The Neuroprotective Effects of Danggui-Shaoyao San on Vascular Cognitive Impairment: Involvement of the Role of the Low-Density Lipoprotein Receptor-Related Protein

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Abstract

Effective drugs for treating dementia are still rare. Danggui-Shaoyao San (DSS), a traditional Chinese medicine, has been widely used in oriental countries for the treatment of various gynecological diseases. Many studies reported that DSS could ameliorate cognitive impairment. In this study, we aimed to investigate the underlying mechanism of DSS on vascular cognitive impairment (VCI) rats. Chronic cerebral hypoperfusion (CCH) is one of the main causes of VCI. CCH resulted in a chain of pathological process, including neuroinflammation, neuronal apoptosis, and oxidative stress. The most widely used animal model of VCI is permanent bilateral common carotid artery occlusion in rats. In this research, we determined whether DSS attenuated cognitive impairment by targeting I kappa B kinase (IKK)/nuclear factor of kappa B (NF- κ B) signal pathway in VCI rats. Morris water maze and fear conditioning tests results indicated that DSS [7.2 g/(kg·d)] could improve learning and memory ability in VCI rats. We also found DSS significantly elevated the levels of low-density lipoprotein receptor-related protein 1 (LRP1) in the brain of VCI rats and this might indirectly target the IKK/NF- κ B signal pathway to exert inhibitory effect on neuroinflammation, neuronal apoptosis, and oxidative stress in VCI rats. The present researches indicated that DSS might attenuate cognitive impairment by targeting IKK/NF- κ B signal pathway in VCI rats and DSS might be a promising agent on VCI.

Keywords: vascular cognitive impairment, chronic cerebral hypoperfusion, Danggui-Shaoyao San, LRP1, IKK/NF- κ B signal pathway

Introduction

AMONG THE SUBTYPES OF DEMENTIA, vascular cognitive impairment (VCI) accounts for 15%–20% of all cases of dementia,¹ which is not only the second common dementia after Alzheimer disease, but also a potential risk factor contributing to the development of Alzheimer's disease.^{2,3}

The clinical syndromes of VCI include cognitive dysfunction, emotional disorder, and diminish executive capacity. These symptoms have a negative impact on the daily life of patients with VCI.⁴ With the gradual increase in VCI patients, effective treatments are more and more urgent. Many studies have been devoted to the neuroinflammation and oxidative stress owing to chronic inflammation and

long-term cerebrovascular dysfunction deficiency in cognitive altered process; these studies focused on endothelium and neuronal disruption acting as a promoter of neurodegenerative events.⁵ The present investigations focus on neuroprotective mechanisms of the central nervous system to reverse the pathological process of VCI.

Traditional Chinese medicine has been widely applied in preventing and treating diseases for 2000 years, and quantities of Chinese herbal medicine formulas are used to improve cognitive function.^{6,7} Danggui-Shaoyao San (DSS), a Chinese herbal compound, contains the following herbs: *Angelica sinensis*, *Ligusticum chuanxiong* Hort, *Paeonia lactiflora* Pall, *Atractylodes macrocephala*, *Alisma Plantago-aquatica* Linn, and *Poria cocos*, and it has been widely used in oriental

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countries for the treatment of various gynecological diseases.⁸ It has been reported that DSS has an effect on free radical-mediated neurological diseases, exhibits anti-inflammatory and antioxidant activities, and reduces cell apoptosis in the hippocampus.⁹ However, mechanism of DSS on ameliorating the cognitive impairment in VCI still has not been reported. The dihydropyridinic calcium antagonist nimodipine has been proposed as a drug that is able to improve cognition in VCI because of vasoactive and neuroprotective actions.¹⁰ Its effect on age-related microangiopathy in experimental models¹¹ makes nimodipine a potential treatment for small-vessel VCI subtypes.

Low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) is a member of LDL receptor gene family, which clears β -amyloid (A β) peptides in the brain.¹² Many investigations indicate that LRP1 inhibits NF- κ B activity via down-regulating cell surface tumor necrosis factor receptor-1 (TNFR1).¹³ Several experimental studies have shown that oxidative stress and neuroinflammation play critical roles in VCI.^{14,15} In addition, I kappa B kinase (IKK) induced phosphorylation of I κ B, which promotes I κ B degradation and release, finally leading to activation of NF- κ B.^{16,17} IKK/NF- κ B pathway exerts antiapoptosis function through many other proteins, such as Bcl-2. It has also been shown that the levels of Bcl-2 protein are increased in neurons of vulnerable brain regions after global and local cerebral ischemia.^{18–22}

Based on the previous study, we used the bilateral common carotid arteries occlusion (BCCAO) model to explore whether DSS could improve cognitive function through IKK/NF- κ B pathway (we explored the cognitive function of DSS through IKK/NF- κ B pathway).^{23,24} The whole experimental process is given in Figure 1. Our experiment demonstrated the potential mechanism of DSS in ameliorating cognitive impairment in VCI through the IKK/NF- κ B pathway by regulated LRP1.

Materials and Methods

Drugs

The qualitative analysis of DSS formula had been performed in a previous research,²⁵ which contains the following ingredients: *Angelica sinensis*, *Ligusticum chuanxiong* Hort, *Paeonia lactiflora* Pall, *Atractylodes macrocephala*, *Alisma Plantago-aquatica* Linn, and *Poria cocos*, provided by the First Affiliated Hospital of Guangzhou University of Chinese Medicine. The six raw herbs were mixed in the ratio of

3:16:8:4:8:4, decocted in water twice, and the filtrates were mixed into a final concentration of 1.5 g/mL. Nimodipine was purchased from Bayer Healthcare Limited (batch number: H20003010).

Animals and treatment

In accordance with policies and procedures described in the Guidelines for the Care and Use of Laboratory Animals published by the National Research Council, the present experiments involving animals was approved by Guangzhou University of Chinese Medicine Animal Ethics Committee.

Adult female Sprague-Dawley rats (weighing 250–300 g, Guangdong Provincial Institute of Traditional Chinese Medicine, Guangzhou, China) were used in the research. The rats were all maintained and housed in a specific pathogen-free animal room at 22°C $\pm$ 2°C with automatic light cycles (12 hours light/dark) and a relative humidity of 40%–60%. Food and tap water were offered and libitum throughout the study. BCCAO was carried out in the rats according to a previous study.²⁶ In brief, animals were anesthetized using 1% pentobarbital sodium (50 mg/kg, i.m.) and the two common carotid arteries of rats were bluntly separated and permanently occluded with a silk suture. The animals in the sham group underwent identical procedures, except the common carotid arteries that were simply exposed without occlusion.

Six-month-old rats were divided into five groups at random: sham group ($n = 12$), model group ($n = 12$), low-dose DSS group [1.8 g/(kg·d), $n = 12$], high-dose DSS group [7.2 g/(kg·d), $n = 12$], and Nimodipine group [20 mg/(kg·d), $n = 12$]. Rats were treated with distilled water, DSS, and Nimodipine, respectively, once per day for 4 weeks.

Reagents

The reagents were as follows: Rabbit anti-p-IKK (1:2000, ab194519; Abcam), rabbit anti-IKK (1:2000, ab32135; Abcam), rabbit anti-p-I κ B (1:1000, ab32518; Abcam), rabbit anti-I κ B (1:1000, 2859S; Cell Signaling Technology), rabbit anti-p-NF- κ B (1:1000, #3033; Cell Signaling Technology), rabbit anti-NF- κ B (1:1000, #8242; Cell Signaling Technology), rabbit anti-Bcl-2 (1:500, #2876; Cell Signaling Technology), rabbit anti-Bax (1:500, #2772; Cell Signaling Technology), rabbit anti-Caspase-3 (1:500, #9662; Cell Signaling Technology), rabbit anti-LRP1 (1:4000, ab92544; Abcam), rabbit anti-RAGE (1:1000, ab3611;

FIG. 1. Scheme of the experimental design. This is a schematic of the present experiment. FC, fear conditioning; MWM, Morris water maze; SD, Sprague-Dawley.

Abcam), and mouse anti- β -actin (1:60,000, A5441; Sigma); anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (1:1000, #7074; Cell Signaling Technology); anti-mouse IgG, HRP-linked antibody (1:1000, #7076; Cell Signaling Technology); malondialdehyde (MDA, Lot: A003-01; Nanjing Jiancheng Bioengineering Institute), superoxide dismutase (SOD, Lot: A001-01; Nanjing Jiancheng Bioengineering Institute), reactive oxygen species (ROS, Lot: E004; Nanjing Jiancheng Bioengineering Institute); enzyme-linked immunosorbent assay (ELISA) kits: rats amyloid beta peptide 1–42 was purchased from the Wuhan Huamei Bioengineering Institute (Wuhan, China; Lot: R09014760). Rats tumor necrosis factor α (TNF- α) Emax immunoassay kit (6.25 pg/mL/400 pg/mL, Lot: Z01019334) and rats IL-1 β Emax immunoassay kit (62.5 pg/mL/4000 pg/mL, Lot: X24019335) were from CUSABIO.

Morris water maze test

Morris water maze test was carried out based on the method of Morris.²⁷ The pool was divided into four imaginary quadrants spatially. In the target quadrant of the pool, 2 cm below the water surface, a circular and transparent escape platform (10 cm diameter) was placed. We performed the visible platform test (water in the pool is clear and transparent) on the first training day before the hidden platform test. Rats were gently placed into the pool, with drop location changing for each trial randomly. Once the rat reached the platform, the escape latency was recorded. The rats were guided to the platform by the trainer and to remain on the platform for 20 seconds; if they failed to reach the platform within 60 seconds, the escape latency was recorded as 60 seconds. After visible platform test, we dyed the pool black, rats were given a position navigation test (hidden platform test) for 5 days. On the sixth day, we removed the platform, rats were permitted to explore in the pool for 60 seconds. Crossing frequency through the platform position and time spent in the target quadrant that indicated the degree of memory consolidation were measured.

Fear conditioning test

Fear conditioning test (FCT) was carried out as described in a previous study.²⁸ In the first part of the test, each rat was allowed to have free activities in the test box for 300 seconds. The presentation of a 2-Hz pulsating tone (80 dB, 3000 Hz) was persisted for 28 seconds. The tone was followed by a mild foot shock (0.8 mA for 2 seconds) immediately. There were six cycles and each cycle was spaced 20 seconds apart. Each rat was permitted to stay in the chamber for a total of 300 seconds. The second part of test was carried out at 3 hours and at 24 hours after the first part, respectively. In the second part, the mild foot shock (0.8 mA for 2 seconds) was removed. By measuring the duration time that the rat demonstrated “freezing behavior,” we could assess the function of associative memory in rats.

Western blot

The tissues were homogenized and lysed in sample buffer (5% saline solution). The lysate was centrifuged at 12,000 g for 10 minutes at 4°C, then denatured by boiling at 100°C with 1:4 loading buffer. The same amount of protein (30 μ g)

was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferred onto the PVDF membranes. The membranes were soaked in 5% skim milk that dissolved in TBST for 1 hour at room temperature, and then incubated with a primary antibody overnight at 4°C. After washing with TBST, the membranes were incubated with appropriate secondary antibodies coupled to HRP for 1 hour at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) and Chem Doc XRS with Quantity One software (Bio-Rad, Hercules, CA). The bands were scanned, and intensities of the bands were measured using Image-Pro Plus 6.0 analysis software.

Enzyme-linked immunosorbent assay

TNF- α and IL-1 β in cortex homogenates and A β 1–42 were quantified using ELISA kits. The tissues were dissociated using 2% sodium dodecyl sulfate. Then, the homogenates were centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant fractions were collected to detect the levels of TNF- α , IL-1 β , and SDS-soluble A β 1–42. Samples were compared with the standard curves to calculate the levels of TNF- α , IL-1 β , and SDS-soluble A β 1–42 in extracts.

Measurement of MDA, ROS, and SOD

The total protein concentration of the supernatant was determined using a bicinchoninic acid protein assay. Then the supernatant was used to detect the levels of MDA, ROS, and SOD in cortex according to the manufacturer's instructions.

Nissl's staining

Brain slices were washed in xylene and then rehydrated through ethanol and double-distilled water. The sections were soaked in Nissl's dye liquor for 5 minutes at room temperature. Double-distilled water was used to rinse the slices and then the slices were dehydrated through alcohol, cleared in xylene. A light microscope and LEICA QWin Plus were used to analyze slice images.

Statistical analysis

SPSS 19.0 software (SPSS, Inc., Chicago, IL) was used to analysis all experimental values given as mean $\pm$ standard error of the mean. One-way analysis of variance was used to analyze differences in data among the groups, which was followed by Dunnett's significant *post hoc* test for pairwise multiple comparisons. Student-Newman-Keuls test was used when there was homogeneity of variance, and the rank sum test was used when the variance was not uniform. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

DSS improved learning and memory performance in VCI rats

In this study, we used Morris water maze and FCTs to assess the impacts of DSS on learning and memory ability in VCI rats.

In the Morris water maze test, we compared the escape latency of the rats in five experimental groups. We performed

the visible platform test on the first training day before the hidden platform test and the escape latencies of the rats in five experimental groups did not show significant change (Fig. 2A) ($p > 0.05$). The escape latency in the model group was significantly elevated compared with the sham group in the positioning navigation test (Fig. 2B, C) ($p < 0.01$ vs.

sham) and the escape latency of DSS high-dose and Nimodipine treatment groups performed shorter compared with the model group (Fig. 2B, C) ($p < 0.01$ and $p < 0.05$, respectively vs. model). The probe test showed that crossing times and the swimming distance significantly differed among the five experimental groups (Fig. 2D, E). Compared with the model

FIG. 2. DSS ameliorated cognitive impairment of VCI rats in Morris water maze test. (A) Escape latencies in water maze of the visible platform test. (B) Escape latencies during the five consecutive days positioning navigation test in Morris water maze. (C) Representative tracings of the animal's path. (D) Crossing times through the platform location in the probe trial. (E) Swimming distance in the target quadrant in the probe trial. Data are given as mean $\pm$ SEM ($n = 12$ rats per group). $^{##}p < 0.01$ versus the sham group. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the model group. DSS, Danggui-Shaoyao San; SEM, standard error of the mean; VCI, vascular cognitive impairment.

group, the DSS high-dose and Nimodipine groups significantly crossed more times (Fig. 2D) ($p < 0.01$ and $p < 0.05$, respectively vs. model). DSS high-dose and Nimodipine groups also significantly swam longer distance in the target quadrant compared with the model group (Fig. 2E) ($p < 0.05$ and $p < 0.05$, respectively vs. model).

In the FCT, the 3-hour test demonstrated that there were no significant discrepancies in freezing time between any two of the experimental groups (Fig. 3A). However, as the 24 hours test demonstrated, the freezing time of the sham group was significantly longer compared with the model group and the freezing time of the DSS high-dose group was significantly longer compared with the model group either (Fig. 3B) ($p < 0.05$ vs. model).

The results of two behavior tests revealed that DSS improved the learning and memory ability in VCI rats.

DSS treatment did not affect A β 1–42 levels through elevating LRP1 expression in VCI rats

Consistent with the findings of a previous study, LRP1 plays a critical role in brain A β clearance and neuroinflammation regulation in neurons and vascular mural cells. LRP1 also plays an important role in Abeta clearance in astrocytes, pericytes, and endothelial cells as shown before.^{29–32} In these experiments, remarkably decreased expression of LRP1 was observed in the cortex and hippocampus of VCI rats, whereas after treatment with DSS or Nimodipine, the proteins restored to the normal levels (Fig. 4A–D) ($p < 0.01$ and $p < 0.05$, respectively vs. model). Next, we measured the protein levels of the receptor for advanced glycation end products (RAGE), but there was no significant discrepancy between the groups (Fig. 4A–D). To uncover the deep mechanism of DSS in mitigating cognition deficiency in VCI rats, we next measured the levels of A β 1–42. It was elevated in the hippocampus of model group compared with sham group (Fig. 4E) ($p < 0.05$ vs. sham). However, the levels of A β 1–42 had no significant change in treatment groups compared with model group. These results demonstrate that the elevated levels of LRP1 did not have a significant effect on SDS-soluble A β 1–42 levels by DSS treatment in VCI rats.

DSS diminished proinflammatory cytokine production in VCI rats

In addition to the role as an endocytic receptor, LRP1 also functions in cell signaling, regulates gene transcription, and inhibits inflammation.^{33,34} To determine whether LRP1 regulates expression of neuroinflammation, we first determined the levels of proinflammatory cytokine in VCI rats. The expression of TNF- α and IL-1 β significantly increased both in the hippocampus of VCI rats (Fig. 5A, B) ($p < 0.01$ and $p < 0.01$, respectively vs. sham). The levels of TNF- α and IL-1 β in DSS high-dose and Nimodipine groups decreased (Fig. 5A, B) ($p < 0.01$ and $p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively vs. model). In response to chronic cerebral hypoperfusion (CCH), TNF- α and IL-1 β , which are proinflammatory cytokines, were significantly elevated in the hippocampus. The elevated levels of proinflammatory cytokines in VCI rats clearly emerged with a significant increment already in early pathological process of VCI. These features also showed that high levels of LRP1 might inhibit the expression of TNF- α and IL-1 β in VCI rats after treatment with DSS. In addition, a previous study showed that membrane-anchored LRP1 plays a suppressor of inflammation by downregulating cell surface TNF- α and cell signaling through the IKK/NF- κ B pathway.³⁵

DSS regulated IKK/NF- κ B pathway by regulating LRP1 in VCI rats

NF- κ B functions as a major regulator of inflammation, cell survival, and as a mediator of the response to TNF- α .³⁶ To identify candidate signaling cascades involved in the regulation of neuroinflammation expression by LRP1, we examined protein expression of p-IKK, IKK, p-I κ B, I κ B, p-NF- κ B, and NF- κ B. In response to TNF- α , phosphorylated IKK was substantially increased in hippocampus of model group compared with sham group (Fig. 6A, B) ($p < 0.01$ vs. sham) and decreased after the administration of DSS high-dose (Fig. 6A, B) ($p < 0.01$ vs. model). Meanwhile, there was no significant change on IKK (Fig. 6A, B). Moreover, there was an increment in the active form of the phosphorylated I κ B in the hippocampus of model group compared with sham group (Fig. 6A, C) ($p < 0.01$ vs. sham),

FIG. 3. DSS ameliorated cognitive impairment of VCI rats in FCT. (A) Freezing time of 3 hours after anesthesia in FCT. (B) Freezing time of 24 hours after anesthesia in FCT. Data are given as mean $\pm$ SEM ($n = 12$ rats per group). [#] $p < 0.01$ versus the sham group. ^{*} $p < 0.05$ versus the model group. FCT, fear conditioning test.

FIG. 4. DSS restored the levels of LRP1 in VCI rats. (A–D) LRP1 and RAGE protein levels in the cortex and hippocampus after DSS treatment are determined by western blot analysis. (E) ELISA results of Aβ_{1–42} levels in the VCI rats after treatment of DSS. Data are given as mean ± SEM ($n=12$ rats per group). [#] $p<0.05$ and ^{##} $p<0.01$ versus the sham group. ^{*} $p<0.05$ and ^{**} $p<0.01$ versus the model group. ELISA, enzyme-linked immunosorbent assay; LRP1, low-density lipoprotein receptor-related protein 1; RAGE, receptor for advanced glycation end products.

and decreased after the treatment of DSS high-dose group (Fig. 6A, C) ($p<0.01$ vs. model). The total IκB in the hippocampus of VCI rats also decreased after the treatment of DSS high-dose group (Fig. 6A, C) ($p<0.01$ vs. model). To further evaluate the consequence of IKK/NF-κB path-

way, we next measured the expression levels of phosphorylated NF-κB and NF-κB. The phosphorylated NF-κB in VCI rats was elevated compared with sham group (Fig. 6A, D) ($p<0.01$ vs. sham), but sharply decreased after the treatment of DSS high dose (Fig. 6A, D) ($p<0.01$ vs. model).

FIG. 5. DSS inhibited the production of proinflammatory cytokine in VCI rats. (A) ELISA results of TNF- α levels in the hippocampus. (B) ELISA results of interleukin-1 β (IL-1 β) levels in the hippocampus. Data are given as mean $\pm$ SEM ($n = 12$ rats per group). ^{##} $p < 0.01$ versus the sham group. ^{*} $p < 0.05$ and ^{**} $p < 0.01$ versus the model group. TNF- α , tumor necrosis factor α .

These results indicated that IKK-induced phosphorylation of I κ B promotes I κ B degradation and release of activated NF- κ B.^{37,38} Our research predicting the effect of DSS on neuroprotection might be related to LRP1 by inhibiting the activation of IKK/NF- κ B signal pathway.

DSS ameliorated neuronal apoptosis in VCI rats

Based on a previous study, we hypothesized that the brain is in low blood flow perfusion for a long period, which can cause cerebral ischemia, hypoxia, and neuroinflammation, resulting in the neuronal apoptosis and oxidative stress in the cerebrum, eventually leading to VCI. To test this hypothesis, we measured the active forms of the effectors of neuronal apoptosis, including Bcl-2, Bax, and cleaved Caspase-3 in VCI rats. The ratio of Bcl-2/Bax decreased and cleaved Caspase-3 increased in VCI rats in hippocampus (Fig. 7A–D) ($p < 0.01$ and $p < 0.01$, respectively vs. sham). The ratio of Bcl-2/Bax increased and the levels of cleaved Caspase-3 decreased in hippocampus after treatment of DSS (Fig. 7A–D) ($p < 0.01$ and $p < 0.01$, respectively vs. model). As one marker of neurodegeneration in the hippocampus and the neural characteristic structures, the Nissl bodies can reflect the neuron survival state. Nissl's staining showed that neurons were diffusely deteriorated, a great number of Nissl bodies lost in the CA1 subfield of the hippocampal formation in VCI rats (Fig. 7E, F) ($p < 0.01$ vs. sham). In contrast, the neurons in the CA1 area in DSS high-dose group showed less neuronal damage, deeply stained, normal form neurons, and the morphology was well preserved (Fig. 7E, F) ($p < 0.05$ vs. model). These results demonstrated that DSS ameliorates neuronal apoptosis and play to the advantage of neuroprotection in VCI rats.

DSS alleviated the oxidative stress in VCI rats

This study found MDA and ROS activity also increased in VCI rats (Fig. 8A, B) ($p < 0.01$ and $p < 0.01$, respectively vs. sham). After being treated by DSS, the levels of MDA and ROS restored to normal levels (Fig. 8A, B) ($p < 0.01$ and $p < 0.01$, respectively vs. model). To further evaluate the consequence of oxidative stress in VCI rats, we next measured

the expression levels of SOD, which were sharply diminished in VCI rats (Fig. 8C) ($p < 0.01$ vs. sham) and elevated after the treatment of DSS and Nimodipine (Fig. 8C) ($p < 0.05$ and $p < 0.05$, respectively vs. model). These results indicated that DSS could ameliorate oxidative stress in VCI rats. This might be one of the neuroprotection functions of DSS.

Discussion

In this research, we showed the neuroprotective effect of DSS against CCH-induced cognitive impairment in VCI rats. We also found an elevation of LRP1 activation in hippocampus and cortex of VCI rats. This suggested that the neuroprotective effects of DSS might in part be ascribed to enhancing LRP1 expression. We speculated that DSS might mediate IKK/NF- κ B pathway in VCI rats by regulating LRP1 expression. Our results indicate that DSS may be a useful therapeutic agent in CCH-induced cognitive impairment (Fig. 9).

VCI is caused by a range of cardiovascular or cerebrovascular conditions that may lead to cerebral ischemic, hypoperfusion, or hemorrhagic brain lesions with the loss of cognitive functions.^{11,39} Considerable evidence indicates that CCH contributes to the development and progression of dementia through neuroinflammation, oxidative stress, and neuronal apoptosis, instead of the normal functionality of the recovery.⁴⁰ Despite the involvement of different mechanisms in VCI, increasing evidence indicates that neuroinflammation is an important pathological feature and an early event in the pathogenesis of VCI.⁴¹ Emerging data indicated that excessive or uncontrolled inflammation leads to worse clinical outcome in acute cerebrovascular diseases.^{42,43}

LRP1 is abundantly expressed in diverse tissues including brain, liver, and vasculature. A previous study revealed that LRP1 is involved in multiple pathways, including modulation of A β clearance, lipid transport, neuroprotection, and synaptic functions in dementia pathogenesis.^{44,45} LRP1 binds diverse extracellular ligands through cellular trafficking and/or signal pathways to recognize ApoE and A β in the pathogenesis of dementia.¹² In addition, recent work by Yang et al. demonstrated that LRP1 suppresses microglial

FIG. 6. DSS inhibited IKK/NF- κ B pathway in VCI rats. (A) Western blot analysis of IKK/NF- κ B signal pathway associated proteins in VCI rats after treatment of DSS. DSS restored the levels of phosphorylation-IKK, phosphorylation-I κ B, and phosphorylation-NF- κ B proteins in VCI rats. Meanwhile, the levels of total I κ B significantly decreased in the model group compared with sham group. (B–D) Quantification of the band intensities are presented in the adjacent graphs. Data are given as mean $\pm$ SEM ($n=12$ rats per group). [#] $p<0.05$ and ^{##} $p<0.01$ versus the sham group. ^{*} $p<0.05$ and ^{**} $p<0.01$ versus the model group. IKK, I kappa B kinase; NF- κ B, nuclear factor of kappa B.

activation by modulating c-Jun N-terminal kinase (JNK) and NF- κ B signal pathways, as a potential disease-modifying target for the treatment of AD.⁴⁶ LRP1 is expressed by microglia, particularly at sites of inflammation, and may be involved in regulating microglial activation. Silencing LRP1 gene expression in microglia or treating microglia with receptor-associated protein activates NF- κ B and JNK and increases expression of inflammatory cytokines.^{46,47–49} In this study, we measured the A β transport protein in VCI rats, such as LRP1 and RAGE. As a result, the expression of LRP1 decreased and the levels of A β 1–42 were elevated in the model group compared with the sham group, whereas the expression of LRP1 increased after treatment of DSS. These results indicated that DSS elevated the levels of LRP1 in VCI rats.

Previous studies found that LRP1 in microglia directly regulates specific signal pathways critical for inflammatory

responses, because downregulating LRP1 expression in microglia results in an increase of proinflammation cytokines.⁵⁰ These reports suggest elevated LRP1 can inhibit the activity of the IKK/NF- κ B pathway, as a system that may be modulated to either promote or inhibit macrophage inflammatory responses.⁵¹ This feature provides a mechanistic explanation for the anti-inflammatory activity of LRP1, which is evident in mouse models of atherosclerosis.⁵² We hypothesize that under basal conditions, DSS could modulate neuroinflammation, neuronal apoptosis, and oxidative stress by targeting LRP1 in an autocrine pathway, which indirectly suppressed IKK/NF- κ B pathway.

TNF- α is a potent proinflammatory cytokine that transduces the signals generated at TNF receptors to nuclear targets such as AP-1 and NF- κ B, which in turn induce genes involved in chronic and acute inflammatory responses.^{36,53} In response to TNF- α , IL-1 β , and other agonists, the I κ Bs

FIG. 7. DSS ameliorates neuronal apoptosis in VCI rats. **(A, B)** The ratio of Bcl-2 and Bax in the hippocampus. **(C, D)** The levels of cleaved Caspase-3 in the hippocampus. **(E, F)** The Nissl's staining in CA1 area of the hippocampus. Data are given as mean $\pm$ SEM ($n = 12$ rats per group). [#] $p < 0.05$ and ^{##} $p < 0.01$ versus the sham group. ^{*} $p < 0.01$ versus the model group.

FIG. 8. DSS alleviated oxidative stress in VCI rats. (A–C) The levels of MDA, reactive oxygen (ROS), and SOD in the hippocampus. Note that MDA and ROS levels were selectively downregulated and SOD levels were elevated in the hippocampus after treatment of DSS. Data are given as mean $\pm$ SEM ($n = 12$ rats per group). ^{##} $p < 0.01$ versus the sham group. ^{*} $p < 0.05$ and ^{**} $p < 0.01$ versus the model group. MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.

are phosphorylated by the IKK complex, resulting in their ubiquitination, degradation, and nuclear translocation of the freed NF- κ B. Once getting into the nucleus, NF- κ B transcriptional activity can be modulated further through phosphorylation by various protein kinases that are TNF- α and other proinflammation responsive.⁵⁴ In APP/PS1 transgenic rats, the levels of proinflammation reduced astrocytosis, A β oligomerization, and deposition, and enhanced neurogenesis.⁵⁵ Another study has shown that NF- κ B and JNK pathways are both important to modulate the release of inflammatory cytokines and phagocytosis in AD pathogenesis.⁴⁴ Numerous studies have demonstrated the activation of NF- κ B in astrocytes and that an astrocyte proinflammatory pathway may have important consequences for cognitive outcome in neurodegenerative disease.⁵⁶ In this study, we found that high expression of TNF- α and IL-1 β in VCI rats results in an increase of p-IKK, p-I κ B and a decrease of I κ B, meanwhile NF- κ B transcriptional activity is activated by I κ B and various protein kinases. The importance of this process in controlling the activity of the IKK/NF- κ B pathway may be regulated transcription and expression of proinflammatory factor. After the treatment of DSS and Nimodipine, DSS could improve the neural function of VCI rats through the IKK/NF- κ B pathway. Actually, inflammatory factors emerging as key players in dementia increase the expression of cytokines (TNF- α and IL-1 β) and consequently, that of IKK/NF- κ B pathway.^{57,58} Nevertheless, emerging data indicate that the harmful stimulus is no longer present, blockade of inflammation is protective in common neurological diseases.^{59,60}

Neuron apoptosis and oxidative stress are caused by a long time of cerebral ischemic, hypoperfusion, neuroinflammation, and hemorrhagic brain lesions with the decline of cognitive functions.⁶¹ Many studies have demonstrated that the suppression of apoptosis, which is mostly dependent on NF- κ B, augments the inflammatory response to TNF- α . Of importance, neuron apoptosis was mediated by various factors, such as Bcl-2, Bax, Caspases, A β , TNF- α , ROS.⁶² The Bcl-2 protein family, including antiapoptotic protein Bcl-2 and proapoptotic protein Bax, plays a very important role in mitochondria-mediated apoptosis and is crucial to mitochondrial regulators during neuron apoptosis.⁶³ A large number of studies indicated that overexpression of Bcl-2 could inhibit the neuron apoptosis.^{64,65} Meanwhile, pre-clinical study found that the number of neuronal apoptosis in the hippocampus of adult rats was significantly reduced in knockout Bax gene.⁶⁶ Many researches confirmed that Caspase-3 plays a critical role in neuron apoptosis and neuron cell remodeling, including the synaptic plasticity, cut of dendritic spines, and longtime history inhibition effect.^{18–22} In addition, intense-activated Caspase-3 regulated to the proteolytic cleavage of polymeric tau protein in the ultimate apoptosis of cells in AD.⁶⁷ Our work suggested that the expression of cleaved Caspase-3 was increased in the brain tissues of the model group compared with sham group. Treatment of DSS high-dose and Nimodipine significantly reduced the expressions of cleaved Caspase-3 and Bax protein, increased the protein expression of Bcl-2 and the ratio of Bcl-2/Bax. Those results suggest that DSS can improve cognitive function and prevent neuronal injury in VCI rats by inhibiting neuronal apoptosis. In this study, neurons

FIG. 9. Schematic diagram of LRP1-associated pathogenesis in VCI rats. An ischemia/hypoperfusion conditions leads to selective activation of proinflammatory mediators (such as IL-1 β , TNF- α) in the hippocampus, CA1 neuron apoptosis, vascular damage, and a cognitive deficit. Under the hypoperfusion condition in the hippocampus, proinflammatory cytokine induces IKK/NF- κ B pathway, which in turn induce genes involved in chronic and acute inflammatory responses. Furthermore, neuron apoptosis and oxidative stress are mostly dependent on the IKK/NF- κ B pathway in response to the longtime of cerebral ischemic, hypoperfusion, neuroinflammation, and hemorrhagic brain lesions with the loss of cognitive functions. At the same time, DSS improves cognitive function and prevents neuroinflammation of VCI rats by targeting LRP1 led to an inhibiting effect on IKK/NF- κ B pathway.

were impaired owing to CCH but reversed by DSS. Meanwhile, the Nissl's staining also demonstrated the neuroprotective function of DSS.

The present study demonstrated that neuronal apoptosis plays a key role in the pathogenesis of VCI and DSS can significantly inhibit neuronal apoptosis. During the past decades, a vast number of studies provided evidences that increased levels of oxidative stress may lead to cerebrovascular changes.⁶⁸ Based on our study, examining cells under basal conditions and in response to the oxidation reaction, we propose that MDA, ROS, and SOD may participate in this produce, among receptors that are indirectly regulated by IKK/NF- κ B pathway. ROS is a kind of molecules with oxidation ability mainly including the ultra oxygen anion, hydrogen peroxide, hydroxyl free radicals, singlet oxygen, and so on, which widely exists in the body and regulates the body's physiological and pathological process.⁶⁹ Studies have shown that ROS can act as a signaling molecule to mediate cell signal transduction, thus inducing apoptosis.⁷⁰ In addition, the effect of ROS-induced

apoptosis may involve in increasing the ratio of Bax/Bcl-2.⁷¹⁻⁷³ After the cerebral ischemia injury, a large number of free radicals can be produced, damaging the cell membrane of neurons, resulting in lipid peroxidation and a large number of MDA. With the massive consumption of antioxidant enzymes, such as SOD, the body's ability to scavenge free radicals gradually decreases.⁷⁴ Massive accumulation of free radicals direct damage to the brain, result in producing large numbers of free radicals, lipid peroxidation in the brain, causing the brain neurons, mitochondrial membrane injury and energy metabolism disorder, and finally lead to cell death.⁷⁵ This feature demonstrated that the DSS protects against neuronal oxidative stress and improves the learning and memory ability in VCI rats. Based on our current findings, DSS might be a promising anti-inflammatory and antioxidative stress agent for the treatment of VCI.

Taken together, our data clearly demonstrated that treatment with DSS could mitigate cognitive deficiency and inhibit the proinflammation factor. DSS inhibited the IKK/NF- κ B

pathway by elevating levels of LRP1. DSS mitigated CCH-associated cognitive decline and played a neuroprotective role in VCI rats that occur after carotid arteries occlusion. Thus, we propose that DSS can be used as a treatment to control cognitive impairment, inflammatory and oxidative process associated with VCI. Therefore, it might not only contribute to symptomatic improvement, but also play important roles for the treatment of neurodegenerative diseases by interfering with key factors of the disease. However, further mechanisms remain to be explored.

Authors' Contributions

The main article text and experiments were completed by H.C. and T.C. Figures 1 to 8 were all drawn by H.C. and Figure 9 was drawn by T.C. C.Y. and Y.W. prepared the Western blot and staining figures analysis. Q.Z., L.Z., and X.P. supported the behavioral tests and reviewed the describing article. H.Z. and L.L. checked the English grammar. Z.G., H.P., and Q.W. supervised the article and directed final version of all contents. All authors reviewed the final draft of the article and approved it for submission.

Author Disclosure Statement

No competing financial interests exist.

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