Abstract. This study aimed to observe the effects of 3'-daidzein sulfonate sodium (DSS) on ischemia-reperfusion-induced brain injury and to analyze the mechanisms responsible for neuronal apoptosis. Focal ischemias were induced in male Sprague-Dawley rats using middle cerebral artery occlusion. The rats were divided into 5 groups based on sham surgery or real occlusion, and treatment with different doses of DSS (0.5, 1.0 and 2.0 mg/kg) or normal saline (model group), injected preoperatively into the rats with cerebral occlusion. After 2 h of ischemia and 24 h of reperfusion, neurological deficit scores were evaluated using the Longa grade point standard. The infarct volume was measured using a triphenyl tetrazolium chloride staining technique. Blood-brain barrier (BBB) permeability was measured using the Evans blue (EB) content of brain tissues, while electron microscopy was used to observe ultrastructural changes. The expression levels of Bcl-2, Bax and caspase-3 were detected by an immunohistochemical method and western blot analysis. The neurological deficit in rats pre-treated with DSS at all doses decreased significantly (P<0.05) in comparison with the model group, as did the cerebral infarct volume ratios. The brain EB content was significantly reduced by the injection of DSS. The ultrastructural integrity of the rat brain injury and to analyze the mechanisms responsible for neuronal apoptosis. The ultrastructural integrity of the rat brain was significantly preserved in the DSS-treated groups in comparison with the model group. This was concomitant with the reduced swelling of astrocytes and pericytes in the BBB. The immunohistochemistry results revealed that DSS significantly enhanced the expression of Bcl-2, and inhibited the expression of Bax and caspase-3 in the brain in comparison to the model group. The number of apoptotic cells in the groups treated with DSS was reduced in comparison with similar areas in the model group. These findings suggest that DSS within a dosage range of 0.5-2.0 mg/kg provides significant protection from injury to the BBB induced by cerebral ischemia-reperfusion, as it exerts a neuroprotective effect by inhibiting apoptosis.

Introduction

Ischemic cerebral vascular disease is a common disease in clinical neurology, and is caused by problems with blood supply to the brain. Ischemic cerebral vascular disease has high rates of incidence, mortality and recurrence, and is severely damaging to lifestyle and health (1.2). Ultra-early thrombolytic therapy is currently being used to reduce damage to brain tissue in cerebral vascular disease (3); however, the secondary damage caused to the brain following reperfusion has gained much attention (4-7). The exact mechanisms of injury in cerebral ischemia-reperfusion are not yet clear. Findings demonstrate that the possible mechanisms of ischemia-reperfusion injury include the oxygen-derived free radical, nitric oxide (NO), calcium overload in cells, the toxicity of excited amino acids, the inflammatory reaction in local brain tissues and apoptosis (8). Neuronal apoptosis plays an important role in ischemia-reperfusion injury (9,10). There are three types of apoptosis-related genes, including pro-apoptotic genes, anti-apoptotic proteins and bidirectional regulator genes. The Bcl-2 family of proteins consists of both anti-apoptotic proteins, such as Bcl-2, and pro-apoptotic proteins such as Bax, which participate in the regulation of apoptosis (11,12). Caspase-3 has also been identified as an enzyme that can trigger a cell apoptotic cascade reaction (13,14).

We previously found that daidzein appeared to have a local anesthetic action (15). It has also been shown to exert protective effects against myocardial ischemia-reperfusion injury in rats following coronary artery lesion (16). Daidzein has an antagonizing role towards Ca$^{2+}$ (17); it impacts on auto-rhythmicity and contractility of the right atrium in ex vivo rat hearts (18), and it has also been shown to induce the contribution of endothelium-derived hyperpolarizing factor to endothelium relaxation in male rats and in vitro arteries (19,20).

Daidzein is insoluble in water, which results in low oral bioavailability and low efficacy; however, 3’-daidzein sulfonate sodium (DSS) is a newly developed synthetic material with increased water solubility, resulting from modification of the structure of daidzein (an active ingredient of kudzu vine...
There are few studies on the pharmacological effects of DSS (21), and the protective effects of DSS against apoptosis in cerebral ischemia have not yet been reported, to the best of our knowledge. Li et al (22) reported that puerarin downregulated caspase-3 protein expression and upregulated Bcl-2 protein expression, which could play a neuroprotective role. However, that study did not consider whether DSS exerts protective effects on cerebral ischemia, or whether it is functionally active in reducing apoptosis.

Thus, in the present study, we aimed to assess the effects of DSS on blood-brain barrier (BBB) permeability, the induction of apoptosis and the expression of Bcl-2, Bax and caspase-3 detected by immunohistochemical methods and western blot analysis.

Materials and methods

Materials. The Department of Naturally Occurring Drugs and Chemistry, Shenyang Pharmaceutical University (Shenyang, China) provided DSS (C15H907SNa) as a white crystalline powder, with a purity of >99%. The chemical structures of daidzein and DSS are shown in Fig. 1.

Adult male Sprague-Dawley (SD) rats (250-280 g, n=240) were purchased from Hunan Silaike Jingda Laboratory Animal Co., Ltd. (Changsha, China). The rats were maintained on a 12-h light and dark cycle, allowed free access to food and water, and allowed to adapt to laboratory conditions for 7 days before the experiment.

All animal experiments were approved by the Animal Care and Use Committee of Gannan Medical College, and conducted according to the National Institutes of Health guidelines.

Preparation of the rat model of cerebral ischemia-reperfusion injury. The rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneally), and a rat model of middle cerebral artery occlusion (MCAO) was established according to the methods of Longa et al (23). A 4-cm-long, 0.2-mm-diameter nylon thread was slowly inserted anteriorly towards the direction of the internal carotid artery, through an incision in the common carotid artery trunk. The common carotid artery bifurcation was labeled. After inserting the thread for 18-20 mm, a slight resistance was felt, indicating that the tiny anterior cerebral artery had been reached. The blood supply of the middle cerebral artery was blocked for 1 h. The nylon thread was then pulled out, and the arterial stump was tied. Subcutaneous tissue and skin were sutured. In the sham surgery group, the common carotid artery, external carotid artery, and internal carotid artery were exposed and tied. Subcutaneous tissue and skin were sutured. In the sham surgery group, the common carotid artery and the external carotid artery were exposed and tied. Subcutaneous tissue and skin were sutured.

The parietal cortex brain tissue on the ischemic side was removed, and then 2 h in a paraformaldehyde solution, with results set up using a series of paraformaldehyde solution concentrations, with results set up using a series of EB solution concentrations, with results set up using a series of EB solution concentrations, with results set up using a series of EB solution concentrations.

Infarct volume measurement. After the neurological deficit tests, rats were decapitated and their brains, including the cerebellum, lower brain stem and olfactory bulb were removed, washed with normal saline (NS), dried with filter paper and frozen. After freezing, the forebrain was generally contained within the thickness of the 5 brain slices cut along the coronal plane. These sections were incubated in 5 ml of 2% triphenyl tetrazolium chloride solution for 30 min at 37˚C in the dark. After staining, the non-ischemic regions were colored red, while the infarcted regions were white. The sections were then fixed with 10% formaldehyde. The infarct volume was calculated using a DT-200 image analysis system (Nanjing Dongtu Technology Co., Ltd., Nanjing, China).

Evaluation of the permeability of the BBB. BBB permeability was measured according to the Evans blue (EB) content in the brain tissues. After infarct modeling (or DSS administration for DSS groups), the rats were immediately injected with 0.25 ml of EB solution (0.5%, dissolved in NS) via the tail vein. After 24 h, rats were narcotized and then heart perfused with NS. The forebrain tissue on the ischemic side was then removed, and after weighing, tissues were homogenized in 7.5% trichloroacetic acid (3 ml/g, wet weight). The homogenate was then centrifuged at 12,000 x g at 4˚C for 20 min. The optical density (OD) value of the supernatant was read at 620 nm. A calibration curve was set up using a series of EB solution concentrations, with results being indicated by EB brain wet weight (μg).

Observation of neurovascular unit ultrastructure by electron microscopy. The rats were narcotized 24 h after MCAO reperfusion, and then internally fixated with 4% paraformaldehyde. The parietal cortex brain tissue on the ischemic side was removed and cut into 1 mm² cubes, fixated for a further 2 h in a paraformaldehyde-glutaraldehyde solution, and then 2 h in 1% osmic acid at 4˚C. After dehydration by methanol gradients, and displacement by epoxypropane, the tissues were embedded and polymerized in polyphenylene sulfide resin. Semi-thin sections were then prepared, stained with methylene blue-azure, and observed under an optical lens. Ultra-thin sections were then stained with both uranyl acetate and lead citrate, and observed under an electron microscope (Hitachi H-7650; Hitachi, Tokyo, Japan).

Immunohistochemical detection of Bcl-2, Bax and caspase-3 expression. Following 24 h of cerebral ischemia-reperfusion, the rats were anesthetized with 10% chloral hydrate (3.5 ml/kg, intraperitoneally). A perfusion apparatus filled with 200 ml...
of NS was immediately inserted into the left ventricle via the left ventricular apex, and administration was continued with 300 ml of 4% paraformaldehyde over a period of 30 min. Brain tissues from the ischemic side were removed and used to prepare paraffin-embedded sections. These sections were incubated in 4% paraformaldehyde overnight, washed with running water for 24 h, soaked in 50% ethanol overnight, then stored in 70% ethanol, followed by 80% ethanol overnight, followed by soaking in 90% ethanol for 1 h, 95% ethanol for 45 min (twice), and 100% ethanol for 45 min (twice). They were then twice-cleared in xylene, for 20 min each time, before infiltration with wax in a drying machine at a temperature of 60°C for 1 h and 1.5 h, respectively. They were then embedded in paraffin, and the paraffin sections were deparaffinized and cut into 4 µm sections, before dewaxing to water. The expression of Bcl-2, Bax and caspase-3 were examined using an immunohistochemistry kit (Booster Bioengineering Institute, Wuhan, China) used according to the manufacturer’s instructions. The sections were incubated in 3% (v/v) H2O2 and washed in distilled water at room temperature for 10 min (3 times) to eliminate endogenous peroxidase activity.

Antigen retrieval protocol. The sections were soaked in 0.01 M citrate buffer solution (pH 6.0) and boiled using a pressure cooker. The sections were then rinsed one to two times with phosphate-buffered saline (PBS) solution (pH 7.2-7.6), incubated in 1% bovine serum albumin blocking buffer for 20 min at room temperature, and excess liquids were then shaken off without further washing. The samples were then incubated with an appropriate diluted primary antibody [Bcl-2 (sc-492; dilution, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bax (sc-526; dilution, 1:100; Santa Cruz Biotechnology, Inc.); caspase-3 (9664s; dilution, 1:75; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C, before washing in PBS solution (pH 7.2-7.6) 3 times, for 2 min each time. They were then incubated for 20 min at 20-37°C with biotinylated goat anti-rabbit IgG (SABC; SA2002, dilution, 1:100; WuHan Boster Biotechnology Limited Company, Wuhan, China), followed by washing in PBS solution (pH 7.2-7.6) 3 times, for 2 min each time. The sections were incubated with streptavidin-biotin complex (SABC; SA2002; WuHan Boster Biotechnology Limited Company) at 20-37°C for 20 min before washing in PBS solution (pH 7.2-7.6) 4 times, for 5 min each time.

DAB color development. The reagents A, B and C in the DAB color development kit were added to 1 ml distilled water and mixed well. The solution was then added to sections and the reaction was observed under a microscope at room temperature for about 6 min. The sections were then washed in distilled water, stained with hematoxylin, dehydrated, cleared and mounted. Positive cells were identified by yellow-brown granules in the cytoplasm under a light microscope. The percentage of positive neuronal cells was estimated using 10 randomly selected, but representative, high-power visual fields per section.

Western blot analysis of Bcl-2, Bax and caspase-3 protein expression. The total proteins in cerebral ischemia-reperfusion brain were purified, centrifuged, and collected in the supernatant. Proteins were quantified with a bicinchoninic acid assay, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies to Bcl-2 (sc-492; 1:300), Bax (sc-526; 1:300) (both from Santa Cruz Biotechnology, Inc.) and caspase-3 (9664s; 1:500; Cell Signaling Technology, Inc.) overnight at 4°C. This was followed by incubation with horseradish peroxidase secondary antibodies (1:1,000) for 2 h at 37°C. Images were obtained using a gel image analysis system (Universal Hood II, 721BR04565; Bio-Rad, Hercules, CA, USA) and OD was measured using image lab 3.0 software. The ratio of the OD of the target protein to β-actin was used to represent the relative expression levels of the target proteins.

Statistical analysis. Data are expressed as the means ± SEM, and were analyzed using GraphPad Prism version 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). The group means were compared using one-way analysis of variance (ANOVA) and a Q-test. Comparisons between groups were conducted using paired t-tests. A probability value of P<0.05 was used to determine a significant difference.

Results

Effects of DSS on neurological deficits. The neurological deficits in the rats pre-treated with DSS (0.5, 1.0 and 2.0 mg/kg) were significantly lower than those in the model group (P<0.05; Fig. 2).

Effects of DSS on cerebral infarction volume. Triphenyl tetrazolium chloride staining revealed that after 24 h of cerebral ischemia-reperfusion, the infarcted areas of the left cerebral hemisphere, which were mainly in the frontal and parietal cortex, the caudate and the putamen, were white. Normal
tissues were stained red, with part of the brain tissue in the penumbral area of the cerebral ischemia-reperfusion injury present as a transition zone of white to red. Compared with the model group, the cerebral infarction volume ratios exhibited obvious reductions in all 3 of the DSS-treated groups (Fig. 3).

**Effects of DSS on BBB permeability.** As shown in Fig. 4, in comparison with the sham-operated group, BBB permeability and the brain EB content were significantly increased in the model group (P<0.001). The injection of different doses of DSS (0.5, 1.0 and 2.0 mg/kg) resulted in a significantly decreased brain EB content in comparison with the model group. This indicated that DSS attenuated the deterioration of BBB permeability in MCAO-induced cerebral ischemia-reperfusion injury in rats.

**Immunohistochemistry for Bcl-2.** Bcl-2-positive yellow-brown granules were observed in the cytoplasm and some nuclei. Bcl-2-positive cells were slightly decreased in number in the model group in comparison with the sham-operated group. Compared with the model group, the number of Bcl-2-positive cells exhibited a significant increase in the DSS-treated groups; the positive cells were also stained more deeply, indicating greater positivity. DSS treatment was shown to upregulate Bcl-2 expression and inhibit apoptosis following cerebral ischemia-reperfusion injury in rats (Fig. 6).

**Immunohistochemistry for Bax.** Bax-positive yellow-brown granules were located in the cytoplasm and some nuclei. Compared with the sham surgery group, the model group exhibited higher numbers of Bax-positive cells, which indicated that the expression of Bax significantly increased following ischemia-reperfusion injury. The number of Bax-positive cells decreased significantly in the DSS-treated groups compared with the model group. The Bax-positive cells were also more lightly stained, indicating less positivity in the DSS-treated groups. DSS treatment decreased the expression of Bax protein, and inhibited cerebral apoptosis following ischemia-reperfusion injury in rats (Fig. 7).

**Immunohistochemistry for caspase-3.** Caspase-3-positive yellow-brown granules were located in the cytoplasm and
some nuclei. Compared with the sham surgery group, the model group exhibited a large number of caspase-3-positive cells, which indicated that the expression of caspase-3 significantly increased following ischemia-reperfusion injury. The number of caspase-3-positive cells was significantly lower in the DSS-treated groups in comparison with the model group. The positive cells were only lightly stained, indicating that they were only weakly positive. DSS treatment decreased caspase-3 protein expression, and inhibited cerebral apoptosis following ischemia-reperfusion injury in rats (Fig. 8).

Western blot analysis of Bcl-2, Bax and caspase-3

Effect of DSS on Bcl-2 expression. The results of western blot analysis revealed that Bcl-2 expression was significantly reduced in the model group compared with the sham-operated group. A significant increase in Bcl-2 expression was detected in the 1.0- and 2.0-mg/kg DSS-treated groups, compared with the model group (Fig. 9).

Effect of DSS on Bax expression. The results of western blot analysis revealed that in comparison with the sham-operated group, Bax expression was significantly increased in the model
A significant decrease in Bax expression was detected in the 1.0- and 2.0-mg/kg DSS-treated groups in comparison with the model group (Fig. 10).

**Effect of DSS on caspase-3 expression.** The results western blot analysis revealed that in comparison with the sham-operated group, caspase-3 expression was significantly increased in the model group. A significant decrease in caspase-3 expression was detected in the 1.0- and 2.0-mg/kg DSS-treated groups, compared with the model group (Fig. 11).

**Discussion**

Ischemia-reperfusion injury is a complex disorder caused by free radicals, NO, calcium overload, excitatory amino acids, inflammation and cell apoptosis. Many drugs have been used in the treatment of cerebral ischemia reperfusion injury, including calcium antagonists, free radical scavengers (24) and growth factors (25,26). Some traditional Chinese medicines have been shown to exert neuroprotective effects on cerebral ischemic...
injury (27-29). It was recently demonstrated (30) that DSS has a low acute toxicity, with the \( \text{LD}_{50} \) in rats being 1.75 mg/kg, which is equivalent to 115-fold the usual daily adult dose. Compared with other medications, DSS is more effective in the treatment of cerebral ischemia-reperfusion injury and is also less toxic; DSS can protect neurons from injury or deterioration.

Cerebral ischemia-reperfusion injury is a complex process involving many factors, with an increase in neurological deficit scores, cerebral blood volume and edema. In the present study, the neurological deficit and the cerebral infarction volume were significantly reduced in the DSS-treated groups (0.5, 1.0 and 2.0 mg/kg) (P<0.05). We also found that DSS can pass through the BBB, relieve perivascular edema of the BBB, maintain the integrity of the vascular wall and reduce ischemia-reperfusion-induced damage.

Neuronal apoptosis plays an important role in ischemia-reperfusion injury. Cell apoptosis can be divided into 4 phases: i) apoptotic signal transduction; ii) activation of apoptotic gene expression; iii) triggering of the execution of cell apoptosis; and iv) removal of apoptotic cells. Apoptosis-related genes can be divided into 3 categories: anti-apoptotic genes, such as Bcl-2; pro-apoptotic genes, such as Bax; and bidirectional regulator genes (31).

Bcl-2 can regulate the activating factor of caspase, inhibit cell damage induced by reactive oxygen species, and change the nuclear-cytoplasmic traffic in cell-cycle regulatory proteins CDK2, CDC2 and p53 (32). The co-expression of Bcl-2 with the gene encoding p53 can delay the apoptosis induced by p53. The synergy between Bcl-2 and Myc prevent movement of p53 into the nucleus, and block p53-induced apoptosis (33). Bcl-2 prevents mitochondrial permeability transition and the release of cytochrome \( c \) from mitochondria into the cytoplasm, it inhibits apoptosis by altering the calcium current of intracellular organelles, and promotes the maintenance of calcium homeostasis (34). Bcl-2 can directly combine with inactive CED-4 human homolog Apaf-1 and block caspase (35).

It is known that Bcl-2 and Bax are apoptosis-regulating proteins that have opposing apoptotic activities. Bcl-2 can form heterodimers with Bax, and the ratio of Bcl-2:Bax could reflect the level of apoptosis; lower Bcl-2:Bax ratios promote apoptosis and higher Bax:Bcl-2 ratios inhibit apoptosis. Bax can induce release of cytochrome \( c \), Bax is involved in the regulation pathway of Bcl-xL by combining with it (36,37). In our study, we observed low number of Bcl-2-positive cells in the model group in comparison with the sham-operated group. The number of Bcl-2-positive cells was higher in the DSS-treated groups than in the model group, and a high number of Bax-positive cells was observed in the model group in comparison with the sham-operated group. The number of Bax-positive cells decreased significantly in the DSS-treated groups compared with the model group.

Caspase-3 is a key enzyme that is involved in the execution of apoptosis and leads to disintegration of the cell. It has been reported that caspase-3 is directly involved in cell apoptosis following cerebral ischemia, by cleaving DNA repair proteins, cytoskeletal proteins, and other related caspase substrate
proteins, thereby leading to cerebral ischemia reperfusion injury (38). In our study, a large number of caspase-3-positive cells was observed in the model group compared with the sham-operated group. The number of caspase-3-positive cells in the DSS-treated groups decreased significantly compared with that in the model group. Our findings suggest that DSS at a dose range of 0.5-2.0 mg/kg can significantly promote the expression of Bcl-2, inhibit the expression of caspase-3 and Bax, and reduce the Bcl-2:Bax ratio. DSS exerts a neuroprotective effect on cerebral ischemia-reperfusion by regulating the expression of Bcl-2, Bax and caspase-3.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (NSFC; nos. 81160399, 81560583) and from the implement plan of Natural Science Foundation of China (NSFC; nos. 81160399, 81560583) and from the implement plan of Natural Science Foundation of China (NSFC; nos. 81160399, 81560583).

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