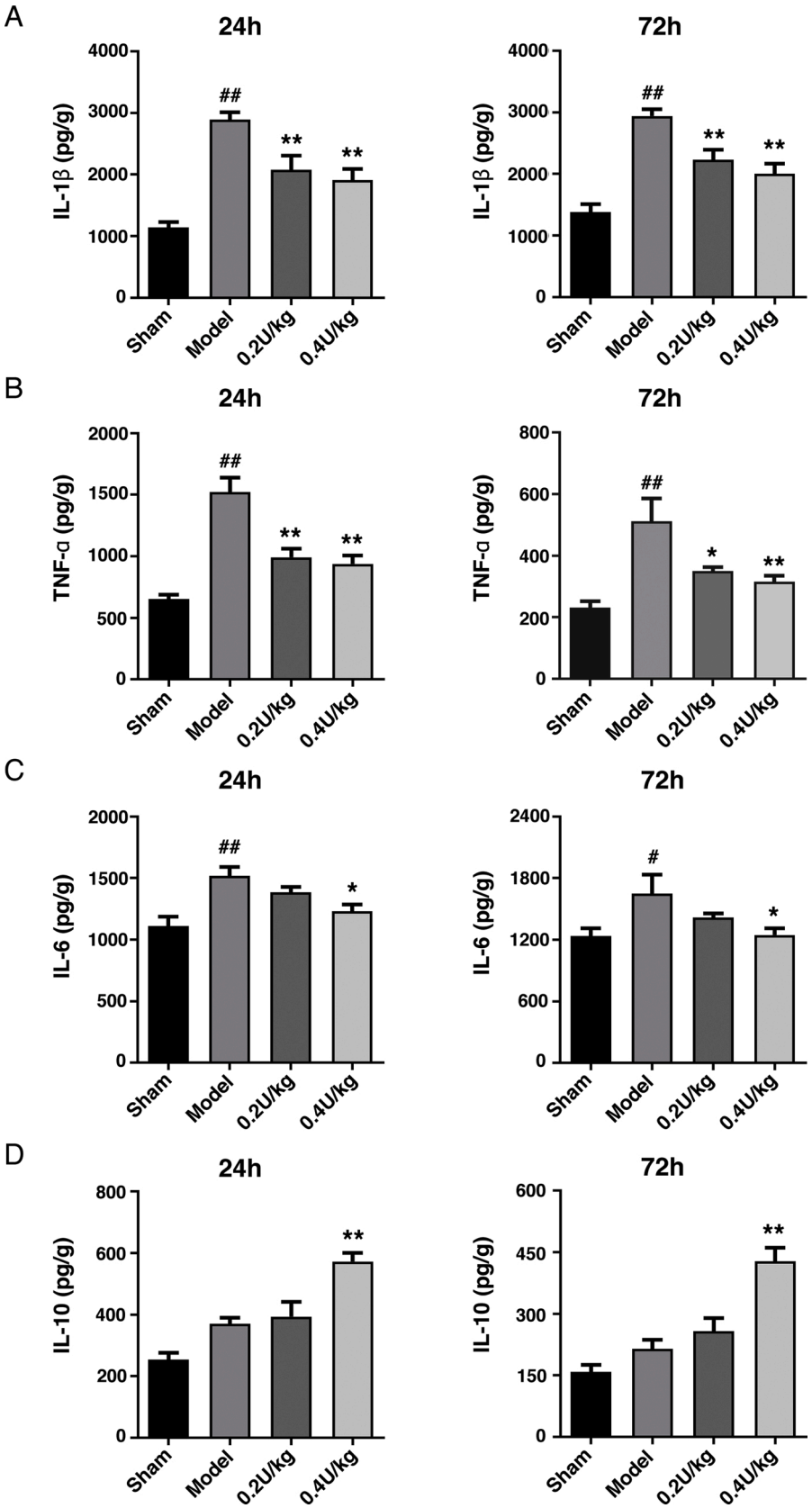

Fig. 4. Effects of HCA treatment on inflammatory response post-TBI.
Expression of IL-1β **(A)**, TNF-α **(B)**, IL-6 **(C)**, and IL-10 **(D)** measured in the ipsilateral hemispheres from indicated treatment groups at 24 h post-TBI (n = 7 per group) and at 72 h post-TBI (n = 8 per group). Data are presented as the means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs model group.

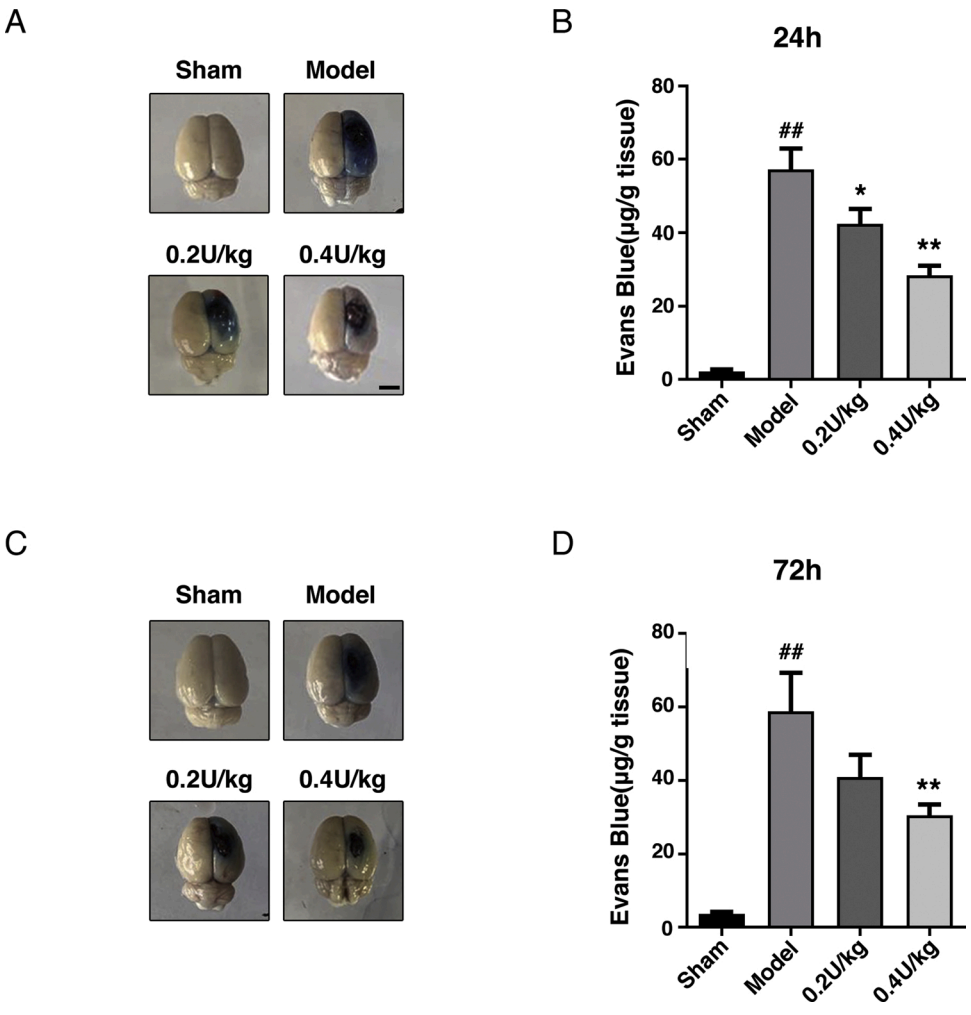

Fig. 5. Effects of HCA treatment on Evans blue extravasation post-TBI.
(A) Representative images of brain tissues from indicated treatment groups at 24 h post-TBI. Blue area indicates extravasation of Evans blue dye. Scale bars, 5 mm. **(B)** Quantification of the Evans blue dye contents measured in the ipsilateral hemispheres from the indicated treatment groups at 24 h post-TBI (n = 14–17 per group). **(C)** Representative images of brain tissues from indicated treatment groups at 72 h post-TBI. Blue area indicates extravasation of Evans blue dye. Scale bars, 5 mm. **(D)** Quantification of the Evans blue dye contents measured in the ipsilateral hemispheres from the indicated treatment groups at 72 h post-TBI (n = 13–14 per group). Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs model group.

Inflammation is one of the most prominent secondary effects in response to TBI. The inflammatory responses triggered by TBI are essential for repairing brain damage and defending against pathogens, while the overproduction of proinflammatory cytokines and long-term imbalance in inflammatory profiles in the injured brain are thought to exacerbate secondary brain damage (Russo and McGavern, 2016). Here, we report that post-TBI treatment with HCA suppressed the induction of the proinflammatory cytokines IL-1β, TNF-α and IL-6 while increasing the level of the anti-inflammatory cytokine IL-10 in the injured brain in TBI rats, indicating that HCA has therapeutic potential to balance the expression of inflammatory substances. It has been reported that intracranial hemorrhage as well as the resulting coagulation abnormalities are risk factors associated with poor prognosis in TBI patients (Abdelmalik et al., 2016; Folkerson et al., 2018). One of the important reasons is that coagulation factors, involving the tissue factor-FVIIa complex, thrombin, and FXa, participate in the coagulation cascade and enhance the inflammatory response (Festoff and Citron, 2019). HCA plays a role in hemostasis by neither affecting the fibrinolytic system nor activating the coagulation cascade, thereby avoiding the induction of inflammation. Despite HCA modulating cytokine expression in parallel with reduced hemoglobin in the injured hemisphere, further work is needed to establish a direct causal relationship between hemostasis and cytokine release.

The BBB is a selectively permeable barrier that is crucial in maintaining CNS microenvironment homeostasis, and BBB breakdown is a hallmark of TBI (Price et al., 2016). The pathological alteration of the BBB can also evolve into hemorrhagic lesions. In addition to the direct mechanical impact, the disrupted delicate balance of local inflammatory cytokines is a major cause that compromises the BBB in TBI, resulting in a significant exacerbation of the initial injury. Moreover, the increased infiltration of blood-derived substances into brain tissue further aggravates proinflammatory cytokine release. The BBB is composed mainly of microvascular endothelial cells that are fused by junctional proteins, among which ZO-1, occludin and claudin-5 are known to be critical for BBB integrity (Komarova et al., 2017). Multiple studies have demonstrated that IL-1β and TNF-α induce an increase in BBB permeability by downregulating tight junction proteins in endothelial cells (Chen et al., 2018; Thome et al., 2019; Wong et al., 2019). To further understand the therapeutic effects of HCA on TBI-induced brain injury, we next evaluated BBB integrity in TBI rats. Consistent with the alteration of the inflammatory response, a single administration of HCA alleviated Evans blue extravasation and the loss of BBB-associated tight junction proteins in injured hemispheres, indicating that HCA exerts a protective role on BBB integrity. However, it should be noted that although 0.2 U/kg HCA reduced IL-1β and TNF-α production at 24 h post-TBI, no significant enhancement of tight junctions was observed at 0.2 U/kg HCA treatment compared to model group. This complicated result indicates that there are factors involved in disrupting tight junctions that can be reversed by HCA treatment at the dose of 0.4 U/kg, but not the 0.2 U/kg. In addition to inflammatory cytokines, multiple regulators and pathways have been reported in mediating tight junction protein expression (Cong and Kong, 2020). In further study, we will examine more indicators to comprehensively evaluate the beneficial effects of HCA on TBI.

The mechanisms underlying the hemostasis effects of HCA have been extensively investigated (Li et al., 2018). HCA exerts procoagulant activities by processing fibrinogen into fibrin without activating FXIII,

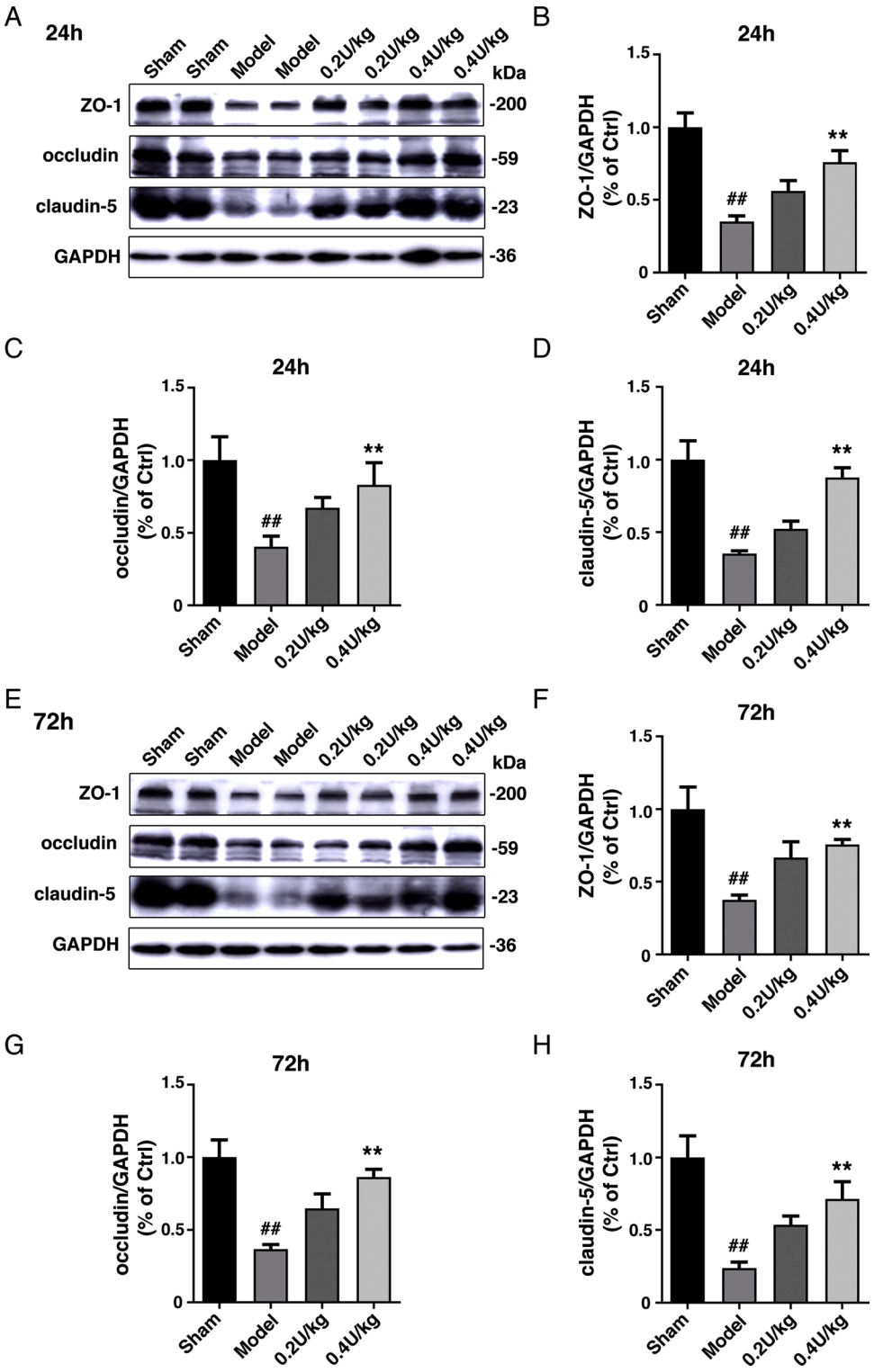

Fig. 6. Western blots analyses of tight junction protein expression post-TBI.
(A) Western blot analysis of ZO-1, occludin and claudin-5 in the ipsilateral hemispheres from the indicated treatment groups at 24 h post-TBI. **(B–D)** Quantification of ZO-1 **(B)**, occludin **(C)** and claudin-5 **(D)** expression from the indicated treatment groups at 24 h post-TBI. GAPDH was used as loading control. n = 6 per group. **(E)** Western blot analysis of ZO-1, occludin and claudin-5 in the ipsilateral hemispheres from the indicated treatment groups at 72 h post-TBI. **(F–H)** Quantification of ZO-1 **(F)**, occludin **(G)** and claudin-5 **(H)** expression from the indicated treatment groups at 72 h post-TBI. GAPDH was used as loading control. n = 6 per group. Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs model group.

producing more easily absorbed segments that reduce the risk of thrombosis. The intracerebral hemorrhage induced directly by TBI is responsible for intracranial hematoma formation, neuroinflammation and BBB disruption. Thus, we deduce that the function of HCA in promoting the hydrolysis of fibrinogen might be involved in the neuroprotection on TBI. Further investigations are needed to clarify the mechanisms contribute to the beneficial effects of HCA against TBI.

HCA is isolated from the snake venom of *Agkistrodon acutus*. As a biologically extracted protein, whether multiple administrations will cause an allergic reaction has not been examined by experiments. In preclinical or clinical trials, no allergic reactions occurred with a single administration of HCA. This study is the first time to investigate the HCA therapeutic effects in the field of TBI. Considering the safety and half-life of HCA, we adopted a single dose of HCA and assessed its effects during the acute phase of TBI. Our results suggest that post-injury HCA exerts a neuroprotective effect and HCA has potential clinical applications in the treatment of acute TBI. Whether multiple administration contribute to long-term recovery will be carried out in future study. Moreover,

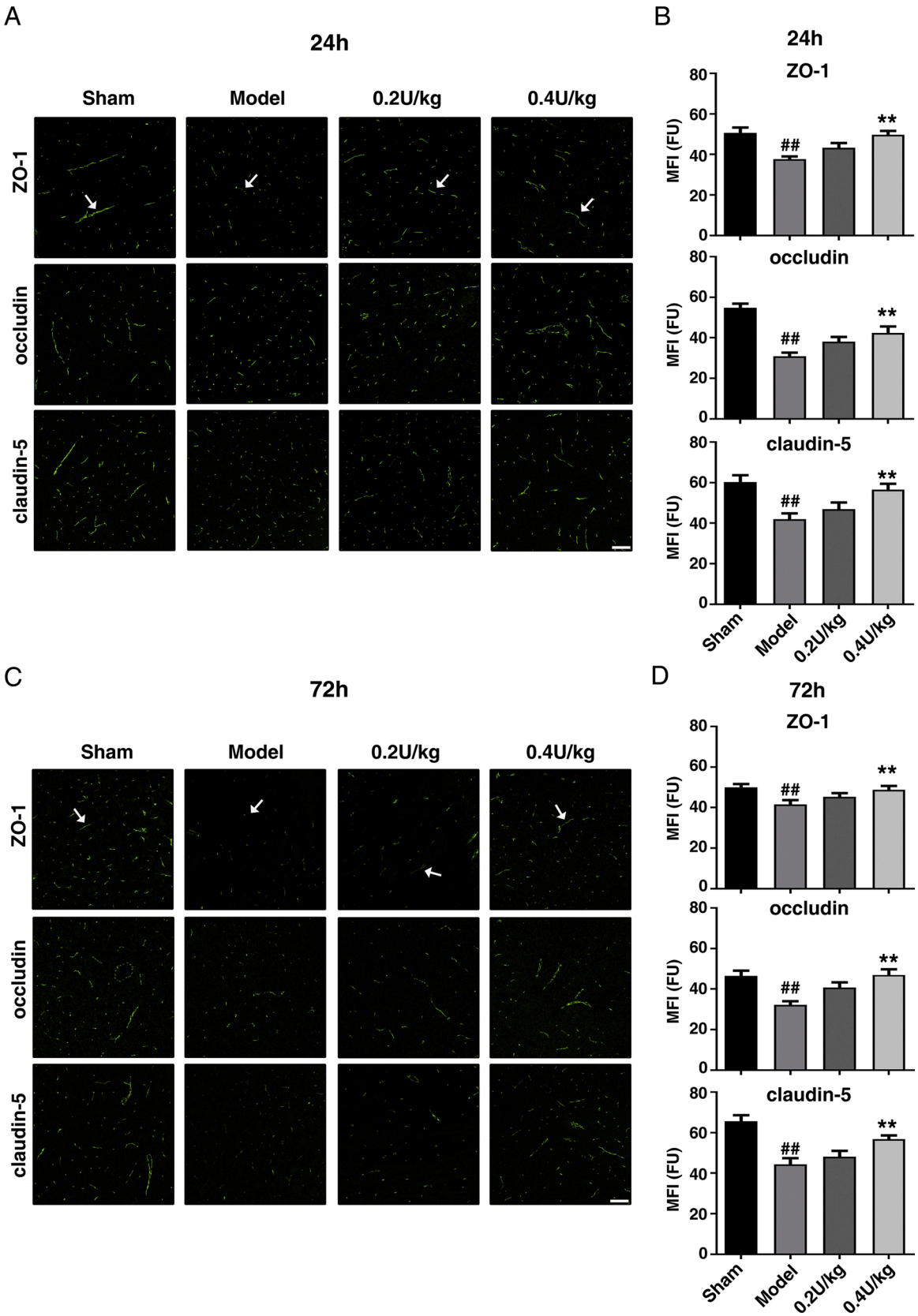

Fig. 7. Immunofluorescence staining analyses of tight junction protein distribution post-TBI.
(A) Representative images of ZO-1, occludin and claudin-5 in ipsilateral hemispheric brain from the indicated treatment groups at 24 h post-TBI. Scale bars, 75 μm. **(B)** Quantification of ZO-1, occludin and claudin-5 mean fluorescence intensity (MFI) from the indicated treatment groups at 24 h post-TBI. n = 3 per group. **(C)** Representative images of ZO-1, occludin and claudin-5 in ipsilateral hemispheric brain from the indicated treatment groups at 72 h post-TBI. Scale bars, 75 μm. **(D)** Quantification of ZO-1, occludin and claudin-5 MFI from the indicated treatment groups at 72 h post-TBI. n = 3 per group. Data are presented as the means ± SEM. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs model group.

comparing the results from single dose and repeated administrations, and different time points help to investigate mechanisms underlying HCA protective effects on TBI.

Together, our study shows that treatment with HCA protects against brain injury in an in vivo TBI model. HCA exerts neuroprotective effects and modulates the inflammatory response and BBB integrity. Since HCA has been widely used clinically in various surgeries with rapid onset and few side effects, it may be possible to expand the clinical applications of HCA. Moreover, the novel function of HCA in addition to hemostasis may provide new insights and the development of novel therapeutic agents for TBI.

Author contributions

Jingshu Tang and Ying Peng designed the experiments; Jingshu Tang and Yuying Kang conducted the majority experiments; Jingshu Tang, Longjian Huang and Ying Peng developed the methodology;Xinhong Feng and Lei Wu analyzed the data; Jingshu Tang drafted the manuscript; Ying Peng conceived and supervised the project; Jingshu Tang and Ying Peng participated in data analysis and wrote and finalized the manuscript writing.

Declaration of Competing Interest

All authors declare that there is no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.brainresbull.2021.01.023.

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