

Original Article

Saikosaponin a protects TBI rats after controlled cortical impact and the underlying mechanism

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Received June 22, 2015; Accepted August 1, 2015; Epub January 15, 2016; Published January 30, 2016

Abstract: The inflammatory response plays a significant role in neuronal cell death and functional deficits after Traumatic brain injury (TBI). Importantly, anti-inflammatory agents have neuroprotective effects. To date, however, no studies have investigated the neuroprotective effects of Saikosaponin a (SSa) after TBI. In the present study, rats with controlled cortical impact (CCI) were used to investigate the neuroprotective effects of SSa. The results showed that SSa reduced body weight loss, improved neurological functions and cognition, and reduced brain edema and blood brain barrier permeability after CCI. Moreover, SSa inhibited aquaporin-4 (AQP-4), matrix metalloprotein-9 (MMP-9), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (c-JNK), tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). The reduction in the loss of occludin mediated by SSa may partially account for its neuroprotective effects. Together, our results suggest that SSa appears to counteract the inflammatory response and neurological function deficits after TBI and possibly via an anti-inflammatory response and inhibition of the MAPK signaling pathway.

Keywords: Saikosaponin a, controlled cortical impact, neuroprotection, inflammation, mitogen-activated protein kinase

Introduction

Traumatic brain injury (TBI) is a serious public health problem. The inflammatory response plays a significant role in neuronal cell death and functional deficits after TBI [1]. Cerebral inflammation after TBI is related to glial activation, leukocyte recruitment, and the upregulation and secretion of mediators such as chemokines and cytokines [2]. Our strategy is to identify pharmacological compounds that specifically target cerebral inflammation for the treatment of TBI, and some anti-inflammatory agents have demonstrated neuroprotective effects in laboratory research [3]. The activation of mitogen-activated protein kinase (MAPK) mediates inflammatory cytokine release [4]. Therefore, MAPK is well recognized as the target of anti-inflammatory agents.

Bupleurum, with a Chinese name Chaihu, is a kind of traditional Chinese medicine, which plays an important role in the treatment of common cold with fever, hepatitis, inflammatory diseases and so on [5]. Saikosaponin, which belongs to the Bupleurum family, is the major chemical constituent isolated from Bupleurum. Saikosaponin has been shown to play a role in anti-epilepsy, anti-convulsion, anti-inflammatory and immune-regulation [6-8]. In addition, saikosaponin was shown to modulate matrix metalloproteinases (MMPs) in endothelial cells [9]. Among saikosaponins, saikosaponin a (SSa, **Figure 1**) is known as the major active constituent, which is usually used as a herbal medicine for the treatment of hepatitis, inflammation, and bacterial or viral infections [10].

The aim of the present study is to determine whether the administration of SSa after TBI

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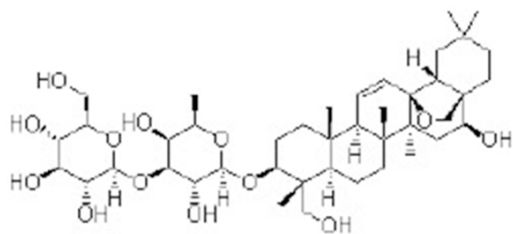


Figure 1. The Structure of SSa.

alters secondary injury cascades in an experimental model. We also investigated its anti-inflammatory mechanism by focusing on the inflammatory signaling pathway. Therefore, to determine the protective role of SSa against TBI, a controlled cortical impact (CCI) [11] model was used to investigate the effects of SSa on body weight loss, neurological deficits, brain edema, blood brain barrier (BBB) permeability, inflammatory factors, and related protein levels in the cortical injury.

Materials and methods

Materials

Saikosaponin a (purity>98%, obtained from National Institutes for Food and Drug Control) was dissolved in 0.9% physiological saline as a stock solution, stored at -20°C, and diluted with medium before each experiment. Working solutions were prepared before each experiment. Other reagents were purchased from Sigma (St. Louis, MO, USA).

Animals

All procedures that involved animals were conducted under the National Guidelines for Care and Use of Laboratory Animals and the Animal Care Guidelines issued by the Animal Experimental Committee of Beijing Neurosurgical Institute. Male adult Sprague-Dawley rats (weight, 295-325 g) were purchased from Beijing Vital River Experimental Animals Technology Ltd. (Beijing, China). The rats were housed in cages and maintained at 24°C with a normal 12 h/12 h light-dark schedule (lights on at 7 AM). The rats had free access to food and water until 24 hours before TBI in the experiments. The rats were randomly assigned to three groups treated with SSa or vehicle: (1) the sham group; (2) the vehicle-treated group: CCI+

vehicle; (3) the SSa group: CCI+SSa. Neurological severity scores (NSS) were evaluated 24 h, 48 h and 7 days after CCI. All rats were sacrificed 24 h or 7 days after CCI for further analysis. Water content, BBB integrity, tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) levels were assayed 24 h and 7 days after CCI. Aquaporin-4 (AQP-4), matrix metalloprotein-9 (MMP-9), occludin, p38 MAPK, and c-Jun N-terminal kinase (c-JNK) levels were determined 24 h after CCI. The body weights were measured before CCI and at 7 days after CCI in all animals, and the change of body weight was expressed as the body weight at 7 days after CCI minus that before TBI (Δ body weight).

With the rats under 10% chloral hydrate anesthesia (400 mg/kg), experimental CCI was induced using a modified CCI model to cause TBI in the animals [11]. Rectal temperature was continuously monitored and maintained at 37 \pm 0.5°C by a negative-feedback-controlled heating pad during the entire experiment. Animals were placed on a stereotaxic frame and secured using two ear bars and an incisor bar. A midline incision was created. A 6-mm diameter craniotomy was performed on the right side midway between the bregma and the lambda, and the medial edge of the craniotomy was located 1.0 mm lateral to the midline. A single impact device (PCI 3000, Hatteras Instruments, Inc., USA) was used to deliver an impact at a velocity of 2.0 m/sec with 2.5 mm deformation and a dwell time of 150 ms using an impactor tip 4 mm in diameter. After CCI, the removed skull section was immediately replaced and sealed with bone wax; the incision was closed with interrupted 4-0 silk sutures. The sham group underwent the same procedure as the injured rats except for the impact. Following TBI, the rats in the sham group were administered 2 ml/kg of 0.9% physiological saline intravenously at 15 min after the operation. The rats in the vehicle and SSa groups were administered 2 ml/kg of 0.9% physiological saline and 20 mg/kg of SSa intravenously, respectively, 15 min after CCI, then the same dose of SSa or saline was administered daily up to 3 days after CCI. All animals were monitored carefully for at least 4 hours after surgery and then daily. The dose and dosing schedule were decided based on our preliminary experiments (5 mg/kg, 10 mg/kg and 20 mg/kg of SSa).

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Table 1. Modified Neurological Severity Score

Motor tests	5
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on floor (normal=0; maximum=2)	2
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Sensory tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 s)	3
Attempts to balance on beam but falls off (>40 s)	4
Attempts to balance on beam but falls off (>20 s)	5
Falls off; no attempt to balance or hang on to beam (<20 s)	6
Reflex absence and abnormal movements	3
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Maximum points	16

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13-16, severe injury; 7-12, moderate injury; 1-6, mild injury.

Neurobehavioral evaluation

In all animals, neurobehavioral tests were performed before CCI and at 24 h, 48 h and 7 days after CCI by an investigator who was blinded to the experimental groups. Neurological function was measured in terms of neurological severity scores (NSS), a scale of 0-16 (normal score 0; maximal deficit score 16) that assesses functional neurological status based on the presence of certain reflexes and the ability to perform motor and behavioral tasks (**Table 1**) [12].

Measurement of water content in traumatic brain tissue

Brain edema was evaluated through measurements of tissue water content. Water content in the CCI hemisphere was measured by the wet-dry weight method as described previously [13]. Briefly, rats were sacrificed 24 h or 7 days after CCI under 10% chloride hydrate anesthesia. Then, the right and left hemispheres were

separated, and the wet weight of the right hemisphere was immediately obtained. The tissue was then dried in an oven at 120°C for 24 h and weighed again to obtain the dry weight. Tissue water content (%) was calculated as $\text{Water content (\%)} = (\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$.

Evaluation of blood-brain barrier (BBB) integrity

BBB integrity was investigated by assessing the extravasation of Evans blue dye (EBD) as previously described [14]. Briefly, rats were sacrificed 24 h or 7 days after CCI under 10% chloride hydrate anesthesia. EBD (2% in saline) was slowly administered intravenously (4 mg/kg) and allowed to circulate for 2 h prior to sacrifice. The rats were then perfused with 0.9% physiological saline to wash away any remaining dye in the blood vessels until clear perfusion fluid was obtained from the right atrium. After the animals were decapitated, the brains

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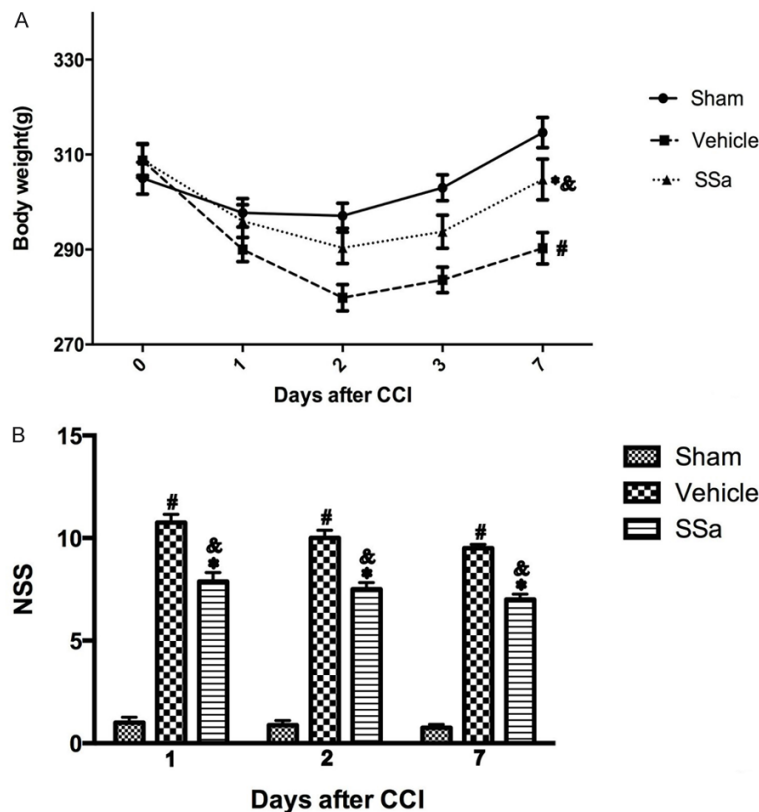


Figure 2. Effects of SSa on the body weight loss and NSS after 7 days of CCI. Vehicle or SSa was injected intravenously 15 min after CCI, up to 3 days. Data were presented as mean \pm SEM (n=8). NSS: neurological severity scores; CCI: Controlled cortical impact; SSa: Saikosaponin a. (#P<0.01 vs. sham-operated rats; &P<0.05 vs. sham-operated rats, *P<0.05, **P<0.01 vs. vehicle-treated rats).

were removed. The right hemisphere was dissected and incubated in 5 ml of formamide (Lot SHBC0489V, Sigma, St. Louis, MO, USA) at room temperature for 3 days. The solutions were then centrifuged for 15 min at 12,000 \times g. The supernatant solution was collected, and EBD tissue levels were assessed using a multi-functional microplate reader (St. Louis, MO, USA) at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Sample values were compared with EBD standards mixed with the solvent (0.625-20 μ g/ml). The hemisphere was dried in a 120 $^{\circ}$ C incubator for 24 h, and the dry weight was obtained after cooling. EBD levels in the hemisphere were expressed as μ g/mg dry weight.

Neuroinflammation

IL-6 and TNF- α levels in the ipsilateral cortex were assayed at 24 h or 7 days after CCI using the MSD multi-array assay system for rodent

cytokines (BMS622, BMS625; eBioscience, San Diego, CA, USA) according to the kit directions.

Western blot

The ipsilateral cortex tissues were homogenized in RIPA buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1% Triton X-100) containing PMSF (R&D Systems Inc., Minneapolis, MN, USA). The homogenates were rocked at 4 $^{\circ}$ C for 30 min and centrifuged at 12,000 \times g for 20 min. Equal amounts of protein (50 μ g of total protein) were loaded into each lane and subject to SDS-PAGE under reducing conditions. The samples were then electroblotted onto nitrocellulose filter membranes (Millipore Inc., MA, USA). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) at room temperature for 2 h and incubated overnight at 4 $^{\circ}$ C with anti-AQP-4 antibody (1:500; Abcam, Cambridge, UK), anti-MMP-9 antibody

(1:1000; Abcam, Cambridge, UK), anti-occludin antibody (1:250; Abcam, Cambridge, UK), anti-p38 MAPK antibody (1:200; Abcam, Cambridge, UK), or anti-c-JNK antibody (1:2000; Abcam, Cambridge, UK). The membranes were washed TBST three times for a total 15 min. The horseradish peroxidase (HRP)-linked secondary antibody (1:5000 dilution) was incubated with the membrane for 1 h at room temperature. Excess antibody was removed with thrice TBST washes for a total of 15 min before incubation in ECL (Bio-Rad) for 1 min. The images were quantified using the Bio-Rad Quantity One software. The rats in the sham group were considered experimental controls for comparison with the experimental groups.

Cognition testing

Long-term beneficial effects of SSa on impairment of cognition were determined up to 7 days after CCI in rats. Cognitive function was assessed using a Morris water maze test, as

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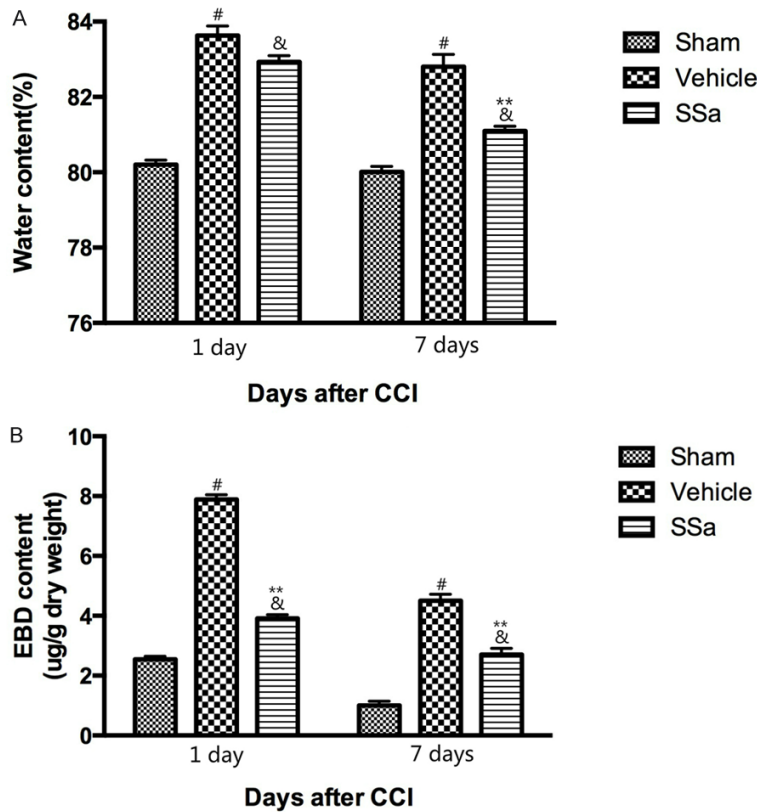


Figure 3. Effects of SSa on the percent of water content (A) and the contents of EBD (B) in injured hemisphere after CCI. Vehicle or SSa was injected intravenously 15 min after CCI, up to 3 days. (A) water content (n=8); (B) EBD content (n=8). Data were presented as mean \pm SEM. CCI: Controlled cortical impact; EBD: Evan's blue dye. (##P<0.001 vs. sham-operated rats; &P<0.05 vs. sham-operated rats, *P<0.05, **P<0.01 vs. vehicle-treated rats).

described previously [15]. Briefly, using a water maze pool [16], on post-operative day (POD) 1 (orientation), the rats were acclimated to the testing room for 30 min. Each rat was tested with four consecutive trials per day for four consecutive days from POD 1 to POD 7. If a rat failed to find the platform within 60 sec, it was guided there (latency recorded 60 sec). In each case, the rat was allowed to remain on the platform for 10 sec. At the end of the experiment, the rats were dried and kept warm. MWM training was recorded using the ZS Dichuang image tracking system (Beijing, China) [16].

Statistical analysis

All data were described as the mean \pm standard error of the mean (SEM). Statistical analysis was performed with SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). Comparing the differences between the sham,

saline-treated, and SSa-treated groups were statistically evaluated by one-way ANOVA with Student's test or Dunnett's test (TNF- α , IL-6, AQP-4, MMP-9, occludin, p38 MAPK and c-JNK levels; brain edema; BBB permeability). Neurological deficits and body weight outcomes were determined using two-way repeated measures ANOVA. Statistical significance was set as P<0.05.

Results

The effects of SSa on body weight loss and NSS after CCI

No significant differences in body weights were noted among all rats before the induction of CCI and sham-operated rats after CCI. CCI rats treated with vehicle solution exhibited increased weight loss compared with sham-operated rats (**Figure 2A**). CCI rats treated with SSa exhibited a marked reduction in weight loss after CCI (**Figure 2A**). No significant differences in neurological deficits were

noted among all rats before the induction of CCI and sham-operated rats after CCI; these rats performed normally. Vehicle-treated rats exhibited significant neurological deficits at 24 h, 48 h and 7 days after CCI (**Figure 2B**).

The effects of SSa on water content and BBB integrity in injured hemisphere after CCI

Figure 3 indicates that the water content percent (%) (**Figure 3A**) and the concentration of EBD (ug/g dry weight) (**Figure 3B**) in vehicle-treated rats were significantly increased 24 h and 7 days after CCI compared with sham-operated rats. SSa treatment significantly reduced the water content percent and the EBD concentration in the injured hemisphere 7 days after CCI (SSa decreased water content 1 day after CCI, although not statistically significant, p>0.05).

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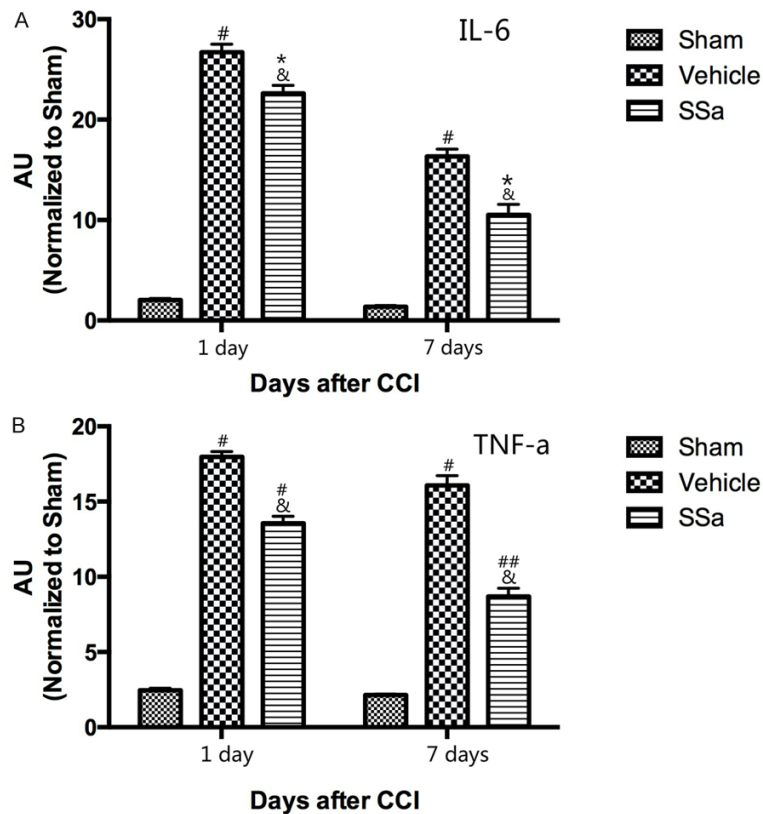


Figure 4. Effects of SSa on the levels of IL-6 and TNF- α in injured hemisphere after CCI. Vehicle or SSa was injected intravenously 15 min after CCI, up to 3 days. A. IL-6 level; B. TNF- α level. Data were presented as mean \pm SEM (n=10). (##P<0.001 vs. sham-operated rats; &P<0.05 vs. sham-operated rats, *P<0.05, **P<0.01 vs. vehicle-treated rats).

The effects of SSa on neuroinflammation after CCI

A significant increase of the levels of IL-6 and TNF- α was noted in the ipsilateral cortex 24 h after CCI (**Figure 4**). Rats treated with SSa exhibited significantly reduced levels of both IL-6 and TNF- α compared with vehicle-treated rats (p<0.05).

The effects of SSa on AQP-4, MMP-9, vehicle, p38 MAPK and c-JNK levels

Compared with vehicle-treated rats, SSa treatment resulted in reduced AQP-4 and MMP-9 levels (**Figure 5**). Occludin levels were increased with SSa treatment compared with vehicle-treated rats (**Figure 5**). SSa treatment markedly decreased the levels of p38 MAPK and c-JNK compared with vehicle-treated rats (**Figure 5**).

The Long-term beneficial effects of SSa: The observation of long-term beneficial effects up

to 7 days suggested that SSa improves cognitive function after TBI (**Figure 6**).

Discussion

In this study, we demonstrated the first time that SSa-treatment attenuated the destruction of the BBB and improved functional recovery after TBI. The therapeutic effects were associated with SSa-induced AQP-4, MMP-9, and inflammatory inhibition, maybe through inhibiting the MAPK signaling pathway.

The functional impairment noted after TBI may be attributed to the early involvement of MMPs in secondary pathogenesis after TBI. Animals treated with an MMP inhibitor within the first 3 days after TBI exhibited less disruption of the BBB and significant functional recovery compared with vehicle-treated rats [17]. Water channels (aquaporins) allow water to cross cell membranes [18]. Specifically, cellular edema is markedly

reduced when the AQP-4 gene is knocked-down in mice [19]. In our study, SSa treatment significantly decreased the level of AQP-4 and MMP-9.

The increased expression of AQP4, MMPs, and inflammatory mediators as well as increased free radical generation during TBI can damage the BBB [20]. The disruption of tight junctions (TJs) and barrier integrity plays a significant role in the pathogenesis of BBB damage in brain injury [20]. Occludin is the major structural proteins of TJs, and its expression is closely related with BBB integrity and brain edema [21]. SSa treatment markedly reduced the loss of occludin.

Neuroinflammation is a component of the secondary injury cascade. Neuroinflammation exerts a negative effect on brain cells and also plays a role in brain edema. A previous study demonstrated that anti-inflammatory agents

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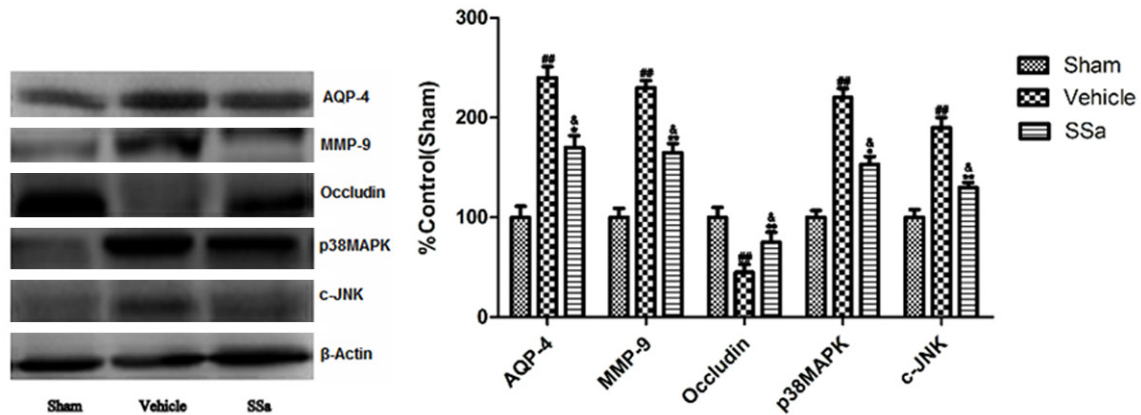


Figure 5. Effects of SSa on the levels AQP-4, MMP-9, occludin, p38 MAPK and c-JNK protein in injured hemisphere after CCI. SSa showed a significant change compared to the vehicle-treated rats. The level was measured by Western blot analysis using actin as a standard control. Data were presented as mean \pm SEM (n=10). (###P<0.001 vs. sham-operated rats; &P<0.05 vs. sham-operated rats, **P<0.01 vs. vehicle-treated rats).

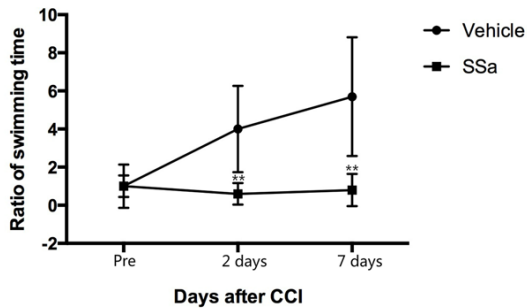


Figure 6. Long-term beneficial effects of SSa. Vehicle or SSa was injected intravenously 15 min after CCI, up to 3 days. The Morris water maze test was performed before and after CCI. Results are expressed as a ratio of swimming time at each time point against the pre-injured value. The results are the mean \pm SEM of 8 rats. (###P<0.001 vs. sham-operated rats; &P<0.05 vs. sham-operated rats, **P<0.01 vs. vehicle-treated rats).

modulate inflammatory mediators, such as IL-6 and TNF- α , that result from TBI and alter the pathophysiology after TBI [6, 22]. SSa significantly reduced the levels of neuroinflammation in the cortex after CCI, resulting in reduced cerebral edema and neurological improvement and supporting previous studies that demonstrated the effect of saikosaponin on the expression of proinflammatory cytokines [6, 22].

The MAPK pathway is associated with inflammatory mediators that are induced by these cytokines. The most important components of the MAPK family are p38MAPK and c-JNK [23].

MAPKs regulate inflammatory mediators, including IL-6 and TNF- α [24]. In our study, we demonstrated that p38 MAPK and c-JNK levels were increased in vehicle-treated rats. However, p38 MAPK and c-JNK levels were significantly reduced in SSa-treated rats, demonstrating that SSa may inhibit the MAPK signaling pathway in TBI. Thus, we hypothesize that SSa-mediated neuroprotection may be associated with its inhibitory effect on the MAPK signaling pathway.

Bupleurum maybe plays a role on the treatment of post-stroke depression (PSD) through inhibiting the apoptosis by means of intervention with reducingBax protein expression and increasing the expression of Bcl-2 [25]. Saikosaponin played an anti-apoptotic role through differential regulation of mitochondrial and nuclear GR translocation [26]. However, the other research has showed that SSa can also inhibit the proliferation of cancer cells in a dose-dependent manner because of its apoptotic effect [27, 28]. In the future, we will detect whetherthe anti-apoptotic effect of SSais as a potential mechanism for its neuroprotection. Because of the absence of severe side effects, SSa may be applicable for the treatment of TBI.

Conclusion

In conclusion, we demonstrate that SSa counteracts the inflammatory response and neurological function deficits after TBI, possibly through an anti-inflammatory response and

inhibition of the MAPK signaling pathway. SSa may serve as an effective therapeutic target for patients with TBI-induced brain edema and neurological function deficits.

Acknowledgements

The authors report no conflict of interests related to this study or the findings specified in this paper. This study was supported by National Natural Science Foundation of China (No. 81171144, No. 81471238, No. 81341059, No. 81371610), Beijing Nova program (No. 2012033) and National Key Technology Research and Development Program of the Ministry of Science and Technology of China (2013BAI09B03).

Disclosure of conflict of interest

None.

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