3’-Daidzein sulfonate sodium improves mitochondrial functions after cerebral ischemia/reperfusion injury

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Graphical Abstract

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Abstract

3’-Daidzein sulfonate sodium is a new synthetic water-soluble compound derived from daidzein (an active ingredient of the kudzu vine root). It has been shown to have a protective effect on cerebral ischemia/reperfusion injury in rats. We plan to study the mechanism of its protective effect. 3’-Daidzein sulfonate sodium was injected in rats after cerebral ischemia/reperfusion injury. Results showed that 3’-daidzein sulfonate sodium significantly reduced mitochondrial swelling, significantly elevated the mitochondrial membrane potential, increased mitochondrial superoxide dismutase and glutathione peroxidase activities, and decreased mitochondrial malondialdehyde levels. 3’-Daidzein sulfonate sodium improved the structural integrity of the blood-brain barrier and reduced blood-brain barrier permeability. These findings confirmed that 3’-daidzein sulfonate sodium has a protective effect on mitochondrial functions after cerebral ischemia/reperfusion injury, improves brain energy metabolism, and provides protection against blood-brain barrier damage.

Key Words: nerve regeneration; 3’-daidzein sulfonate sodium; cerebral ischemia/reperfusion injury; infarct volume; anti-oxidation; mitochondria; mitochondrial membrane swelling; mitochondrial membrane potential; superoxide dismutase; malondialdehyde; glutathione peroxidase; blood-brain barrier; neural regeneration

Introduction

Ischemic stroke is a major disease with high morbidity, mortality, and disability rates, which can be harmful to human health. During the rescue and treatment of ischemic disease, tissue injury has been shown not to be caused by ischemia itself, but takes place following restoration of the blood supply, termed ‘ischemia/reperfusion (I/R) injury’ (Kanoski et al., 2007). Cerebral I/R injury refers to severe brain dysfunction when the blood supply is returned to tissue after a period of ischemia (Cheng et al., 2009). There are a variety of factors involved in this pathological process, including excitatory amino acid poisoning, oxidative stress, intracellular calcium overload, inflammatory reactions, and apoptosis (Ildan et al., 2001; Turley et al., 2005). At present, mitochondria are considered a subcellular target for ischemic injury, and have been investigated as potential targets for drug development (Armstrong, 2007).

3’-Daidzein sulfonate sodium (DSS) is a new synthetic water-soluble compound derived from daidzein (an active ingredient of the kudzu vine root) (Figure 1). In our previous studies, we found that DSS exhibits various pharmacological activities, such as anti-oxidation, anti-arrhythmia, and anti-hypoxia, and also can protect against myocardial I/R injury (Zeng et al., 2006a, b, 2009a, b; Zhong et al., 2008, 2011b; Huang et al., 2009; Li et al., 2010a, b). DSS also has a protective effect on cerebral I/R injury in rats (Li et al., 2009; Zeng et al., 2009d, 2010, 2013; Zhong et al., 2011a). To explore the possible protective mechanisms of DSS against cerebral I/R injury, the main objective was to investigate DSS effects on mitochondrial functions after cerebral I/R injury in rats.
Materials and Methods

Experimental animals
Healthy specific pathogen-free male Sprague-Dawley rats weighing 260–280 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., China (certificate No. SCXK (jing) 2012-0001). All experiment procedures comply with the guidelines of the Principles of Laboratory Animal Care, formulated by the National Institute of Health and the Legislation of China for the use and care of laboratory animals. Efforts were made to minimize the number of animals used and the suffering of the experimental animals. The rats were housed in a specific pathogen-free animal house, and allowed free access to food and water. The rats were allowed to adapt to laboratory conditions for 7 days before the experiment. Rats were randomly divided into a sham-operated group, MCAO group, and 0.5, 1.0, and 2.0 mg/kg-DSS treated groups, with six rats in each group.

Establishment of rat middle cerebral artery occlusion (MCAO) models
A rat MCAO model was established according to the methods of Longa et al. (1989). Reperfusion was performed after 90 minutes of ischemia. Sprague-Dawley rats were fasted for 12 hours before anesthesia with an intraperitoneal injection of 10% chloral hydrate (350 mg/kg). The surgical region was disinfected with 70% alcohol. A midline neck incision was made, and the soft tissues over the trachea were gently retracted with a retractor. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated from the vagus nerve. Typically, the CCA bifurcates into the ECA and ICA, which flow toward the cranial and facial regions, respectively. Afterwards, the ICA bifurcates into the middle cerebral artery (MCA) and pterygopalatine artery. Two closely spaced permanent knots were then placed at the distal part of the ECA (below the suprathyroid artery) to prevent the backflow of blood. A microvascular clamp was placed in the ICA and transiently proximal to the CCA junction. The tied section of the ECA was dissected using microscissors to insert the monofilament and reach the CCA junction, and a knot was placed below the arteriotomy site in the ECA. The microvascular clamp placed in the ICA was removed for filament insertion. The filament was carefully inserted from the CCA junction, up to 18–20 mm, into the MCA. After confirmation of MCA blockage, the rat model allowed a blood supply from the CCA. After 90 minutes, the filament was carefully withdrawn until the tip was near the arteriotomy. Following filament removal, the knot was tightened in the ECA. When reperfusion was confirmed, the neck was sutured using surgical thread. To relieve pain and discomfort during the postoperative period, topical lidocaine gel was applied to the incision region, and the rat received 1.0 mL of normal saline subcutaneously as fluid replenishment after surgery. At 24 hours after surgery, the rats were sacrificed and analyzed. In the sham-operated group, the CCA, ECA, and ICA were exposed and isolated. The MCA was not occluded in the sham-operated group. During surgery, room temperature was maintained at 23–25°C.

Drug administration
We then evaluated permeability of the blood-brain barrier (BBB) at 10 minutes, 8 hours, and 16 hours after occlusion. Rats in the 0.5, 1.0, and 2.0 mg/kg-DSS treated groups received three injections of DSS (0.5, 1.0, or 2.0 mg/kg; Department of Naturally Occurring Drugs and Chemistry, Shenyang Pharmaceutical University, China) via the tail vein. The sham-operated group and MCAO group were administered the same volume of physiological saline at the same time points via the tail vein. In the other experiments, at 10 minutes after occlusion, rats in the 0.5, 1.0, and 2.0 mg/kg-DSS-treated groups received an injection of DSS (0.5, 1.0, or 2.0 mg/kg) via a sublingual intravenous injection. The sham-operated group and MCAO group were administered the same volume of physiological saline at the same time points via the sublingual vein.

Determination of infarct volume
Infarct volume was measured using the 2,3,5-triphenyl tetrazolium chloride method (Bederson et al., 1986a; Zausinger et al., 2000; Li et al., 2010a). At 24 hours after cerebral ischemia and reperfusion, the rats were sacrificed and brains were quickly removed. Each brain was cut into 2-mm coronal slices, resulting in five sections in total. Sections were incubated in 2% 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA; Shanghai Chemical Reagent Company Import repacking, Shanghai, China, batch No. 110710) at 37°C for 30 minutes and then fixed with 10% formalin for 12 hours. Changes in color of brain tissue were observed. Normal brain tissue was red, while infarcted brain tissue was pale.

Neurological deficit scores
Neurological deficits were assessed in accordance with the Longa’s method at 24 hours after cerebral ischemia and reperfusion (Bederson et al., 1986b; Longa et al., 1989). A modified scale was used for neurological assessment: 0, no deficit; 1, failure to extend left forepaw fully; 2, circling to the lateral side; 3, falling to the contralateral side; and 4, no spontaneous walking, with a loss of consciousness. In the sham-operated group, no deficit was detected, with a score of 0. I/R rats which scored 1–3 points were included in the experiments.

Extraction of rat brain mitochondria
At 24 hours after cerebral ischemia and reperfusion, rats were decapitated, and the brains were quickly removed. The cerebral cortex on the damaged side was harvested, placed in a precooled small beaker, and washed three times with ice-cold separation buffer containing 0.25 M sucrose, 10 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid, and 250 μg/mL bovine serum albumin, pH 7.4. After removing the blood vessels and congestion, the brain tissues were cut into pieces with small scissors. These brain tissues were manually homogenized ten times in 10 mL/g separation buffer in a Potter-Elvehjem glass homogenizer (Botong Chemical Technology Co., Shanghai, China). The homogenates were
Figure 1 Chemical structures of 3′-daidzein sulfonate sodium.

Figure 2 Effects of DSS on neurological function in rats with cerebral ischemia/reperfusion injury.
Lower Longa neurological scores indicate better neurological function. Data are expressed as the mean ± SEM (n = 6), and were analyzed by one-way analysis of variance. I: MCAO group, II–IV: 0.5, 1.0, and 2.0 mg/kg DSS-treated groups, respectively. *P < 0.05, vs. MCAO group.

DSS: 3′-Daidzein sulfonate sodium; MCAO: middle cerebral artery occlusion.

Figure 3 Effects of DSS on cerebral infarct volume in rats with cerebral ischemia/reperfusion injury.
Infarct volume of the left cerebral hemisphere, showing damage mainly in the frontal and parietal cortices, the caudate, and the putamen, which are white. Normal tissues are stained red. Part of the brain tissue reveals a transition zone of white to red, which is the penumbral area of cerebral ischemia/reperfusion injury. (A) Sham-operated group; (B) MCAO group; (C) 0.5 mg/kg DSS-treated group; (D) 1.0 mg/kg DSS-treated group; (E) 2.0 mg/kg DSS-treated group. DSS: 3′-Daidzein sulfonate sodium; MCAO: middle cerebral artery occlusion.

Figure 4 Effects of DSS on mitochondrial swelling in the rat brain. Data are expressed as the mean ± SEM (n = 6), and were analyzed by one-way analysis of variance. #P < 0.05, vs. sham-operated group; *P < 0.05, **P < 0.01, vs. MCAO group. I: Sham-operated group; II: MCAO group; III–V: 0.5, 1.0, and 2.0 mg/kg DSS-treated group. DSS: 3′-Daidzein sulfonate sodium; OD: optical density; MCAO: middle cerebral artery occlusion.

Figure 5 Effects of DSS on mitochondrial membrane potential in rat brain.
Data are expressed as the mean ± SEM (n = 6), and were analyzed by one-way analysis of variance. #P < 0.05, vs. sham-operated group; *P < 0.05, **P < 0.01, vs. MCAO group. I: Sham-operated group; II: MCAO group; III–V: 0.5, 1.0, and 2.0 mg/kg DSS-treated groups, respectively. DSS: 3′-Daidzein sulfonate sodium; MCAO: middle cerebral artery occlusion.

Figure 7 Effects of DSS on BBB permeability in rats with cerebral ischemia/reperfusion injury.
BBB permeability was measured according to EB content in brain tissue. Higher EB content represents greater BBB permeability. Data are expressed as the mean ± SEM (n = 6), and were analyzed by one-way analysis of variance. #PPP < 0.001, vs. sham-operated group; **P < 0.01, ***P < 0.001, vs. MCAO group. I: Sham-operated group; II: MCAO group; III–V: 0.5, 1.0, and 2.0 mg/kg DSS treated groups, respectively. DSS: 3′-Daidzein sulfonate sodium; MCAO: middle cerebral artery occlusion; BBB: blood-brain barrier; EB: Evans blue.
centrifuged at 1,000 × g for 10 minutes. The supernatants were collected and centrifuged at 12,000 × g for 10 minutes. The mitochondria were precipitated (Zhang et al., 2003), and washed once with separation buffer, and centrifuged at 12,000 × g for 10 minutes. All steps were carried out at 4°C. The concentration of mitochondrial protein was measured using the Coomassie brilliant blue (Bradford, 1976). The mitochondrial pellets were suspended in buffer containing 280 mM sucrose, 0.5 mM ethylenediamine tetraacetic acid and 10 mM Tris-HCl (pH 7.4) at a protein concentration of 10 mg/mL.

Mitochondrial swelling assay
The fresh mitochondria were preserved at 4°C without freeze-thawing until use. The total volume of reaction buffer was 200 µL (250 mM sucrose, 5 mM KH$_2$PO$_4$, 3 mM sodium succinate, pH 7.2), which included 100 µg mitochondrial protein. Optical density (OD) values at 520 nm (OD$_{520}$) were recorded within 10 minutes using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) after mixing the reagents. The temperature was maintained constantly at 25°C during this process. Mitochondrial swelling was determined in all groups by measuring changes in OD$_{520}$. When the swelling degree of mitochondria increased, the OD$_{520}$ value decreased. Decreased OD$_{520}$ values indicated mitochondrial swelling. A lower OD value indicated a higher degree of swelling (Lee et al., 2002).

Detection of mitochondrial membrane potential
Rhodamine 123 (Rh123) is a specific fluorescent dye with a positive charge, which can be absorbed by the mitochondrial membrane; the absorption rate is proportional to the membrane potential. The change in fluorescence intensity of Rh123 reflects the changes in mitochondrial membrane potential in cells. A weak fluorescence intensity indicates a low mitochondrial membrane potential (Zhang et al., 2008). The detection was performed in 96-well plates. A total of 150 µL of membrane potential reaction buffer and 1 µL of 1 mM Rh123 (Sigma-Aldrich) were added to each well. The excitation wavelength was 488 nm and emission wavelength was 530 nm. The fluorescence value was detected at 37°C, 50 µL of the mitochondrial protein suspension (approximately 10 mg/mL) was added and mixed, and fluorescence values of Rh123 taken up by mitochondria within 10 minutes were detected using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Mitochondrial membrane potential was calculated using the Nernst equation: \[ \Delta \psi = 61.54 \log \frac{[\text{Rh123}]}{[\text{Rh123}]} \] where [Rh123]$_{in}$ is the matrix volume of 1 µL/mg mitochondrial protein was considered. [Rh123]$_{out}$ represents the fluorescence value measured 10 minutes after addition of mitochondria.

Measuring mitochondrial malondialdehyde (MDA) content, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activity
A SOD assay kit (Nanjing Jiancheng Biological Reagent Co., Ltd., Nanjing, Jiangsu Province, China) was used for the SOD assay. MDA levels were analyzed for lipid peroxidation products using a rat MDA ELISA kit (Nanjing Jiancheng Biological Reagent Co., Ltd.). GSH-Px activities were detected using a rat GSH-Px enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Biological Reagent Co., Ltd.).

Evaluation of BBB permeability
BBB permeability was measured according to Evans blue content in the brain tissues (Jiao et al., 2011). After surgery (or DSS administration for DSS groups), the rats were immediately injected with 0.25 mL Evans blue solution (Sigma-Aldrich; 0.5%) dissolved in normal saline via the tail vein. After 24 hours, the rats were anesthetized and the heart was perfused with normal saline. The forebrain tissue on the ischemic side was then removed. After weighing, tissues were homogenized in 7.5% trichloroacetic acid (3 mL/g). The homogenate was then centrifuged at 12,000 × g at 4°C, for 20 minutes. The OD value of the supernatant was read at 620 nm and detected using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A calibration curve was set up using a series of Evans blue solution concentrations, with results indicated by Evans blue content (µg) in brain wet weight (g).

Observation of neurovascular unit ultrastructure by electron microscopy
Rats were anesthetized 24 hours after reperfusion, and then internally fixed with 4% paraformaldehyde. The brain was removed, and parietal cortex brain tissue on the ischemic side was removed and cut into 1-mm cubes, fixed for an additional 2 hours in 2% paraformaldehyde-glutaraldehyde solution, and then 2 hours in 1% osmic acid at 4°C. After dehydration in an ethanol gradient, and displacement by epoxypropane, the tissues were embedded and polymerized in polyphenylene sulfide resin. Semithin sections were then prepared, stained with methylene blue-azure, and observed under an optical lens. Ultrathin sections were then stained with both uranyl acetate and lead citrate, and observed under a transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis
Data are expressed as the mean ± SEM, and were analyzed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Differences were compared between groups with one-way analysis of variance. \( P < 0.05, P < 0.01 \) was considered statistically significant.

Results
Effects of DSS on neurological scores in rats with cerebral I/R injury
Longa neurological scores were significantly lower in the 0.5, 1.0, and 2.0 mg/kg-DSS treated groups than in the MCAO group \( (P < 0.05; \text{Figure 2}) \).

Effects of DSS on cerebral infarct volume in rats with cerebral I/R injury
Triphenyl tetrazolium chloride staining showed significantly
Effects of DSS on mitochondrial swelling following cerebral I/R injury in the rat brain

A lower OD value indicated a higher degree of swelling. Compared with the sham-operated group, mitochondrial swelling significantly increased (OD540 nm value significantly decreased) in the MCAO group (P < 0.05). In the three DSS treated groups, mitochondrial swelling significantly decreased (OD540 nm value significantly increased) compared with the MCAO group (P < 0.05 or P < 0.01; Figure 4).

Effects of DSS on mitochondrial membrane potential in the rat brain after cerebral I/R injury

After mitochondria were loaded with fluorescent dye Rh123, the fluorescence intensity of mitochondria was higher in DSS treated groups than in the MCAO group (P < 0.05 or P < 0.01; Figure 5).

Effects of DSS on mitochondrial oxidative stress following cerebral I/R injury

Compared with the sham-operated group, mitochondrial SOD and GSH-Px activities were significantly decreased, and mitochondrial MDA contents were significantly increased, in the MCAO group (P < 0.05). Compared with the MCAO group, mitochondrial SOD and GSH-Px activities were significantly increased, and mitochondrial MDA contents were significantly decreased, in DSS treated groups (P < 0.05 or P < 0.01; Figure 6).

Effects of DSS on BBB permeability in rats with cerebral ischemia/reperfusion injury

As shown in Figure 7, compared with the sham-operated group, Evans blue contents significantly increased in the MCAO group (P < 0.001). Injection of different doses of DSS (0.5, 1.0, or 2.0 mg/kg) resulted in significantly decreased Evans blue content compared with the MCAO group (P < 0.001).

Effects of DSS on BBB ultrastructure in rats with cerebral ischemia/reperfusion injury

In the sham-operated group, the BBB remained intact, with intact endothelial cells and a vascular wall structure. The perivascular astrocytic foot processes and pericytes exhibited no swelling, and the vessel lumen was not affected. In the MCAO group, however, the perivascular astrocytes exhibited obvious swelling, including cytoplasmic vacuolation, edematous fluid, swelling of perivascular foot processes, separation from basement membranes, and narrowing of the lumen. Compared with the MCAO group, astrocytic swelling was significantly ameliorated in the 0.5 mg/kg-DDS treated group. Although pericyte swelling was still observed, the vessel lumen recovered and blood flow was restored. In the 1.0 and 2.0 mg/kg-DDS treated groups, pericyte and perivascular astrocyte swelling in the BBB was significantly ameliorated (Figure 8).

Discussion

Cerebral I/R injury refers to the severe brain damage that occurs after restoration of blood supply following cerebral ischemia. Studies have shown that cerebral I/R injury is a complex pathophysiological process (Tian, 2015; Yan et al., 2015). The interruption of blood flow to the brain and reperfusion leads to irreversible brain tissue damage (Gao et al., 2015; Feng et al., 2016). It is a fast process that involves a number of aspects, such as energy depletion, increased release of excitatory amino acids, intracellular calcium overload, free radical damage, acidosis, inflammatory cytokines damage, and apoptosis-related gene activation (Ildan et al., 2001; Turley et al., 2005). These aspects are not isolated from one another and can overlap or be associated with each other, subsequently leading to neural necrosis or apoptosis (Kotake et al., 2005). Energy metabolism disorders play a key role in neuronal damage. Mitochondrial dysfunction is one of the main causes of direct cerebral ischemia damage and delayed neuronal necrosis, which is also the key factor in initiating cell apoptosis (Abe et al., 1995, 1996; Kristián and Siesjö, 1998). Significant changes in mitochondrial structure occur during the early stages of apoptosis. First, mitochondrial membrane structural damage appears and mitochondrial swelling occurs, which is gradually aggravated (Yang et al., 2008). Second, mitochondrial transmembrane potential decreases (Shi and Qing, 2011). The mitochondrial membrane potential is an important indicator that reflects mitochondrial functions. Membrane potential changes affect proton pump functions, thereby affecting the formation of adenosine triphosphate (Zhang et al., 2008). Decreased mitochondrial transmembrane potential is considered an early step in the apoptosis cascade reaction, and it occurs before nuclear apoptosis (such as chromatin condensation and DNA fragmentation). Once the mitochondria collapse, cell apoptosis becomes irreversible (Dzeja et al., 2001). Thus, finding effective drugs to protect mitochondria, improve cellular energy metabolism, and inhibit apoptosis, will be important in treating cerebral I/R injury. In this study, DSS was shown to reduce mitochondrial swelling in the brain following cerebral I/R injury in rats. An increased mitochondrial membrane potential, which is beneficial for recovery of mitochondrial functions, also played an important role in reducing neuronal damage after cerebral I/R. These results suggested that the protective mechanisms of DSS on cerebral ischemic injury were associated with improved mitochondrial functions.

Following cerebral ischemia, especially during the reperfusion period, secondary-induced oxygen-free radicals increase, which is an important factor in causing neuronal injury (Try- stman et al., 1991). The reduced generation of free radicals or complete removal protect against ischemic brain injury (Abe et al., 1988). SOD and GSH-Px are intracellular antioxidants that increase scavenging of free radicals; they are also enzyme regulators of free radical balance in the body and can mobilize or activate the endogenous antioxidant system, thereby pre-
vent or reducing damage by free radicals. This further reduced MDA content, which results in decreased lipid peroxidation (Keller et al., 1998). Together, these results suggest that DSS reduces brain damage caused by oxygen-free radicals.

The BBB is composed of capillary endothelial cells, intercellular tight junctions, a basal membrane, and astrocytes. The BBB blocks the transport of certain substances from the blood into the central nervous system to maintain a stable internal environment (Butt et al., 1990; Ballabh et al., 2004; Abbott et al., 2006). I/R injury is strongly associated with secondary damage of BBB structure and function; decreased BBB function and increased BBB permeability can cause serious vascular edema and other complications (Yang and Rosenberg, 2011). Results from the present study showed that DSS can pass through the BBB, and a dose of 0.5–2 mg/kg DSS protected against BBB damage following MCAO-induced I/R injury in rats. DSS reduced Evans blue leakage into the brain, showing a protection of BBB integrity. Observations of BBB ultrastructure showed that DSS significantly improved perivascular edema and helped to maintain intact vascular structures.

Author contributions: JZ and WY were responsible for the study design, paper authorization and obtained funding. QC, ZHH and HX were responsible for the course of experiments and statistical analysis. QL and XL were responsible for the preparation of experimental apparatus and reagents. All authors approved the final version of the paper.

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References