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Therapeutic effect of dexmedetomidine on intracerebral hemorrhage *via* regulating NLRP3

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Abstract. – OBJECTIVE: To investigate the protective effect of dexmedetomidine on intracerebral hemorrhage (ICH) mice and the underlying mechanism.

MATERIALS AND METHODS: The *in vivo* ICH model was induced by the injection of autologous blood in C57BL/6 mice. Mice were randomly assigned into control group (no specific treatment) and treatment group (dexmedetomidine administration). Mouse neuronal deficits were evaluated by modified neurological severity scores (mNSS) and Corner Turn Test. ICH volume and cerebral edema at the hemisphere of lesions were determined. Inflammatory response at post-ICH was examined by flow cytometry and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The integrity and permeability of blood brain barrier (BBB) were detected by Western blot and Evans Blue extravasation.

RESULTS: Dexmedetomidine treatment markedly alleviated the clinical symptoms of ICH mice, and decreased ICH volume and cerebral edema. QRT-PCR data revealed down-regulated levels of NLRP3, ASC, Caspase-1, and IL-1β in mice of the treatment group relative to controls. Moreover, dexmedetomidine administration decreased contents of CD11b+CD45int, IL-6, and IL-1β, but elevated TGF-β content in treatment group. The Evans Blue leakage was reduced in mice of the treatment group. Both protein and mRNA levels of Claudin-5 and ZO-1 were upregulated in the treatment group.

CONCLUSIONS: Dexmedetomidine remarkably inhibits the activation of inflammasome, alleviates secondary cerebral injury and inflammation, and protects the integrity and permeability of BBB at post-ICH.

Key Words:
ICH, Cerebral edema, Inflammasome, Microglia.

Introduction

Intracerebral hemorrhage (ICH) is the second common type of stroke, accounting for 10-15% of all stroke cases globally. In China, ICH ranks 20-30% of stroke cases[1]. The neurological damage secondary to ICH is mainly divided into primary and secondary injury. Increasing evidence suggested the crucial role of the inflammatory cascade in ICH. It not only destroys the blood brain barrier (BBB), but it also accelerates the formation and expansion of cerebral edema. Inflammatory response at post-ICH initiates the oxidative stress, neuronal apoptosis, and necrosis, thus aggravating neurological damage[2,3].

Microglia are activated by a variety of signaling pathways within a few minutes after ICH, which immediately release inflammatory cytokines to aggravate brain damage, such as tumor necrosis factor-α (TNF-α), matrix metalloproteinase-9 (MMP-9), interleukin-1β (IL-1β), etc.[4]. Recent studies have shown that intracellular NOD-like receptors (NLRs) are capable of activating microglia and participating in immune inflammatory responses[5]. The inflammasome NLRP3 is a protein complex that binds to ASC and pro-Caspase-1[6,7]. NLRP3 is mainly expressed in myeloid cells and remains an extremely low expression in normal brain tissues. Under the stimulation of brain injury, it is activated and mostly expressed in microglia[8]. Current studies have reported that NLRP3 activation at post-ICH induces Caspase-1 and releases IL-1β and IL-18, which in turn accelerates neutrophil infiltration to attenuate cerebral inflammation[9,10]. NLRP3 also induces the increase in vascular endotheli-

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al permeability and aggravates cerebral edema. In theory, the inhibition of NLRP3 activation at post-ICH exerts a neuroprotective role to reduce brain inflammatory response, cerebral edema and neurological damage.

Dexmedetomidine is a highly selective adrenergic α2 receptor agonist, which is extensively applied for anesthesia, sedation, analgesia, and anti-anxiety[11]. Dexmedetomidine exerts a neuroprotective role in brain injury. It can effectively inhibit the activation of NLRP3 after brain trauma, reduce the release of pro-inflammatory factors and prevent neutrophil infiltration. Moreover, dexmedetomidine suppresses microglial activation and protects BBB damage[12,13]. A great number of *in vivo* and *in vitro* experiments all demonstrated the neuroprotective role of dexmedetomidine at post-ICH[14-16]. The pharmacological functions of dexmedetomidine mainly include reduction of cerebral ischemia-reperfusion, inhibition of inflammatory response, activation of anti-apoptotic pathways and suppression of neuronal autophagy[17-19].

This study established the ICH mode in mice *via* injection of autologous blood. We aimed to explore whether dexmedetomidine could effectively inhibit the NLRP3 activation and the specific mechanism in alleviating the inflammatory response at post-ICH.

Materials and Methods

Experimental Animals

Male adult C57BL/6 mice (aged 8-10 weeks old, 20-25 g in weight) were housed in a standard environment with free access to drink and food. Mice were randomly assigned into control group and treatment group, with 30 mice in each. After ICH procedures, 25 μg/kg/day of dexmedetomidine or isodose vehicle was intraperitoneally administrated for three days consecutive. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center.

Establishment of ICH Model in Mice

ICH mouse model was induced by injection of autologous blood into the caudate nucleus. Mice were anesthetized with 5% of chloral hydrate by intraperitoneal injection, and the areas around the eyelids were disinfected with iodophor. 30 μL of blood sample was drawn from the medial canthus of mouse using the glass capillary and transferred to heparin-infiltrated microinjector pre-fixed to the syringe pump. The mouse was fixed on the brain's stereotaxic apparatus, and the bregma was located. At 0.5 mm anterior and 2.3 mm rightward to the bregma, the bone window was created with 1 mm in depth. 5 μL of autologous blood in fixed microinjector was injected into the brain parenchyma at 1 μL/min through the bone window for 3 mm and maintained there for 5 min. Subsequently, the microinjector was further punctured for 0.7 mm in depth, injected with 25 μL of blood and maintained for 20 min. The microinjector was withdrawn in three times, with a 5 min interval. Finally, the bone window was closed with bone wax and sutured. The whole procedures were conducted in a constant temperature room at 24°C. Mouse body temperature, blood pressure, and blood oxygen were closely monitored.

Neurological Deficit Evaluation

After anesthesia awareness, mouse motor, movement, sensation, balance and reflex functions were evaluated by the modified neurological severity scores (mNSS). The mNSS ranged from 0 to 18 scores, and higher scores indicated worse neurological deficits. Limb coordinate function was evaluated by the Corner Turn Test. The number of times that mice turned to the injured side was recorded. Corner Turn Test was repeated at least for 10 times, with 30 s interval. Finally, the ratio of turning numbers of the total numbers was calculated.

Determination of ICH Volume

The ICH volume was calculated using the Image Pro Plus software (Silver Springs, MD, USA). Briefly, mice were anesthetized with 10% of chloral hydrate at the third day of ICH. After exposure of mouse heart, the perfusion needle (25G) was inserted into the left ventricle from the right atrial appendage and at least 20 mL of cold PBS was perfused, followed by perfusion of 20 mL of cold 4% paraformaldehyde. Color change of liver from dark to pale indicated an effective perfusion. Subsequently, the mouse brain was extracted and sliced into 1 mm in depth. ICH volume = ICH area × layer thickness.

Determination of Cerebral Edema

Mice were anesthetized with 10% of chloral hydrate at the third day of ICH. The brain tis-

sue was divided into the hemisphere of lesion, contralateral hemisphere of lesion and the cerebellum. Tissues were placed in the pre-weighed tinfoil (A), and weighed (B). Wet weight = B-A. The brain tissues wrapped in the tinfoil were dried in the oven at 100°C for 24 h and weighed (C). Dry weight = C-A. Finally, cerebral edema index = (wet weight – dry weight) / wet weight × 100%.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissues and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Primer sequences of NLRP3, caspase-1, IL-1β, ASC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were searched from Gen Bank, and relative plasmids were synthesized by Primer 5.0. The relative level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Evans Blue Extravasation

2 mL/kg of Evans Blue (2%) was intraperitoneally injected in mice at 48 h of post-ICH. 24 hours later, mice were sacrificed for harvesting brain tissues. Tissues were weighed and prepared for brain homogenate, which was suspended in 5 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) for 72 h incubation at 60°C, and centrifuged at 1000 rpm for 5 min. The supernatant was collected to record the absorbance at 450 nm to 570 nm. The content of Evans Blue leakage per gram of brain tissue was calculated.

Western Blot

Total protein from brain tissues was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), it was blocked in 5% (of) skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The Mann-Whitney U test was used to analyze intergroup differences. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated a significant difference.

Results

Dexmedetomidine Improved Mouse Neuronal Dysfunction at Post-ICH

At day 1 and day 3 after ICH, clinical symptoms of ICH mice were evaluated by mNSS score and Corner Turn Test. The lower mNSS score was observed in mice of treatment group 1 and 3 days after ICH (Figure 1A, 1B). Meanwhile, the ratio of corner turning was reduced at day 1 and day 3 in treatment group relative to controls (Figure 1C, 1D). It is proved that dexmedetomidine administration improved neuronal dysfunction at post-ICH.

Dexmedetomidine Reduced ICH Volume and Cerebral Edema

At day 3 after ICH, ICH volume and cerebral edema were recorded. As the data revealed, ICH volume and cerebral edema at the hemisphere of lesion remarkably decreased in treatment group (Figure 1E, 1F).

Dexmedetomidine Suppressed NLRP3 Activation at Post-ICH

QRT-PCR data showed that mRNA levels of NLRP3, ASC, Caspase-1, and IL-1β were markedly upregulated after ICH, which were downregulated after dexmedetomidine administration in ICH mice (Figure 2A-2D). It is suggested that dexmedetomidine effectively suppressed the NLRP3 activation.

Dexmedetomidine Suppressed Microglia Activation and Inflammatory Response

At day 3 after ICH, monocells were extracted from lesioned brain tissues for flow cytometry analysis. Dexmedetomidine administration remarkably decreased the $CD11b^{+}CD45^{int}$ level, demonstrating the inhibited microglia activation (Figure 3A). Conversely, TGF-β level was upregulated in treatment group, indicating the suppressed inflammatory response after dexmedetomidine administration (Figure

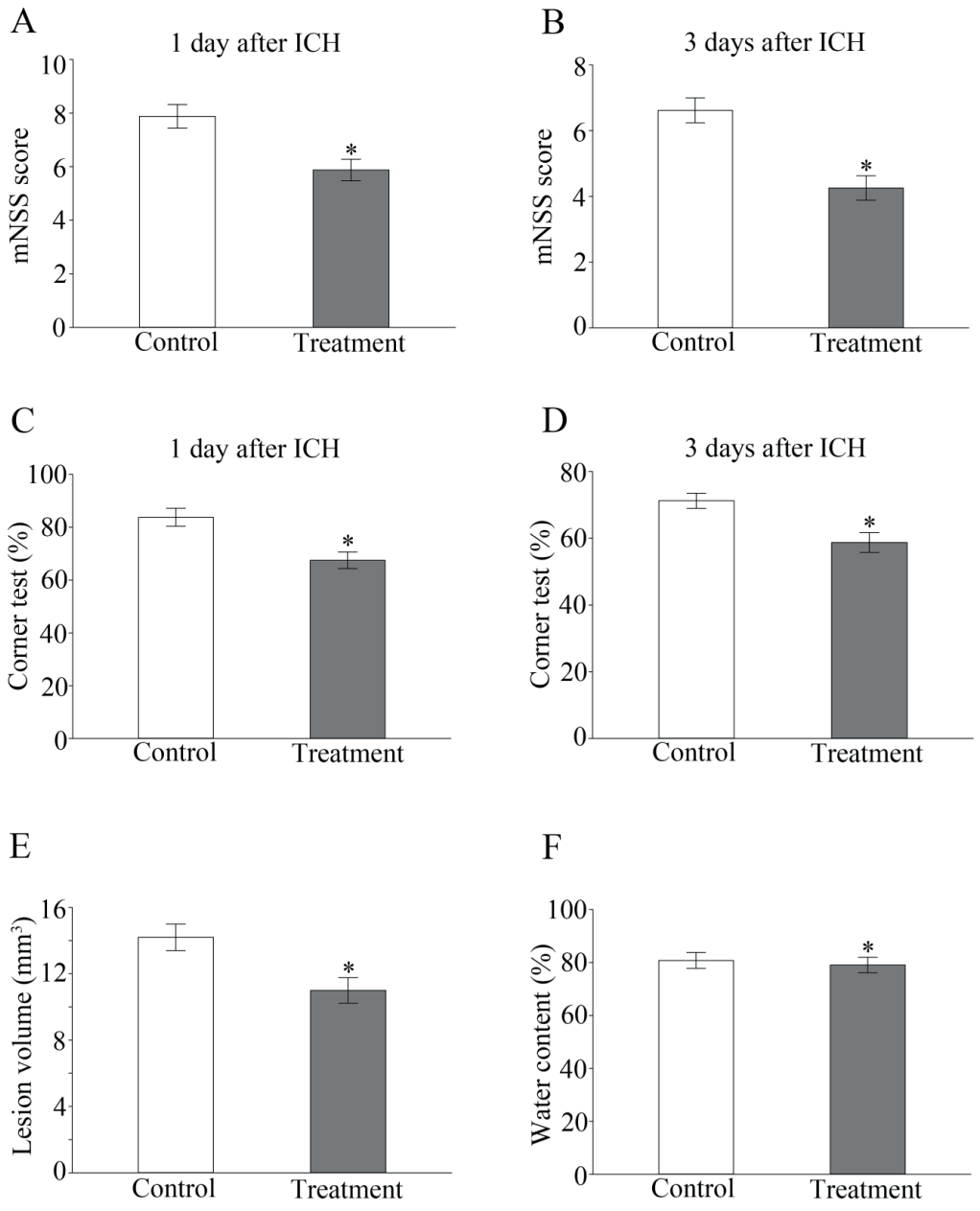

Figure 1. Dexmedetomidine improved mouse neuronal dysfunction at post-ICH. ***A,*** mNSS score at day 1 after ICH in mice of control and treatment group. ***B,*** mNSS score at day 3 after ICH in mice of control and treatment group. ***C,*** Corner Turn test at day 1 after ICH in mice of control and treatment group. ***D,*** Corner Turn test at day 3 after ICH in mice of control and treatment group. ***E,*** Lesion volume at day 3 after ICH in mice of control and treatment group. ***F,*** Cerebral edema at day 3 after ICH in mice of control and treatment group. *$p<0.05$, *vs.* Control group.

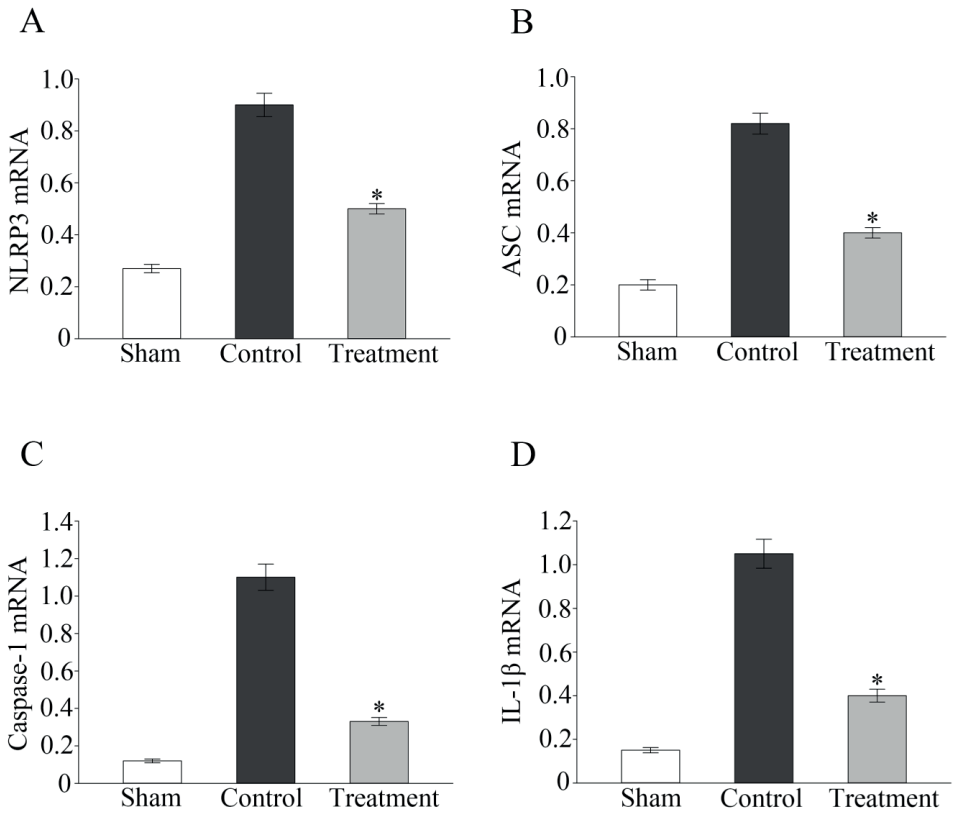

Figure 2. Dexmedetomidine suppressed NLRP3 activation at post-ICH. ***A,*** Relative level of NLRP3 in mouse brain tissues of control and treatment group. ***B,*** Relative level of ASC in mouse brain tissues of control and treatment group. ***C,*** Relative level of Caspase-1 in mouse brain tissues of control and treatment group. ***D,*** Relative level of IL-1β in mouse brain tissues of control and treatment group. *$p<0.05$, *vs.* Control group.

3B). Meanwhile, IL-6 and IL-1β levels were down-regulated in treatment group (Figure 3C, 3D).

Dexmedetomidine protected the integrity and permeability of BBB at post-ICH

Compared with control group, the Evans Blue leakage was remarkably reduced in treatment group (Figure 4A). Protein levels of Claudin-5 and ZO-1 were upregulated in treatment group, as well as their mRNA levels (Figure 4B-4D). It is believed that dexmedetomidine could maintain the integrity and permeability of BBB in ICH mice.

Discussion

Cerebrovascular disease becomes the leading cause of death in our country instead of cardiovascular disease and cancer, which severely endangers human health[20]. With the aging of the population, the incidence of cerebrovascular disease has increased year by year. ICH accounts for about 10-15% of cerebrovascular diseases, manifesting a high mortality and disability[21,22].

Primary injury of ICH is mainly caused by the damage of brain tissue structure due to mechanical compression and mass effect of hematoma. Besides, secondary injury at post-ICH has resulted from cytotoxicity, excitotoxicity, inflammation and oxidative stress[23,24]. Effective control of secondary injury at post-ICH is of significance in the neuroprotective treatment of ICH. Nowadays, inflammatory cascade has been identified to be crucial in ICH. It induces oxidative stress, neuronal apoptosis and neuronal dysfunction at post-ICH[25-27]. Microglia are immediately activated by ICH, which in turn release inflammatory factors[28]. It is reported

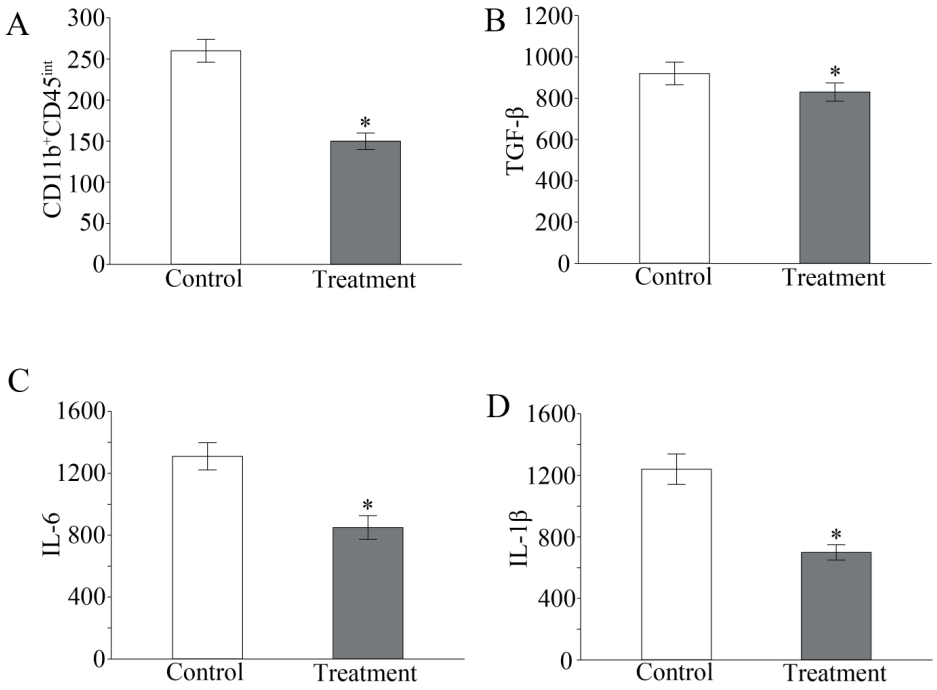

Figure 3. Dexmedetomidine suppressed microglia activation and inflammatory response. ***A,*** Content of $CD11b^{+}CD45^{int}$ in mice of control and treatment group. ***B,*** Content of TGF-β in mice of control and treatment group. ***C,*** Content of IL-6 in mice of control and treatment group. ***D,*** Content of IL-1β in mice of control and treatment group. *$p<0.05$, *vs.* Control group.

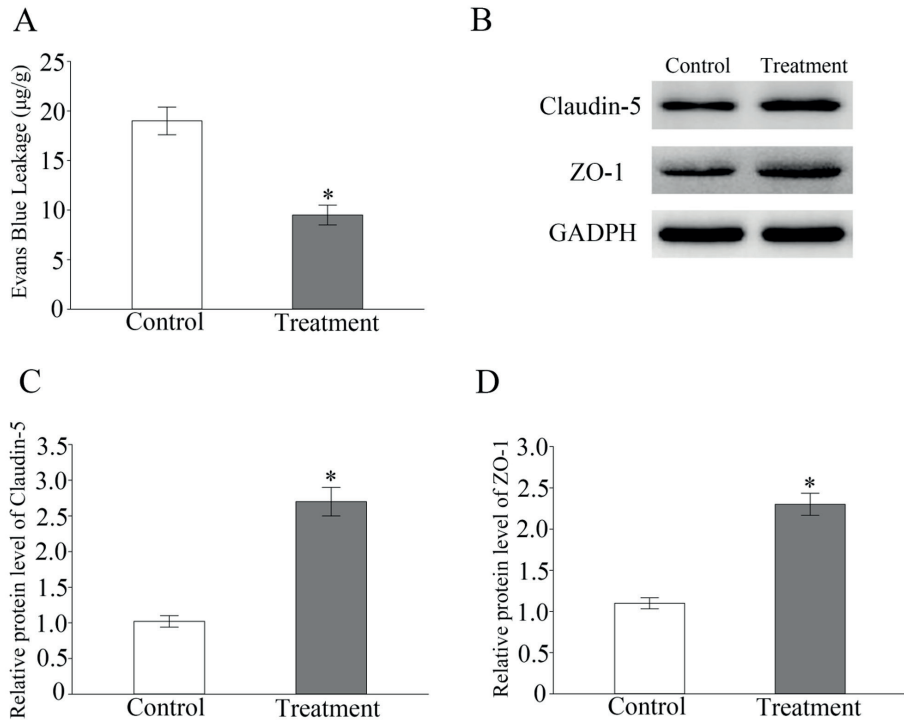

Figure 4. Dexmedetomidine protected the integrity and permeability of BBB at post-ICH. ***A,*** Evans Blue leakage in mice of control and treatment group. ***B,*** Western blot analyses of Claudin-5 and ZO-1 in mice of control and treatment group. ***C,*** Relative level of Claudin-5 in mice of control and treatment group. ***D,*** Relative level of ZO-1 in mice of control and treatment group. *$p<0.05$, *vs.* Control group.

that the activated NLRP3 after ICH induces Caspase-1 to release pro-inflammatory factors, which aggravates cerebral inflammation. Moreover, NLRP3 could stimulate neutrophil infiltration to the CNS, and expand the inflammatory lesions[29-31]. The increased vascular endothelial permeability due to NLRP3 activation further aggravates cerebral edema[29-31].

As a highly potent and highly selective adrenergic α2 receptor agonist, dexmedetomidine is proved to be able to suppress the inflammatory response and oxidative stress, showing a protective effect on important organs[32-34]. In this study, dexmedetomidine was identified to improve neuronal dysfunction, and reduce ICH volume and cerebral edema at post-ICH. Moreover, the protective effect of dexmedetomidine was confirmed to be related to the NLRP3 inhibition. After dexmedetomidine administration, ICH mice presented decreased levels of pro-inflammatory factors, increased the level of anti-inflammatory factor and suppressed microglia activation.

To sum up, dexmedetomidine suppressed the NLRP3-mediated cerebral inflammation at post-ICH, which contributed to alleviate neuronal dysfunction and cerebral edema. Dexmedetomidine could be applied as a promising drug for the clinical treatment of ICH.

Conclusions

We found that dexmedetomidine remarkably inhibits the activation of inflammasome, alleviates secondary cerebral injury and inflammation, and protects the integrity and permeability of BBB at post-ICH.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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