

## Original Article

# 3'-Daidzein sulfonate sodium provides neuroprotection by promoting the expression of the $\alpha 7$ nicotinic acetylcholine receptor and suppressing inflammatory responses in a rat model of focal cerebral ischemia

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**Abstract:** In a previous study using a rat model of focal cerebral ischemia/reperfusion (I/R) injury, we found that 3'-Daidzein sulfonate sodium (DSS), a derivative of daidzein, exerts neuroprotective effects by alleviating brain edema and reducing levels of interleukin (IL)-6. The present study was designed to further examine the potential mechanisms of the neuroprotective properties of DSS in a rat model of cerebral I/R injury. We found that treatment with DSS ameliorated neurological deficit, infarct size, and cerebral water content in rats with cerebral I/R injury. Moreover, treatment with DSS significantly reduced the levels of IL-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  in serum and in the ischemic penumbra. Additionally, DSS treatment increased the expression of nicotinic acetylcholine receptor alpha 7 ( $\alpha 7$ nAChR), and inhibited the expression of glial fibrillary acidic protein, phosphorylated p65 nuclear factor  $\kappa$ B, and phosphorylated inhibitor of NF- $\kappa$ B $\alpha$ , suggesting that DSS provides neuroprotection by suppressing inflammatory responses after focal cerebral I/R injury. Notably,  $\alpha$ -bungarotoxin, an antagonist of  $\alpha 7$ nAChR, reversed the neuroprotective effects of DSS after cerebral I/R injury, suggesting that inhibition of  $\alpha 7$ nAChR expression is sufficient for reversal of the neuroprotective effects of DSS. In conclusion, we found that DSS treatment provides neuroprotection by promoting  $\alpha 7$ nAChR expression in a rat model of focal cerebral ischemia, thus establishing  $\alpha 7$ nAChR as a potential therapeutic target in cerebral I/R injury.

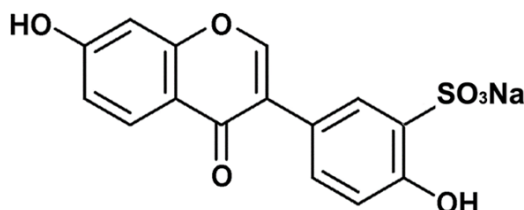
**Keywords:** 3'-Daidzein sulfonate sodium, cerebral ischemia/reperfusion, pro-inflammatory cytokines, nicotinic acetylcholine receptor alpha 7

## Introduction

Molecular mechanisms that underlie central nervous system (CNS) injury by ischemic stroke are quite complex, primarily due to the activation of multiple pathways associated with inflammation, oxidative stress, energy metabolism and excitatory amino acid toxicity, free radical formation, calcium overload, and mitochondrial dysfunction [1, 2]. Cerebral inflammation plays an important role in the pathogenesis and progression of ischemic stroke [3]. There is increasing evidence that the levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6, are upregulated after cerebral ischemia [4]. In addition, the inflammation-related transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B), is

markedly upregulated and activated in ischemic brain regions [5]. Therefore, anti-inflammatory treatments have been proposed to reduce ischemic cerebral damage and improve clinical outcomes after ischemic insult.

Release of acetylcholine (ACh), the primary neurotransmitter elicited by the vagus nerve, significantly attenuated the release of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-18) in lipopolysaccharide-stimulated human macrophages [6]. This physiological mechanism is commonly known as the cholinergic anti-inflammatory pathway (CAP), and is mediated by the activity of nicotinic acetylcholine receptor alpha 7 ( $\alpha 7$ nAChR) [7]. A previous study reported the expression of  $\alpha 7$ nAChRs on the surface of hippocampal astrocytes; activation of these re-



**Figure 1.** Chemical structure of 3'-Daidzein sulfonate sodium (DSS).

ceptors inhibited inflammatory responses [8]. Similarly, treatment with PNU-120596, which activates the expression of  $\alpha 7$ nAChRs, significantly improved neuronal survival in cerebral ischemia by augmenting the neuroprotective effects of endogenous choline/Ach [9]. Therefore,  $\alpha 7$ nAChR appears to be an important anti-inflammatory target for therapeutic intervention.

3'-Daidzein sulfonate sodium (DSS) is a novel, synthetic, water-soluble compound, which was developed by modifying the structure of daidzein, an active ingredient of the kudzu vine root. We previously showed that DSS exerted protective effects in a rat model of focal cerebral I/R injury by reducing brain edema, improving mitochondrial function, and inhibiting IL-6 expression and neuronal apoptosis in brain tissue [10-12]. Nevertheless, possible mechanisms involved in the activity of DSS after cerebral I/R injury have not been fully elucidated.

In the present study, we aimed to further investigate the mechanisms that mediate the neuroprotective properties of DSS. Therefore, we assessed the effects of DSS on the production of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ), as well as its effects on the regulation of glial fibrillary acidic protein (GFAP, an astrocyte marker),  $\alpha 7$ nAChR expression levels, and NF- $\kappa$ B signaling pathway components, in focal cerebral I/R injury. Additionally, we performed treatment with  $\alpha$ -bungarotoxin ( $\alpha$ -BuTX), an  $\alpha 7$ nAChR antagonist, to elucidate the role of  $\alpha 7$ nAChRs in DSS-mediated neuroprotection in a cerebral I/R injury model.

## Materials and methods

### DSS

DSS, a white crystalline powder, was provided by the Shenyang Pharmaceutical University Department of Naturally Occurring Drugs and Chemistry (China); it exhibited a purity of >99%.

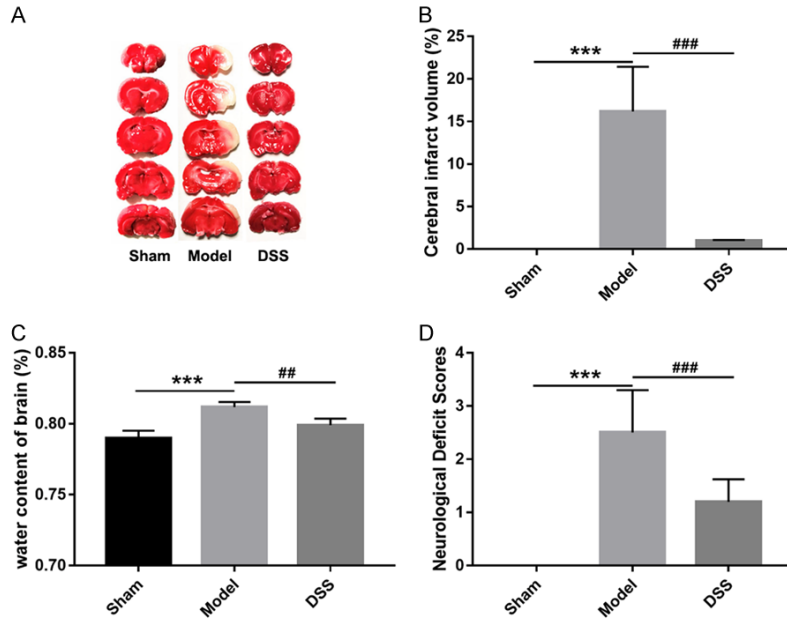
The compound was diluted to the required concentration with double-distilled water before use in experiments. The chemical structure of DSS is shown in **Figure 1**.

### Animals

Adult male Sprague-Dawley rats (weight = 250-280 g) were purchased from Hunan Silaike Jingda Laboratory Animal Co., Ltd. (China). The rats were maintained on a 12-hour light/dark cycle and given free access to food and water; they were adapted to these conditions for at least 7 days before experiments were performed. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US NIH (Publication No. 85-23, revised 1985) and were approved by the Experimental Animal Ethics Committee of Gannan Medical University (Permit No. SYXK (Gan) 2012-0006). In accordance with these guidelines, efforts were made to minimize animal suffering and to reduce the number of animals used.

### Focal cerebral I/R model and animal grouping

The rats used in our experiments were divided randomly into 5 groups: sham surgery, cerebral I/R injury (model group), 2.0 mg/kg DSS treatment (DSS group) [10, 11], 2.0 mg/kg DSS plus 0.5  $\mu$ M  $\alpha$ -BuTX (DSS +  $\alpha$ -BuTX group) [13], and 2.0 mg/kg DSS plus normal saline treatment (DSS + vehicle group). The rats were anesthetized with an intraperitoneal (ip) injection of 10% chloral hydrate (350 mg/kg). Transient focal cerebral ischemia was induced as previously described by Longa *et al.* [14]. In brief, the right carotid rejoin was exposed through a ventral midline cervical incision. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated. A 4-cm length of fish nylon thread, with 0.28-mm-diameter body and 0.38-mm-diameter tip, was introduced from the ECA into the ICA until mild resistance was felt (18-20 mm). In this manner, the thread occluded the origin of the middle cerebral artery (MCA). Two hours after occlusion of the MCA, the thread was removed to allow reperfusion of the ischemic area. Sham-operated animals were subjected to the same surgical procedures, but the MCA was not occluded. The rectal temperature was maintained at 37-38°C with a heat lamp and heating pad during the operation. The room temperature was controlled within the range of 25-27°C throughout the experimental proce-



**Figure 2.** Effects of DSS treatment on brain infarct size, neurologic deficit scores, and water content rate in an ischemia/reperfusion (I/R) injury rat model. A and B. Brain infarct size was measured by TTC staining after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. C. The cerebral water content was calculated by the wet and dry weight method after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. D. Neurological deficit scores were assessed by Longa's scores after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model and without DSS treatment in the sham group. Data are expressed as mean  $\pm$  SD. \*\*\*P<0.001 versus the sham group. ##P<0.01 and ###P<0.001 versus the I/R model group.

dure. Ten minutes after the induction of ischemia, the drug treatment groups were administered 2.0 mg/kg DSS, with or without 0.5 ml/kg  $\alpha$ -BuTX, via the sublingual vein. The sham surgery and model groups were administered normal saline (1 ml/kg body weight) via the sublingual vein.

#### Evaluation of neurological deficit scores

The neurological function of the rats was evaluated at 24 hours after reperfusion by using a 5-point rating scale as follows: 0, no neurologic deficit; 1, failure to extend left forepaw fully (a mild focal neurologic deficit); 2, circling to the contralateral side (a moderate focal neurologic deficit); 3, falling to the contralateral side (a severe neurologic deficit), and 4, no spontaneous walk and unconsciousness.

#### Cerebral infarct volume measurement

Twenty-four hours after the assessment of neurological deficit, rats were deeply anesthetized

and sacrificed; fresh brains were rapidly removed, harvested, and frozen at  $-20^{\circ}\text{C}$  for 20 minutes. The brains were then coronally sectioned into 2-mm-thick sections with a stainless-steel mold and stained in a 0.5% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) solution for 10 minutes at  $37^{\circ}\text{C}$  in the dark. After staining, non-ischemic regions were colored red, while the infarct regions were white. The sections were then fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS. Stained slices were photographed with a digital camera. The infarct area was quantified by a DT-200 image analysis system. The infarct volume was calculated by using the previously reported formula for corrected infarct volume [11]. Total infarct volumes were calculated as the sum of all section volumes multiplied by section thickness (2 mm). Infarct

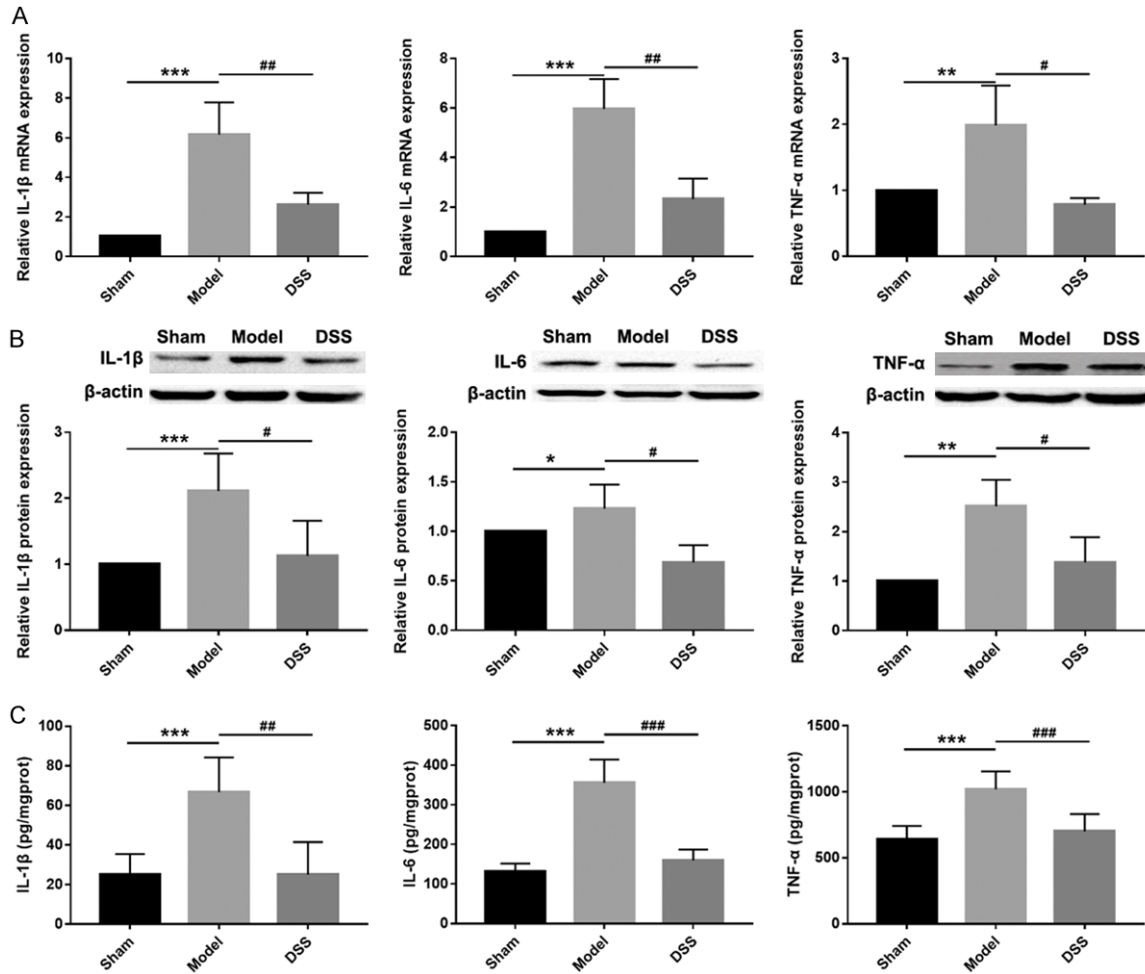
volume data were expressed as a percentage of total brain area.

#### Determination of brain water content

Twenty-four hours after reperfusion, rats were anesthetized with 10% chloral hydrate (350 mg/kg, ip) and their brains were removed. The cerebellum, lower brain stem, and olfactory bulb were subsequently dissected out, washed with normal saline, and dried with filter paper. The forebrain samples were then weighed to obtain the wet weight and were dried at  $105^{\circ}\text{C}$  for 24 hours before obtaining the dry weight measurements. Brain water content (%) was calculated as follows:  $[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%$ .

#### Radioimmunoassay

Twenty-four hours after reperfusion, rats were anesthetized. Blood was obtained via a vein in the abdominal cavity and centrifuged at  $4^{\circ}\text{C}$  for 10 minutes at  $3,000 \times g$ ; resulting serum was



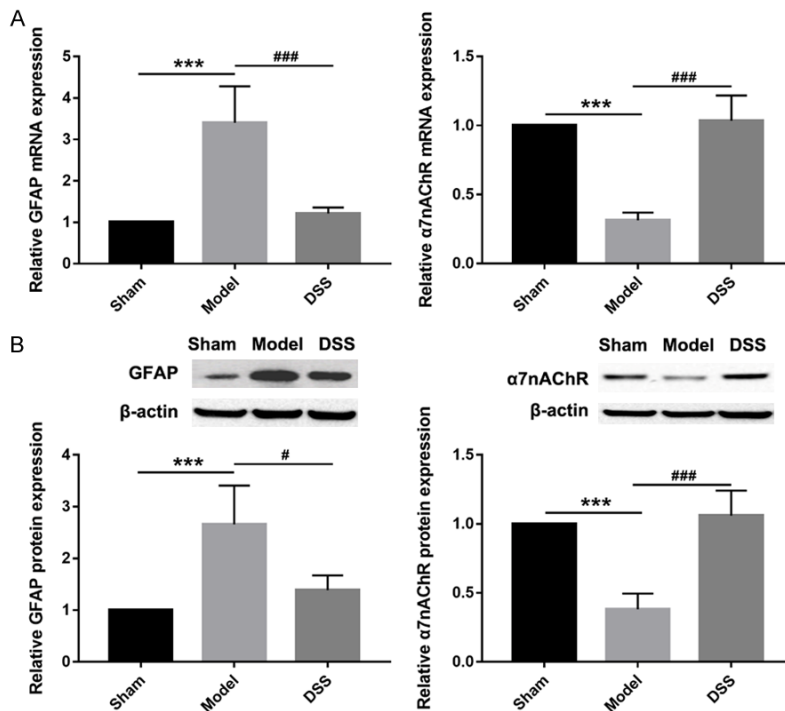
**Figure 3.** Effects of DSS treatment on the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in an I/R injury rat model. A. The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the ischemic penumbra were measured by qRT-PCR after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. B. The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the ischemic penumbra were evaluated by western blotting analysis after 24 hours of treatment with or without 2.0 mg/kg DSS treatment in the injury model and without DSS treatment in the sham group. C. The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in serum were measured by radioimmunoassay after 24 hours of treatment with or without 2.0 mg/kg DSS treatment in the injury model, and without DSS treatment in the sham group. Data are expressed as mean  $\pm$  SD. \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 versus the sham group. # $P$ <0.05, ## $P$ <0.01, and ### $P$ <0.001 versus the I/R model group.

stored at  $-80^{\circ}\text{C}$ . The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the serum were determined by using commercially available radioimmunoassay kits (Beijing Furui Bioengineering Co., Beijing, China), in accordance with the manufacturer's protocol.

#### Western blotting analysis

After 24 hours of reperfusion, rats were anesthetized. The ischemic penumbra portion of the cortex was rapidly isolated from the right fore-brain on ice, then rapidly placed in liquid nitrogen. Protein samples were generated from fro-

zen cortex homogenized with lysis buffer (Tris-HCl (pH 7.5) 50 mM, NaCl 250 mM, EDTA 10 mM, NP-40 0.5%, Leupeptin 10  $\mu\text{M}$ , PMSF 1 mM, and NaF 4 mM). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated with primary antibodies against  $\alpha 7$ nAChR, GFAP (Santa-Cruz Biotechnology, Santa Cruz, CA, USA), nuclear factor- $\kappa\text{B}$ -p65 (p65-NF- $\kappa\text{B}$ ), phosphorylated p65 nuclear factor  $\kappa\text{B}$  (p-p65-NF- $\kappa\text{B}$ ), inhibitor of NF- $\kappa\text{B}$  ( $\text{I}\kappa\text{B}\alpha$ ), p- $\text{I}\kappa\text{B}\alpha$  (Cell Signaling Technology, Beverly, MA, USA), and  $\beta$ -actin (Zhongshanjingqiao, Beijing, China). After



**Figure 4.** Effects of DSS treatment on the expression of GFAP and  $\alpha 7$ nAChR in an I/R injury rat model. A. The expression levels of GFAP and  $\alpha 7$ nAChR in the ischemic penumbra were measured by qRT-PCR after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. B. The expression levels of GFAP and  $\alpha 7$ nAChR in the ischemic penumbra were evaluated by western blotting analysis after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. Data are expressed as mean  $\pm$  SD. \*\*\* $P < 0.001$  versus the sham group. # $P < 0.05$  and ### $P < 0.001$  versus the I/R model group.

the membranes were washed with Tris-buffered saline + Tween, horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, rabbit anti-goat IgG, and goat anti-mouse IgG; Zhongsanjingqiao) were applied. The blots were developed by using ECL western blotting detection reagents (Thermo Fisher Scientific, Waltham, MA, USA). The images were obtained by super sensitivity chemiluminescence imaging system (Universal Hood II, Bio-Rad, Hercules, CA, USA). Densitometry analysis of bands was performed with the Image Lab 3.0 software. Results were expressed as the ratio of target protein gray value to  $\beta$ -actin gray value. Images of three independent experiments in each group.

#### Real-time quantitative PCR (qRT-PCR)

Total RNA from the ischemic penumbra of the cortex was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. The quantity and

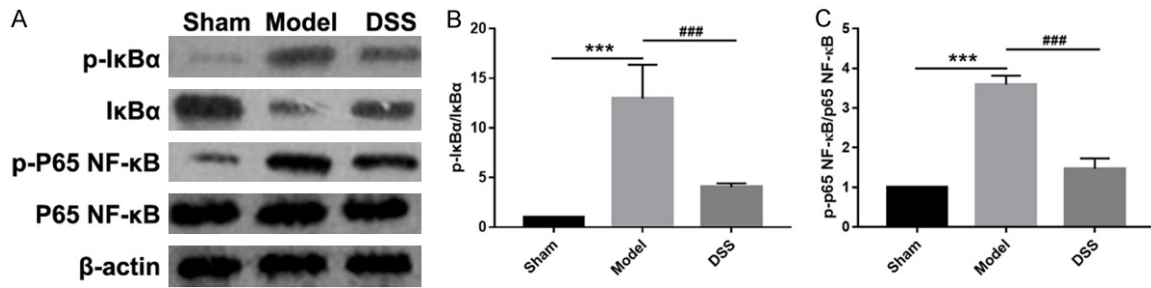
quality of the total RNA were determined by using an Automatic Microplate Reader (Thermo Fisher Scientific). cDNA was synthesized from 2  $\mu$ g total RNA with the M-MLV First-Strand Kit (Invitrogen) and used as the template for subsequent real-time PCR. The PCR cycling conditions were 95°C for 5 minutes, followed by two-step cycling conditions: 40 cycles of 95°C for 20 seconds, 60°C for 60 seconds, followed by a melting curve from 60°C to 95°C. This protocol was performed on the ViiATM 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR was performed with the following primers: IL-1 $\beta$  forward primer 5'-CACCTCAATGGA-CAGAACATAAGC-3' and reverse primer 5'-TCTTCTTT-GGGTATTGTTTGGGA-3', IL-6 forward primer 5'-AGC-ATACAGTTTGTGGACATT-3' and reverse primer 5'-CA-ACATTCATATTGCCAGTTCT-3', TNF- $\alpha$  forward primer 5'-

CCACCACGCTCTTCTGTCTACTG-3' and reverse primer 5'-GGGCTACGGGCTTGCTACTC-3',  $\alpha 7$ nAChR forward primer 5'-GGCTCTGCTGGTATTC-TTGCTG-3' and reverse primer 5'-TGGTGCTG-GCGAAGTATTGTG-3', GFAP forward primer 5'-GCAAGAAACAGAAGAGTGGTATCG-3' and reverse primer 5'-CTCTCCAAGGACTCGTTCGTG-3', GAPDH forward primer 5'-CCCATCACCATCTT-CCAGGAG-3' and reverse primer 5'-GTTGTCA-TGGATGACCTTGGC-3'. GAPDH was used as an endogenous control. Relative expression was calculated in terms of the fold change by using the  $2^{-\Delta\Delta Ct}$  method. All experiments were performed in triplicate.

#### Statistical analysis

Data were analyzed by using the Statistical Package for the Social Sciences (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA). All experimental data were expressed as the mean  $\pm$  standard deviation (SD). Comparisons of the sham model and DSS-treated groups were per-





**Figure 5.** Effects of DSS on the expression of p-IkB $\alpha$ , IkB $\alpha$ , p-p65 NF- $\kappa$ B, and p65 NF- $\kappa$ B in an I/R injury rat model. A. Representative images depicting the expression of p-IkB $\alpha$ , IkB $\alpha$ , p-p65 NF- $\kappa$ B, and p65 NF- $\kappa$ B in the ischemic penumbra, evaluated by western blotting analysis, after 24 hours of treatment with or without 2.0 mg/kg DSS in the injury model, and without DSS treatment in the sham group. B. The ratio of p-IkB $\alpha$ /IkB $\alpha$  was calculated in each of the three groups. C. The ratio of p-p65 NF- $\kappa$ B/p65 NF- $\kappa$ B was calculated in each of the three groups. Data are expressed as mean  $\pm$  SD. \*\*\*P<0.001 versus the sham group. ###P<0.001 versus the I/R model group.

formed by using a one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test. DSS-treated groups with or without  $\alpha$ -BuTX were compared by using an independent-sample test. P<0.05 was considered statistically significant.

## Results

### DSS reverses neurological deficits following cerebral I/R injury

Twenty-four hours after treatment, measurements were performed of brain infarct size, neurologic deficit scores, and water content rates (Figure 2). At 24 hours after cerebral I/R, the infarction areas of the right cerebral hemisphere were colored white, mainly in the frontal and parietal cortex and caudate putamen. Normal brain tissue appeared red and portions of the tissue represented transition zones of white to red from the penumbral area of the cerebral I/R injury. Compared with the sham group, the brain infarct size, neurologic deficit scores, and water content rates were elevated in the injury model group. Brain infarct size, neurologic deficit scores, and water content rates in the DSS-treated group were significantly lower than in the model group.

### Treatment with DSS lowers levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ during cerebral I/R injury

As shown in Figure 3, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels in the collected serum and in the ischemic penumbra were significantly greater in the injury model group, compared with the sham group. After treatment with DSS, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels were significantly reduced, compared with the injury model group.

### DSS suppresses GFAP expression and promotes $\alpha 7$ nAChR expression regulated by cerebral I/R injury

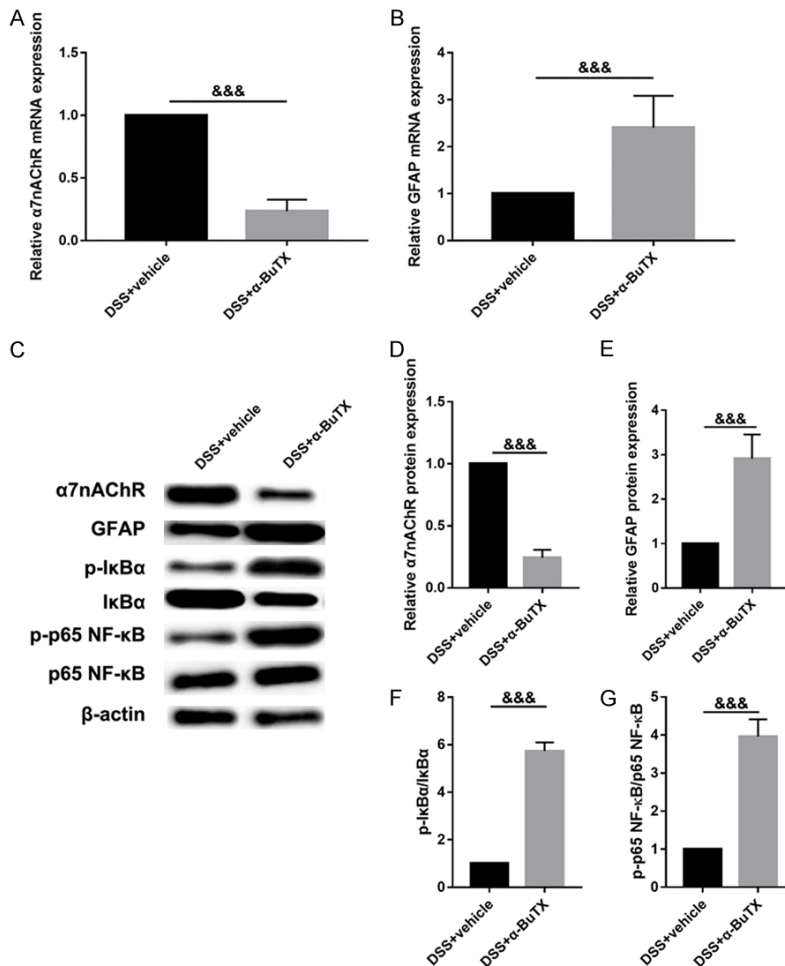
As shown in Figure 4, mRNA and protein expression levels of GFAP in the ischemic penumbra were significantly increased in the injury model group compared with the sham group. mRNA and protein expression levels of GFAP in DSS treatment group were significantly decreased compared with the injury model group. mRNA and protein expression levels of  $\alpha 7$ nAChR were significantly reduced in the ischemic penumbra compared with the sham group. mRNA and protein expression levels of GFAP in DSS treatment group were significantly increased compared with the injury model group.

### Treatment with DSS reduces the expression of NF- $\kappa$ B during cerebral I/R injury

As shown in Figure 5, p-IkB $\alpha$  and p-p65 NF- $\kappa$ B expression levels in the ischemic penumbra were significantly greater in the injury model group, compared with the sham group. In addition, careful analysis of the injury model group revealed that p-IkB $\alpha$  and p-p65 NF- $\kappa$ B expression levels were significantly reduced after treatment with DSS.

### Inhibition of $\alpha 7$ nAChR expression reverses the neuroprotective effects of DSS in cerebral I/R injury

To study the effects of DSS-mediated activation of  $\alpha 7$ nAChR activity, expression of  $\alpha 7$ nAChR was inhibited via treatment with its antagonist,  $\alpha$ -BuTX. After 24 hours of treatment,  $\alpha 7$ nAChR expression in the DSS +  $\alpha$ -BTX group was significantly reduced, compared with the DSS +



**Figure 6.** Inhibition of  $\alpha 7$ nAChR expression by  $\alpha$ -BuTX treatment reverses the effect of DSS on GFAP, p-IkB, and p-p65 NF- $\kappa$ B levels in a cerebral I/R injury rat model. A and B. The expression levels of GFAP and  $\alpha 7$ nAChR were measured by qRT-PCR after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the ischemic penumbra of the injury rat model. C. Representative images of the expression of  $\alpha 7$ nAChR, GFAP, p-IkB $\alpha$ , IkB $\alpha$ , p-p65 NF- $\kappa$ B, and p65 NF- $\kappa$ B, evaluated by western blotting analysis, after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the ischemic penumbra of the injury rat model. D and E. The relative expression levels of  $\alpha 7$ nAChR and GFAP were evaluated by western blotting analysis after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the ischemic penumbra of the injury rat model. F and G. The ratios of p-IkB $\alpha$ /IkB $\alpha$  and p-p65 NF- $\kappa$ B/p65 NF- $\kappa$ B were calculated after treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, for 24 hours in the ischemic penumbra of the injury rat model. Data are expressed as mean  $\pm$  SD. \*\*\* $P$ <0.001 versus the DSS + vehicle group.

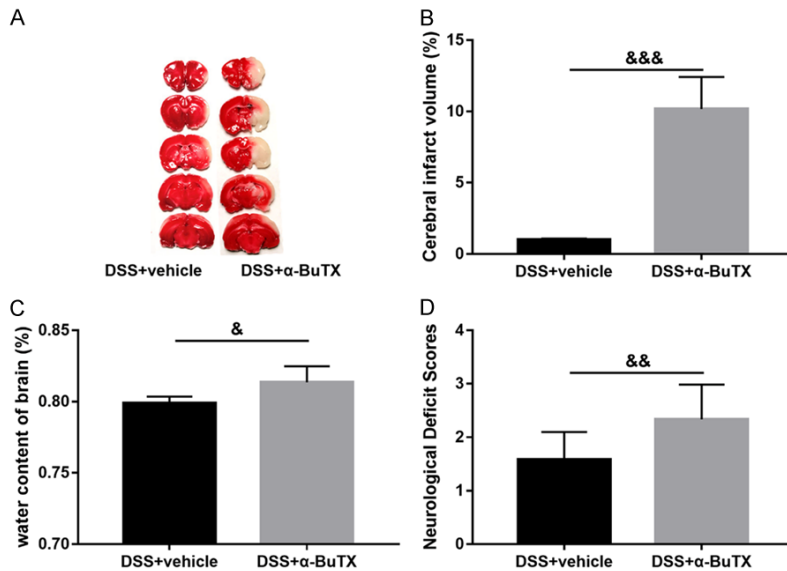
vehicle group, suggesting that  $\alpha$ -BuTX inhibited the expression of  $\alpha 7$ nAChR (Figure 6A, 6C and 6D). Additionally, the expression levels of GFAP, p-IkB, and p-p65 NF- $\kappa$ B in the DSS +  $\alpha$ -BTX group were significantly greater than those in the DSS + vehicle group (Figure 6B, 6C and 6E-G). Compared with the DSS + vehicle group, brain infarct size, neurologic deficit scores, and

water content rates were significantly elevated in the DSS +  $\alpha$ -BTX group (Figure 7). Furthermore, the levels of the inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the DSS +  $\alpha$ -BuTX group were significantly greater than those in the DSS + vehicle group (Figure 8).

## Discussion

In our study, we demonstrated that peripheral and CNS inflammatory response signals were activated after cerebral I/R injury. Inflammatory cytokines are known to be involved in the process of cerebral I/R injury, and play an important role in the development of CNS disorders [15]. It is well-known that the levels of pro-inflammatory cytokines (e.g., IL-1 $\beta$  and TNF- $\alpha$ ) increase in serum and in the affected hemisphere of the brain within hours after ischemic insult. Subsequently, these cytokines enhance the expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells and leukocytes; this facilitates the adhesion and transendothelial migration of leukocytes. Through these mechanisms, acute cerebral ischemia triggers an inflammatory cascade, which causes brain edema and further neuronal injury.

Accordingly, inhibiting the expression of IL-1 $\beta$  and TNF- $\alpha$  appears to reduce ischemic brain damage [16]. Consistent with these previous findings, the present study showed that cerebral ischemia induced the production of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ; moreover, it markedly enhanced brain infarct size, neurologic deficit scores, and water content rates, promoting neurological deficits. Importantly,



**Figure 7.** Inhibition of  $\alpha 7$ nAChR expression by  $\alpha$ -BuTX reverses the effects of DSS on brain infarct size, neurologic deficit scores, and water content rate in an I/R injury rat model. A and B. Brain infarct size was measured by TTC staining after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg  $\alpha$ -BuTX. C. Cerebral water content was calculated by the wet and dry weight method after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg  $\alpha$ -BuTX. D. Neurological deficit scores were assessed by Longa's scores after 24 hours of treatment with 2.0 mg/kg DSS, with or without 0.5 ml/kg  $\alpha$ -BuTX. Data are expressed as mean  $\pm$  SD. &P<0.05, &&P<0.01, and &&&P<0.001 versus DSS + vehicle group.

the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , as well as the elevations in brain infarct size, neurologic deficit scores, and water content rates, were effectively suppressed by treatment with the DSS compound. Therefore, our results suggest that DSS treatment suppresses pro-inflammatory cytokines and subsequently improves neurological deficits.

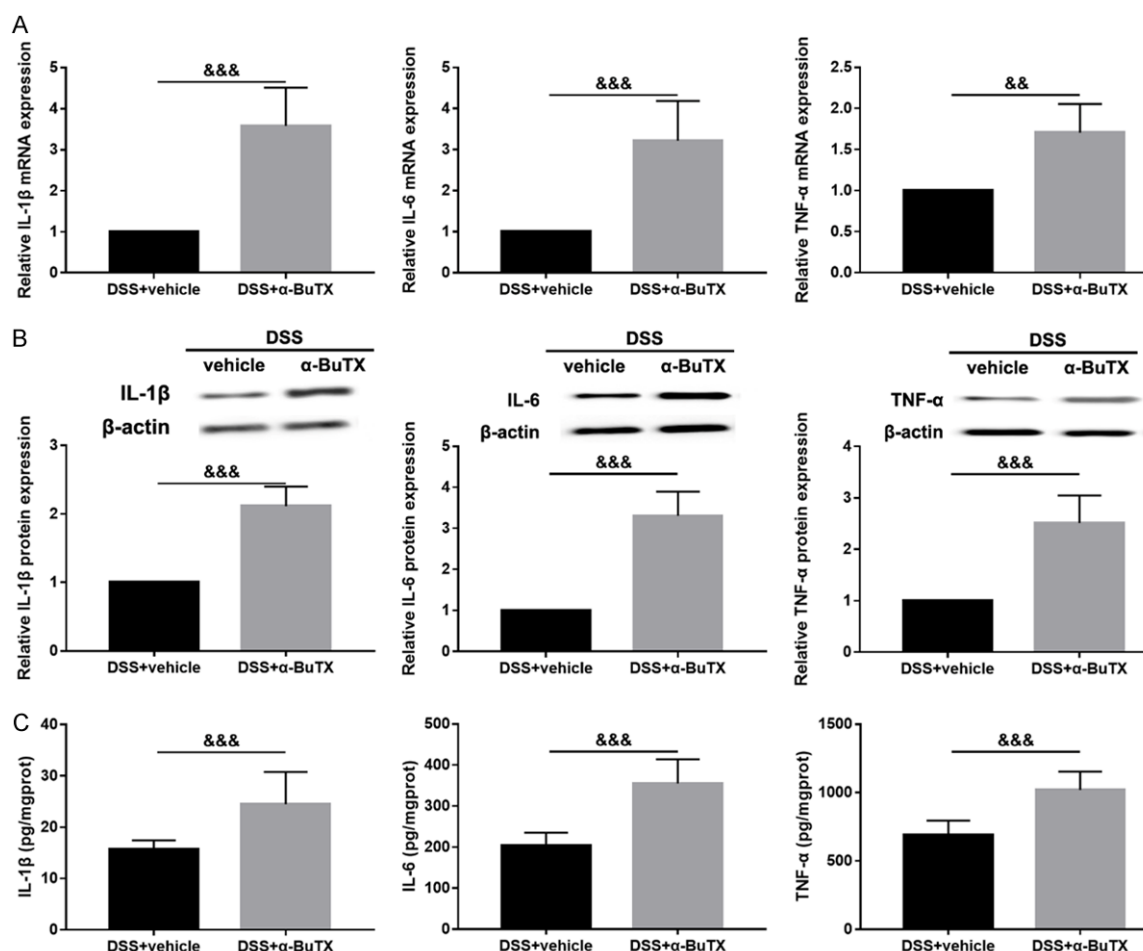
During the early stages of I/R cerebral injury, astrocytes are able to protect neurons from injury via housekeeping mechanisms [17]. However, after substantial progression of brain ischemia, astrocytes are rapidly over-activated, thus promoting morphological transformations and elevating the expression of pro-inflammatory cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , and IL-6), which aggravates the injury and exacerbates the formation of brain edema [18]. Therefore, the inhibition of excessive astrocyte activation is an attractive target to induce neuroprotective effects after I/R injury [19]. GFAP is an astrocyte marker that is detected after cerebral I/R injury. In the present study, we demonstrated a clear increase in the expression of GFAP after cerebral I/R injury; this was alleviated after DSS treatment. Our results thus suggest that

DSS exerts neuroprotective effects through the inhibition of excessive astrocyte activation, thereby attenuating the release of pro-inflammatory cytokines.

NF- $\kappa$ B is an important transcription factor involved in many physiological and pathological processes, such as inflammation and apoptosis [20]. Activated NF- $\kappa$ B translocates from the cytoplasm into the nucleus, where it is associated with the production of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8. This begins a vicious cycle of increased levels of TNF- $\alpha$  and IL-1 $\beta$ , which further activate NF- $\kappa$ B and enhance the production of pro-inflammatory cytokines. Vagus nerve stimulation has been shown to attenuate the disruption of tight junctions in the intestinal epithelium of endotoxemic mice; this is mediated by suppressing p65 NF- $\kappa$ B translocation and  $\alpha 7$ nAChR activation through inhibition of NF- $\kappa$ B [21]. In the present study, I/R cerebral injury decreased  $\alpha 7$ nAChR expression levels, promoted the expression of p-I $\kappa$ B $\alpha$  and p-p65 NF- $\kappa$ B protein in the ischemic penumbra. Notably, treatment with DSS was sufficient to reverse these changes. These results suggest that the activation of  $\alpha 7$ nAChR may exert an anti-inflammatory effect in cerebral I/R injury by inhibiting the activation of NF- $\kappa$ B and downstream production of pro-inflammatory cytokines. By using the antagonist,  $\alpha$ -BuTX, we found that the inhibition of  $\alpha 7$ nAChR expression levels was sufficient to increase the expression of both p-I $\kappa$ B $\alpha$  and p-p65 NF- $\kappa$ B, which are activated through the NF- $\kappa$ B signaling pathway. This antagonist-mediated inhibition promoted the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ; it also enhanced brain infarct size, neurologic deficit scores, and water content rates, reversing the neuroprotection obtained through treatment with DSS.

In summary, we showed that treatment with DSS effectively suppresses the NF- $\kappa$ B signaling pathway, inhibits the pro-inflammatory respon-





**Figure 8.** Inhibition of  $\alpha 7$ nAChR expression by  $\alpha$ -BuTX reverses the effects of DSS on the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in an I/R injury rat model. **A.** The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured by qRT-PCR after 24 hours of treatment with 2.0 mg/kg of DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the ischemic penumbra of the injury rat model. **B.** The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were evaluated by western blotting analysis after 24 hours of treatment with 2.0 mg/kg of DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the ischemic penumbra of the injury rat model. **C.** The levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured by radioimmunoassay after 24 hours of treatment with 2.0 mg/kg of DSS, with or without 0.5 ml/kg of  $\alpha$ -BuTX, in the serum of the injury rat model. Data are expressed as mean  $\pm$  SD. &&P<0.01, and &&&P<0.001 versus DSS + vehicle group.

se and astrocyte activation, and provides neuroprotection; these effects are mediated through increased  $\alpha 7$ nAChR expression in a rat model of focal cerebral ischemia. Thus,  $\alpha 7$ nAChR may provide a therapeutic target against cerebral I/R injury and should be further evaluated.

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## Disclosure of conflict of interest

None.

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