Original Article Salvianolic acid B reduces brain inflammation and oxidative stress after experimental intracerebral hemorrhage

Pi-Da Hao1*, Liang-Bo Guo2*, Jie Yang3*

¹Deparement of Neurology, Dongda Hospital, Shanxian, Heze 274300, Shandong Province, China; ²Deparement of Neurosurgery, Dongda Hospital, Shanxian, Heze 274300, Shandong Province, China; ³Department of Critical care Medicine, Dongda Hospital, Shanxian, Heze 274300, Shandong Province, China. ^{*}Equal contributors.

Received June 23, 2017; Accepted January 4, 2018; Epub August 15, 2018; Published August 30, 2018

Abstract: Salvianolic acid B, a phenolic acid ingredient from Salviae miltiorrhiza (Danshen), exerts neuroprotective properties through its anti-inflammation, anti-oxidation and anti-apoptosis actions, but its role in intracerebral hemorrhage (ICH) remains unclear. This study evaluates the effect of Salvianolic acid B on brain injury using the rat experimental ICH model. The results suggest that intraperitoneal administration of Salvianolic acid B (12 mg/ kg body weight/day) exerted the neuroprotective effect against ICH-induced brain injury at 72 hours, as shown by attenuated neurological deficit and brain edema, decreased the IL-1 β , IL-6, TNF- α staining positive cells and the expression level in the brain tissue, lowered the ROS and MDA and increased the GSH and SOD, reduced the TUNEL staining positive cells. In summary, these results indicate that Salvianolic acid B treatment attenuates brain injury induced by ICH via reducing brain inflammation and oxidative stress.

Keywords: Intracerebral hemorrhage, Salvianolic acid B, inflammation, oxidative stress, cell apoptosis, IL-1 β , IL-6, TNF- α , brain injury, ROS

Introduction

Intracerebral hemorrhage (ICH) is a subtype of hemorrhagic stroke, occurs when the diseased blood vessels of brain parenchyma suddenly rupture. Primary brain injury following ICH dues to hematoma formation and mechanical damage [1]. Secondary brain injury after ICH caused by the intra-parenchymal blood components, such as red blood cells and immunoglobulins, which subsequently activates oxidative stress and inflammatory pathways and contributes to the brain edema and neurological deficits [1]. The inflammatory response following ICH begins when the blood enter in the brain parenchyma, then causes the resident microglial activation. Firstly, activated microglia clears the hematoma through the phagocytosis, while excessive microglial activation aggravates primary brain injury by releasing proinflammatory cytokines, chemokines and free radicals. These cytokines and chemokines cause infiltration and activation of blood-derived leukocytes and

macrophages, which contributes to ICH-induced brain injury by producing proinflammatory mediators and reactive oxygen species (ROS) [2]. Hence, anti-inflammatory and antioxidant therapy provide neuroprotective effect after ICH [3, 4].

Salvianolic acid B (SAB, molecular formula is $C_{36}H_{30}O_{16}$) is a bioactive hydrophilic extract from Salviae miltiorrhiza (Danshen), which has been assigned as the marker component of Salviae miltiorrhiza in the Chinese Pharmacopoeia [5]. Salvianolic acid B is a phenolic acid ingredient and has strong pharmaceutical activity, which is used to treat cardiocerebral vascular diseases and prevent cancer [5]. Moreover, Salvianolic acid B has neuroprotective effects against ischemia brain injury by reducing lipid peroxides and free radicals [6, 7], and by suppressing the platelet activation and neuroinflammation [8]. However, it is not yet known whether Salvianolic acid B affects neuroinflammation and oxidative stress after ICH.

In this study, we aim to investigate the hypotheses: Salvianolic acid B attenuates inflammation, oxidative stress and cell apoptosis following ICH in rats.

Materials and methods

Animals and experimental design

Adult male Sprague-Dawley rats (270-300 g, Animal Centre of Lukang, Jining, China) were group-housed in cage at a constant temperature and humidity on a 12 hours light-12 hours dark cycle. All Experimental procedures were performed in accordance with the National Institutes of Health (NIH) guideline.

The experimental groups consisted of the sham group (n = 18), ICH+vehicle group (n = 18), and the ICH+Salvianolic acid B (SAB) group (n = 18). At 1 hour after ICH, rats received intraperitoneally injection of Salvianolic acid B (0.2 ml, 12 mg/kg body weight/day) or vehicle (saline) of equal volume. Time and dosage of Salvianolic acid B administration was based on prior study in rat model of transient middle cerebral artery occlusion [9]. At 72 hours after surgery, neurological scores were assessed, then all rats were killed under the anesthetized by chloral hydrate. Six rats in each group were for Immunofluorescence and TUNEL staining. Six rats in each group were for molecular biochemical analysis. Six rats in each group were for detecting brain water content and ELISA assay.

Experimental ICH model

Rat ICH model was produced as described in previous study [10]. In brief, rat was anesthetized by chloral hydrate (400 mg/kg body weight, i.p) and then fixed to the stereotactic frame. The 1.0 μ l volume of saline containing collagenase IV (0.3 U, Sigma, USA) was injected into the caudatum (lateral, 3.0 mm; posterior, 0.5 mm; depth, 6.0 mm) for 10 min using a 30 gauge needle attached to a Hamilton syringe. The needle was stayed 10 min and withdrawn following injection. The burr hole was sealed with the bone wax.

Neurological scores

At 72 hours after ICH, the modified neurological severity score (mNSS) method was used to evaluate neurological scores as described in previ-

ous study [10]. Briefly, mNSS method was comprised of motor, sensory, abnormal movements and reflexes absent that was scored from normal (score 0) to maximal deficit (score 18). Two 'blinded' investigators evaluated the score.

Measurement of brain water content

At 72 hours after ICH, under anesthesia with chloral hydrate, the infused hemispheres were taken and weighted quickly to determine the wet weight, then dried completely to determine the dry weight. The brain water content = (wet weight - dry weight)/wet weight × 100%.

Immunofluorescence and TUNEL staining

Immunofluorescence and TUNEL staining were performed as described previously [11]. At 72 hours after ICH, under anesthesia with chloral hydrate, rats were transcardially perfused with PBS followed by 4% paraformaldehyde solution. The brains were taken and fixed in 4% paraformaldehyde solution for 12 hours and then in 30% sucrose solution for 72 hours at 4°C. The coronal sections of brain were obtained from the Leica CM1950 cryostat, and permeabilized with 0.2% Triton X-100 solution for 5 min and blocked in 5% goat serum for 1 hour at room temperature. Next, coronal sections were incubated with (IL-1 β , IL-6 and TNF- α antibody; 1:100, Santa Cruz Biotechnology) for 15 hours at 4°C. Next day, the sections were washed with PBS and then incubated anti-mouse IgG-TRITC or anti-rabbit IgG-FITC for 2 hour at room temperature. The coronal sections were washed with PBS and cover slipped with the antifading solution. For TUNEL staining, the coronal section was used In Situ Cell Death Detection Kit with Fluorescein (Roche, Germany) following the manufacturer's instructions. All pictures were captured from a fluorescent microscope (Olympus, Japan) under the constant parameters.

Western blots

At 72 hours after ICH, under anesthesia with chloral hydrate, the brain tissue around the hematoma was dissected out and homogenized in RIPA lysis buffer (Beyotime, China) and then clarified by centrifuging for 10 min at 4°C. After determination of the protein content with the Bradford Protein Assay Kit (Beyotime, China), the supernatant containing 30 μ g of pro-



Figure 1. Salvianolic acid B improved neurological deficit and brain edema after ICH. (A) Schematic representation of the area for taken for assay (quadrangle for brain water content, ELISA and Western blot, triangle for immunofluorescence and TUNEL staining). (B) mNNS score and (C) brain water content at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group. Value are indicated as mean \pm SD (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001; One-way ANOVA with Bonferroni's multiple comparisons test; n = 6, per group).

tein was subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane by a transfer apparatus at 350 mA for 2 hours. Next, the membrane was blocked with 5% nonfat milk and then incubated with primary antibody (anti-active caspase-3 or caspase-3 antibody, 1:1000, Abcam, USA) for 12 hours at 4°C. After incubating with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature, the membrane were viewed using the chemiluminescence kit (Millipore, USA).

Enzyme linked immunosorbent assay (ELISA)

At 72 hours after ICH, under anesthesia with chloral hydrate, the brain tissue around the hematoma was dissected out and homogenized in saline. The content of IL-1 β , IL-6 and TNF- α were measured using the [Rat IL-1 β ELISA kit (EK3061), Rat IL-6 ELISA kit (EK301B2), and Rat TNF- α ELISA kit (EK3822), MultiSciences, China] following the manufacturer's instructions.

Measurement of ROS (reactive oxygen species), malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity

At 72 hours after ICH, under anesthesia with chloral hydrate, the brain tissue around the hematoma was dissected out and homogenized in saline. The ROS production, MDA content, 3-Nitrotyrosine content and SOD activity were measured using the [Reactive Oxygen Species Assay Kit (S0033), Lipid Peroxidation MDA Assay Kit (S0131), GSH assay kit (S0053), Total Superoxide Dismutase Assay Kit (S0101), Beyotime, China] following the manufacturer's instructions.

Statistical analysis

All values were expressed as mean \pm SD. Oneway ANOVA followed by the Bonferroni's multiple comparisons was used to compare the difference among the sham, vehicle and Salvianolic acid B groups using the GraphPad Software Prism 5.0. *P* < 0.05 was considered significant.

Results

Salvianolic acid B significantly improved neurological deficits and brain edema after ICH

Blood clots were found on striatum at 72 hours after ICH (**Figure 1A**). Compared to the sham group, the average neurological score in the ICH+vehicle group was significantly higher at 72 hours following ICH (**Figure 1B**). The neurological score in the ICH+Salvianolic acid B group significantly decreased when compared with the ICH+vehicle group (**Figure 1B**). In addition, the brain edema was evaluated at 72 hours



Figure 2. Salvianolic acid B reduced the IL-1 β , IL-6 and TNF- α positive cells after ICH. A. Representative images show immunofluorescence staining of IL-1 β , IL-6 and TNF- α at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group, scale bar = 50 µm. B. Quantitative analysis of the IL-1 β , IL-6 and TNF- α positive cells were represented as mean ± SD (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001; One-way ANOVA with Bonferroni's multiple comparisons test; n = 3, per group).

after ICH using the wet/dry weight method. The average brain water content in the ICH+vehicle group was significantly increased as compared to the sham group (**Figure 1B**). However, the brain water content in the ICH+Salvianolic acid B group significantly decreased when compared to the ICH+vehicle group. These results indicate that administration of Salvianolic acid B significantly improved neurological deficits and brain edema after ICH.

Salvianolic acid B suppressed the level of IL-1 β , IL-6 and TNF- α after ICH

Next we measured the level of IL-1 β , IL-6 and TNF- α that are known to be the most important

proinflammatory cytokines using the immunofluorescence staining and ELISA assay at 72 hours after ICH. Statistics results from immunofluorescence staining showed that the number of IL-1 β , IL-6 and TNF- α positive cell in the ICH+ vehicle group was significantly increased as compared with the sham group, but administration of Salvianolic acid B significantly reduced these positive ce-Ils when compared to the ICH+vehicle group (Figure 2). Meanwhile, ELISA assay suggested that the content of IL-1 β , IL-6 and TNF- α in ICH+vehicle group obviously higher than in the sham group, while treatment of Salvianolic acid B significantly reduced these higher level as compared with the ICH+vehicle group (Figure 3). These results suggested that Salvianolic acid B suppressed the neuroinflammation, especially in the release of proinflammatory cytokines.

Salvianolic acid B inhibited oxidative stress after ICH

To explore the possible role of Salvianolic acid B on oxi-

dative stress after ICH, the level of ROS, the oxidative stress damage marker of lipid (MDA), GSH and the key enzyme of antioxidant defense (SOD) were measured at 72 hours after ICH. As shown in Figure 4, ICH significantly increased the ROS production and MDA content as compared with that observed in the sham group, while treatment of Salvianolic acid B significantly reduced these increase when compared to the ICH+vehicle group (Figure 4A and 4B). Meanwhile, ICH significantly decreased the GSH content and SOD activity, while administration of Salvianolic acid B significantly reversed these decrease (Figure 4C and 4D). These finding suggested that Salvianolic acid B inhibited oxidative stress after ICH.



Figure 3. Salvianolic acid B decreased the content of IL-1 β , IL-6 and TNF- α after ICH. ELISA assay shows the concentration of (A) IL-1 β , (B) IL-6, and (C) TNF- α at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group. Value are indicated as mean \pm SD (**P* < 0.05 and ***P* < 0.01; One-way ANOVA with Bonferroni's multiple comparisons test; n = 6, per group).

Salvianolic acid B reduced cell apoptosis after ICH

To investigate the effect of Salvianolic acid B on the cell apoptosis after ICH, we assessed brain sections by TUNEL staining and Western Blots at 72 hours after ICH. Statistics results from TUNEL staining showed that the number of TUNEL positive cells in the ICH+vehicle group was significantly increased as compared with the sham group, but administration of Salvianolic acid B significantly reduced these positive cells when compared to the ICH+vehicle group (**Figure 5A** and **5B**). In addition, Western Blots analysis indicated that administration of Salvianolic acid B significantly reduced the expression of active caspase-3 in comparison with the ICH+vehicle group (**Figure 5C**). These results suggested that Salvianolic acid B reduced cell apoptosis after ICH.

Discussion

This study showed that administration of Salvianolic acid B significantly improves neurological function, attenuates the brain edema at 72 hours after ICH. Also, Salvianolic acid B obviously reduced the positive cells and content of IL-1 β , IL-6 and TNF- α at 72 hours after ICH. Moreover, Salvianolic acid B significantly decrease the ROS level and MDA content, and increase GSH content and the SOD enzyme activity at 72 hours after ICH. Furthermore, Salvianolic acid B significantly reduces the TUNEL staining positive cells and active caspase-3 expression at 72 hours after ICH. These results indicate that Salvianolic acid B attenuates the secondary brain injury induced by ICH through inhibiting the inflammation and oxidative stress.

When ICH occurs, the resident microglia and macrophages were activated within few minutes and lasted for 4 weeks. In the early stage, activated microglia and macrophages have beneficial effects through clearing hematoma via phagocytosis, while excessive activated microglia aggravates secondary brain injury by releasing proinflammatory cytokines IL-1B, IL-6 and TNF- α [4]. IL-1 β , IL-6 and TNF- α have been shown to be increased after experimental ICH [10, 12, 13] and ICH patients [14], and contribute to ICH-induced perihematomal edema formation. Our results showed that Salvianolic acid B significantly reduces the level of IL-1β, IL-6 and TNF- α following ICH. Previous studies have shown that Salvianolic acid B can suppress the microglial activation in lipopolysaccharide-treated microglia-neuron co-culture [15], also inhibited microglia activation after experimental traumatic brain injury in mice [16]. Therefore, our findings plus these evidenc-



Figure 4. Salvianolic acid B suppressed oxidative stress after ICH. (A) ROS production, (B) MDA content, (C) GSH content, and (D) SOD activity at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group. Value are indicated as mean \pm SD (**P* < 0.05 and ***P* < 0.01; One-way ANOVA with Bonferroni's multiple comparisons test; n = 6, per group).



Figure 5. Salvianolic acid B inhibited cell apoptosis after ICH. A. Representative images show TUNEL staining at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group, scale bar = 50 μ m. B. Quantitative analysis of the TUNEL positive cells in there groups. C. Western-blot shows the expression of caspase-3 at 72 hours in the sham group, ICH+vehicle group, and the ICH+Salvianolic acid B (SAB) group. Value are indicated as mean \pm SD (*P < 0.05 and ***P < 0.001; One-way ANOVA with Bonferroni's multiple comparisons test; n = 3, per group).

es indicate that Salvianolic acid B may attenuates the release of pro-inflammatory mediators through the inhibition of microglial activation. These Salvianolic acid B-mediated anti-inflammation effects should be further investigated.

When ICH occurs, excessive reactive oxygen species (ROS) derived from the metabolic product of hemoglobin, the activated microglia and macrophages, and damaged cells, initiates oxidative stress tissue injury in the form of DNA damage, protein oxidation and lipid peroxidation, then causes cell apoptosis and brain blood barrier disruption [17, 18]. Consistent with previous report, we observed significantly the increased production of ROS and MDA that is an oxidative marker of lipid, and decreased the content of GSH and SOD activity after ICH. On the other hand, Salvianolic acid B significantly reversed ICH-induced the increase of ROS and MDA, and restored the ICH-induced the decrease of the GSH and SOD. Previous studies reported that Salvianolic acid B exerts the anti-oxidant effect in hippocampal CA1 neurons after cerebral ischemia [7]. Hence, this evidence and our finding indicate that Salvianolic acid B may against brain injury-induced oxidative stress.

In conclusion, this study shows for the first time that Salvianolic acid B provides neuroprotective effect after ICH. Intraperitoneally injection of Salvianolic acid B (0.2 ml, 12 mg/kg body wei-

ght/day) significantly attenuates the neurological deficit and brain edema, reduced the release of proinflammatory cytokines IL-1 β , IL-6 and TNF- α , and reduced the oxidative stress and cell apoptosis at 72 hours in rat ICH model.

Acknowledgements

This work was supported by fund from the National Natural Science Foundation of China (81270336).

Disclosure of conflict of interest

None.

Address correspondence to: Jie Yang, Department of Critical Care Medicine, Dongda Hospital, Shanxian, Heze 274300, Shandong Province, China. Tel: +8613954078648; E-mail: m13954078648@163. com

References

- [1] Keep RF, Hua Y and Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet Neurol 2012; 11: 720-731.
- [2] Chen S, Yang Q, Chen G and Zhang JH. An update on inflammation in the acute phase of intracerebral hemorrhage. Transl Stroke Res 2015; 6: 4-8.
- [3] Duan X, Wen Z, Shen H, Shen M and Chen G. Intracerebral hemorrhage, oxidative stress, and antioxidant therapy. Oxid Med Cell Longev 2016; 2016: 1203285.
- [4] Zhou Y, Wang Y, Wang J, Anne Stetler R and Yang QW. Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation. Prog Neurobiol 2014; 115: 25-44.
- [5] Cao W, Guo XW, Zheng HZ, Li DP, Jia GB and Wang J. Current progress of research on pharmacologic actions of salvianolic acid B. Chin J Integr Med 2012; 18: 316-320.
- [6] Chen YH, Du GH and Zhang JT. Salvianolic acid B protects brain against injuries caused by ischemia-reperfusion in rats. Acta Pharmacol Sin 2000; 21: 463-466.
- [7] Jiang YF, Liu ZQ, Cui W, Zhang WT, Gong JP, Wang XM, Zhang Y and Yang MJ. Antioxidant effect of salvianolic acid B on hippocampal CA1 neurons in mice with cerebral ischemia and reperfusion injury. Chin J Integr Med 2015; 21: 516-522.
- [8] Wang Y, Chen G, Yu X, Li Y, Zhang L, He Z, Zhang N, Yang X, Zhao Y, Li N and Qiu H. Salvianolic acid B ameliorates cerebral ischemia/ reperfusion injury through inhibiting TLR4/ MyD88 signaling pathway. Inflammation 2016; 39: 1503-1513.

- [9] Xu S, Zhong A, Ma H, Li D, Hu Y, Xu Y and Zhang J. Neuroprotective effect of salvianolic acid B against cerebral ischemic injury in rats via the CD40/NF-kappaB pathway associated with suppression of platelets activation and neuroinflammation. Brain Res 2017; 1661: 37-48.
- [10] Xie RX, Li DW, Liu XC, Yang MF, Fang J, Sun BL, Zhang ZY and Yang XY. Carnosine attenuates brain oxidative stress and apoptosis after intracerebral hemorrhage in rats. Neurochem Res 2017; 42: 541-551.
- [11] Zhang ZY, Sun BL, Liu JK, Yang MF, Li DW, Fang J, Zhang S, Yuan QL and Huang SL. Activation of mGluR5 attenuates microglial activation and neuronal apoptosis in early brain injury after experimental subarachnoid hemorrhage in rats. Neurochem Res 2015; 40: 1121-1132.
- [12] Hua Y, Wu J, Keep RF, Nakamura T, Hoff JT and Xi G. Tumor necrosis factor-alpha increases in the brain after intracerebral hemorrhage and thrombin stimulation. Neurosurgery 2006; 58: 542-550; discussion 542-550.
- [13] Lin S, Yin Q, Zhong Q, Lv FL, Zhou Y, Li JQ, Wang JZ, Su BY and Yang QW. Heme activates TLR4-mediated inflammatory injury via MyD88/TRIF signaling pathway in intracerebral hemorrhage. J Neuroinflammation 2012; 9: 46.
- [14] Castillo J, Davalos A, Alvarez-Sabin J, Pumar JM, Leira R, Silva Y, Montaner J and Kase CS. Molecular signatures of brain injury after intracerebral hemorrhage. Neurology 2002; 58: 624-629.
- [15] Wang SX, Hu LM, Gao XM, Guo H and Fan GW. Anti-inflammatory activity of salvianolic acid B in microglia contributes to its neuroprotective effect. Neurochem Res 2010; 35: 1029-1037.
- [16] Chen T, Liu W, Chao X, Zhang L, Qu Y, Huo J and Fei Z. Salvianolic acid B attenuates brain damage and inflammation after traumatic brain injury in mice. Brain Res Bull 2011; 84: 163-168.
- [17] Qu J, Chen W, Hu R and Feng H. The injury and therapy of reactive oxygen species in intracerebral hemorrhage looking at mitochondria. Oxid Med Cell Longev 2016; 2016: 2592935.
- [18] Zhang ZY, Jiang M, Fang J, Yang MF, Zhang S, Yin YX, Li DW, Mao LL, Fu XY, Hou YJ, Fu XT, Fan CD and Sun BL. Enhanced therapeutic potential of nano-curcumin against subarachnoid hemorrhage-induced blood-brain barrier disruption through inhibition of inflammatory response and oxidative stress. Mol Neurobiol 2017; 54: 1-14.