Protective effects of dehydrocostuslactone on rat hippocampal slice injury induced by oxygen-glucose deprivation/reoxygenation

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Abstract. The present study aimed to investigate the protective effects of dehydrocostuslactone (DHL) against rat hippocampal slice injury caused by oxygen-glucose deprivation/reoxygenation (OGD/R). Rat hippocampal slice injury was induced by OGD/R *in vitro*, and the degree of injury was evaluated through a lactate dehydrogenase (LDH) assay and 2,3,5-triphenyltetrazolium chloride (TTC) staining. The protein expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), cytochrome *c* (cyt-c), apoptotic protease activating factor 1 (apaf-1), caspase-9, caspase-7, caspase-3, sequestosome 1 (SQSTM1) and microtubule-associated protein 1 light chain 3 (LC3) were analyzed through western blot analysis. The results showed that 1, 5 and 10 μM DHL decreased the levels of LDH ($P<0.05$) and increased the A_{490} value of TTC ($P<0.05$). Furthermore, the expression of Bcl-2 was enhanced, and the protein expression levels of Bax, cyt-c, apaf-1, caspase-9, caspase-7, caspase-3, SQSTM1 and LC3 were significantly inhibited ($P<0.05$), compared with those in the OGD/R group. These results suggested that DHL elicited protective effects against hippocampal OGD/R injury, and its underlying mechanism may be associated with inhibiting apoptosis.

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Introduction

Ischemic stroke is a common cause of mortality worldwide (1,2). The main pathogenetic mechanisms of cerebral ischemia include intracellular calcium overload (3,4), excitatory amino acid toxicity, increase in oxygen free radicals (4), inflammatory responses (4,5), autophagy and apoptosis (6). A complex interplay also exists among these factors, for example, autophagy and apoptosis. Although a suitable level of autophagy may provide neuroprotection, the excessive activation of autophagy can induce cell death. Apoptosis is essential in the pathogenesis and prognosis of different cerebrovascular diseases, including chronic and acute ischemia. As a result of hypoxia and lack of glucose, when cells suffer from ischemic stimulation due to an increasing number of reactive oxygen species (ROS) in the cytoplasm (7), including $\cdot O_2^-$, H_2O_2 and $\cdot OH$, in addition to anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and pro-apoptotic protein Bcl-2-associated X protein (Bax) (8,9), the mitochondrial membrane permeability transition pore is opened. This leads to increased mitochondrial membrane permeability and decreased mitochondrial membrane potential. Subsequently, cytochrome *c* (cyt-c) is translocated from the mitochondrial matrix to the cytoplasm, forming an apoptotic complex by combining apoptotic protease activating factor 1 (apaf-1) and caspase-9. Finally, the caspase-dependent apoptotic pathway is activated. Furthermore, other transcription factors, including, p53, tumor necrosis factor-α and nuclear factor-κB, may be involved in the apoptotic process (10,11). Therefore, inhibiting autophagy and apoptosis is a possible target for stroke treatment.

The hippocampus is a region of the brain sensitive to changes in oxygen-glucose levels (12). Rat hippocampal slices are used in *in vitro* experiments, as a mutual connection between neurons and glial cells is maintained (13,14). Hippocampal slices have been used to investigate the effects of anoxia, excitotoxicity and depolarization (15-17). The excitability of CA1 neurons increases when cerebral ischemia occurs. If oxygen-glucose supply is not restored, or therapeutic windows exceed optimal levels, neurons become excited until these cells die (18).

Dehydrocostuslactone (DHL) is a sesquiterpene lactone derived from the medicinal plant *Aucklandia lappa* Dence, belonging to the family Asteraceae (19) and is a major active ingredient of Aucklandiae Radix. The metabolite of DHL can penetrate the blood-brain barrier and exhibit various pharmacological activities, including anti-inflammatory (20), anti-ulcer (19) and antimicrobial properties (21), anti-apoptotic and pro-apoptotic effects on different human cancer cell lines (22,23), and oxidative and endoplasmic reticulum stress responses (24).

In the present study, rat hippocampal slices were subjected to oxygen-glucose deprivation and reoxygenation (OGD/R) and used to investigate whether DHL reduces neuronal apoptosis and autophagy *in vitro*.

Materials and methods

Animals. Male Sprague-Dawley rats (weighing 250-290 g, aged 8-10 weeks old) were purchased from the Experimental Animal Center of Ningxia Medical University [Yinchuan, China; animal license no. SCXK (Ning) 2011-0001]. All rats were maintained in a well-ventilated environment with a 12 h/12 h light/dark cycle at constant room temperature (25±2˚C) and humidity (50-70%). The rats were provided with free access to normal rodent food and water.

Reagents. DHL (cat. no. 34080) was purchased from Chengdu Pusi Biological Technology Co., Ltd. (Chengdu, China). A stock solution was prepared using dimethyl sulfoxide (DMSO) solution and diluted with normal artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 3.00 mM KCl, 2.50 mM $MgSO_4 \cdot 7H_2O$, 2.00 mM $CaCl_2$, 26.00 $NaHCO_3$, 1.25 mM NaH_2PO_4 and 10.00 mM glucose, and glucose-free aCSF (equal mole sucrose instead of glucose) to obtain the required concentration. Low, middle and high DHL concentrations were set at 1, 5 and 10 μM, respectively. Nimodipine injection (cat. no. H20140301) was provided by Bayer Schering Pharma AG (Shanghai, China). The nimodipine concentration was set at 10 μM. 2,3,5-triphenyltetrazolium chloride (TTC; cat. no. T8877; Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) was dissolved in aCSF and stored in the dark. The following substances and kits were utilized in the present study: Lactate dehydrogenase (LDH) assay kit (cat no. A020-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China); total protein extraction and BCA protein quantification kits (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China); primary antibodies against Bcl-2 (cat. no. 2876), Bax (cat. no. 2772), cyt-c (cat. no. 4272), apaf-1 (cat. no. 8723) and β-actin (cat. no. 4970) were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA); primary antibodies against caspase-9 (cat. no. ab2013), caspase-7 (cat. no. ab25900) and caspase-3 (cat. no. ab44976) were procured from Abcam (Cambridge, UK); primary antibodies against sequestosome 1 (SQSTM1; cat. no. bs-2951R) and microtubule-associated protein 1 light chain 3 (LC3; cat. no. bs-8878R) were procured from BIOSS (Beijing, China); horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. ZB-2301) was obtained from ZSGB-BIO (Beijing, China); Z-VAD-FMK, a novel broad-spectrum caspase protease inhibitor (cat. no. C1202) was obtained from Beyotime Institute of Biotechnology (Nantong, China).

Rat hippocampal slice preparation. The rats were anesthetized with 7% chloral hydrate (350 mg/kg, i.p.) and sacrificed by decapitation. Their brains were rapidly excised and placed in an ice-cold bath filled with a 95% O_2/5% CO_2 gas mixture of aCSF for 1 min. The bilateral hippocampus was also rapidly excised. The hippocampal slices were isolated, coronally trimmed, glued to the stage of a vibration slicing machine (LEICA VT 1000S; Leica Microsystems, Inc., Buffalo Grove, IL, USA), and coronally cut to a thickness of 400-μm at 0˚C. The hippocampal slices were divided into eight groups: Control, OGD, OGD+nimodipine (10 μM, dissolved in aCSF and glucose-free aCSF), OGD+DHL (1, 5 and 10 μM dissolved in DMSO, aCSF and glucose-free aCSF, respectively), OGD+Z-VAD-FMK (20 μM), and OGD+Z-VAD-FMK (20 μM)+DHL (10 μM). Each group contained 40 slices. All hippocampal slices were immediately pipetted lightly into an incubator and then recovered for 1 h in oxygenated aCSF at 37˚C.

OGD/R injury model. Following the recovery period, the slices were lightly washed with aCSF and transferred into a 24-well culture plate with four slices per well. The OGD of the experimental groups was induced by glucose-free aCSF. This dissection buffer was bubbled with a 95% N_2/5% CO_2 gas mixture in a 24-well culture plate for 30 min. The control group was incubated in aCSF equilibrated with 95% O_2/5% CO_2 for 30 min (OGD period). Subsequently, the slices were transferred into a new 24-well culture plate containing oxygenated normal aCSF and placed in a 95% O_2/5% CO_2 gas mixture incubator. Finally, the slices were incubated for 1 h at 37˚C. Five treatment groups received nimodipine (10 μM), Z-VAD-FMK (20 μM), DHL (1, 5 and 10 μM), and Z-VAD-FMK (20 μM)+DHL (10 μM) within the OGD/R period. The drugs were dissolved in aCSF and glucose-free aCSF.

LDH activity measurement. LDH is a cytosolic enzyme that can be released into extracellular fluid when cell membranes are disrupted. Therefore, this enzyme is used to evaluate the degree of injury. In the present study, the viability of the rat hippocampal slices was determined following the completion of reoxygenation by measuring the concentration of LDH released into the culture supernatants through spectrophotometry at 450 nm in an ELISA reader in accordance with the manufacturer's protocol.

TTC staining. Following reoxygenation completion, the rat hippocampal slices were transferred into small vials (50 ml) containing 2% (w/v) TTC solution in aCSF aerated vigorously for 10 min at 37˚C. The stained slices were subsequently removed and rinsed twice with normal saline. The slices were dried and weighed. An extraction buffer (DMSO and 100% ethanol prepared 1:1) was added (20 μl/mg) and stored in the dark for 24 h at room temperature. The quantity of the extracted formazan was then measured through spectrophotometry at 490 nm using an ELISA reader.

Western blot analysis. Western blot analysis was performed to detect the relative levels of Bcl-2, Bax, cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3. The slices were treated and grouped as indicated in the previous section. In each group, four slices were collected as one sample following administration of the

respective treatments. All samples were rapidly placed in an ice bath, and total protein extraction was initiated using the total protein extraction kit. The protein extraction fluid, containing lysis buffer (1 ml), PMSF (10 μl), protein inhibitor (1 μl) and phosphatase inhibitor (10 μl), was added in a volume of 0.5 ml to 100 mg of tissue slice in a Pro homogenizer and homogenized 30 times. The homogenate was transferred to a centrifuge tube and centrifuged at 12,000 x g for 10 min at 4˚C to remove cellular debris. The BCA protein assay reagent kit was used to determine the protein concentration. The lysates from the slices were boiled for 5 min. Equivalent quantities (80 μg) of protein samples were subjected to 10% SDS-PAGE and electrically transferred onto PVDF membranes using an electrophoretic transfer system (40D; Beijing Liuyi Biotechnology Co., Ltd., Beijing, China). These membranes were subdivided, and each target protein and loading control protein were analyzed from a single transfer. Nonspecific binding sites were blocked with PBST containing 5% nonfat dry powdered milk for 1 h at room temperature. The membranes were then incubated with primary antibodies against Bcl-2 (1:1,000), Bax (1:3,00), cyt-c (1:1,000) apaf-1 (1:1,000), caspase-9 (1:1,000), caspase-7 (1:500), caspase-3 (1:500), and β-actin (1:1,000) at 4˚C overnight. Immunostained β-actin served as the respective control. The membranes were washed three times with PBST containing 0.1% Tween and re-incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 2 h at room temperature. The bands were then visualized using a Super Signal West Pico Chemiluminescence kit and finally exposed to radiographic films. The resulting bands were analyzed with Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The image intensities of Bcl-2, Bax, caspase-9, caspase-7, caspase-3, SQSTM1 and LC3 were quantified and normalized with β-actin protein band density.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The results are presented as the mean ± standard deviation. Differences among more than two groups were assessed using one-way analysis of variance followed by the least significant difference post hoc test. Differences between two groups were analyzed using an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of DHL on LDH release in OGD/R-injured rat hippocampal slices. The degree of injury on the hippocampal slices exposed to OGD/R was determined through the LDH assay. The OGD/R group showed a significant increase in LDH catalytic activity compared with that of the control group. The LDH concentrations of the DHL-treated groups (1, 5 and 10 μM) were lower than that of the OGD/R group (P<0.05), and this finding was concentration-dependent. No significant differences in the levels of LDH were observed between the nimodipine and DHL groups (1, 5 and 10 μM; P>0.05; Fig. 1).

Effects of DHL on TTC staining of OGD/R-injured hippocampal slices. The TTC staining results were quantified through spectrophotometry at 490 nm using an ELISA reader.

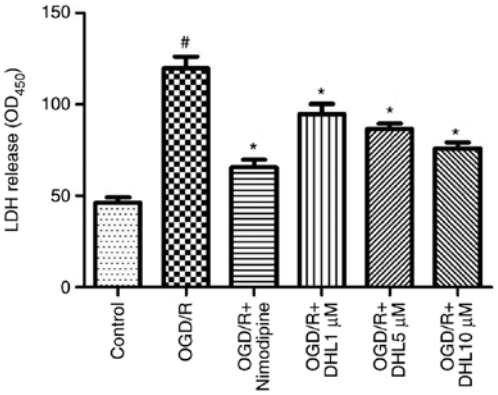

Figure 1. Effect of DHL on LDH release in the culture medium of hippocampal slices subjected to OGD/R. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; LDH, lactate dehydrogenase; OGD/R, oxygen-glucose deprivation/reoxygenation.

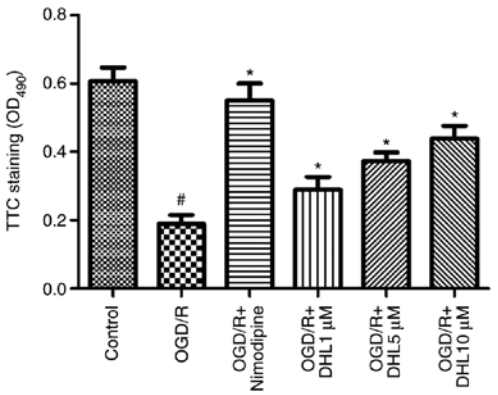

Figure 2. Effect of DHL on TTC staining of OGD/R-injured hippocampal slices. The results showed that DHL increased the OD_{490} values of rat hippocampal slices subjected to OGD/R. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation; TTC, 2,3,5-triphenyltetrazolium chloride.

The OD_{490} of TTC staining was obtained to evaluate the degree of hippocampal slice injury. The results demonstrated that the OD_{490} of the OGD/R group was significantly lower than that of the control group, the DHL-treated groups, and the nimodipine-treated group. The OD_{490} of the DHL (1, 5 and 10 μM) and nimodipine groups were significantly higher than that of the OGD/R group (P<0.05), with no significant difference in OD_{490} was observed between the nimodipine- and DHL-treated groups (1, 5 and 10 μM; P>0.05; Fig. 2).

Effects of DHL on the expression of Bcl-2, Bax, cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3. To investigate the anti-apoptotic effects of DHL on rat hippocampal slice injury induced by OGD/R, the present study determined the expression levels of cyt-c (Fig. 3), apaf-1 (Fig. 4), Bcl-2, Bax (Fig. 5), caspase-9 (Fig. 6), caspase-7 (Fig. 7) and caspase-3 (Fig. 8), and used these as markers of apoptosis in the mitochondria. The results of western blot analysis revealed that the expression level of Bcl-2 in the OGD/R group was significantly lower than that of the control group, whereas the expression level of

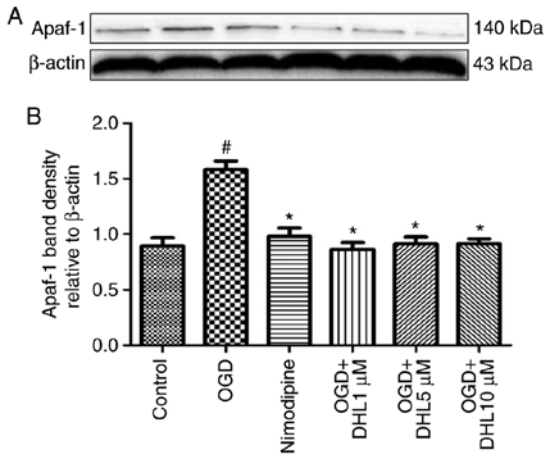

Figure 3. Effects of DHL on the expression of apaf-1. (A) Western blot analysis of apaf-1, with β-actin shown as a loading control; (B) Quantitative analysis of the expression of apaf-1. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. apaf-1, apoptotic protease activating factor 1; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

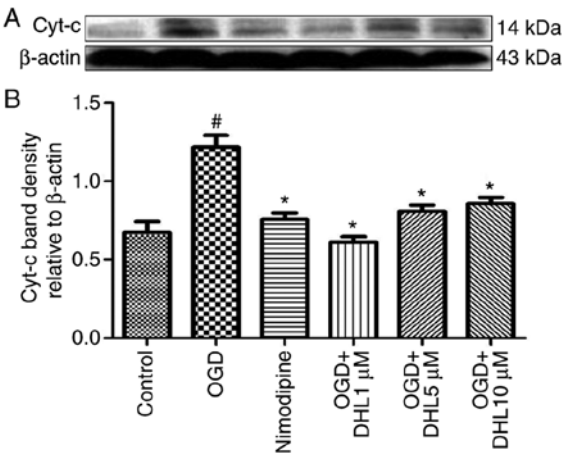

Figure 4. Effects of DHL on the expression of cyt-c. (A) Western blot analysis of cyt-c, with β-actin shown as a loading control; (B) Quantitative analysis of expression of cyt-c. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. cyt-c, cytochrome *c*; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

Bax in the OGD/R group was markedly higher than that of the control group. Treatment with DHL (1, 5 and 10 μM) enhanced the expression of Bcl-2 and significantly inhibited the expression of Bax. The expression levels of cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3 were also significantly increased in the OGD/R group. Treatment with DHL (1, 5 and 10 μM) reduced the expression levels of apaf-1, cyt-c, caspase-9, caspase-7 and caspase-3, however, no significant difference was found in the expression levels of Bcl-2, Bax, cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3 between the nimodipine- and DHL-treated groups (1, 5 and 10 μM; P>0.05).

Effects of Z-VAD-FMK and DHL on the expression of cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3. To compare the anti-apoptotic effects of Z-VAD-FMK and DHL on rat hippocampal slice injury induced by OGD/R, the present study determined the expression levels of cyt-c, apaf-1, caspase-7 (Fig. 9), caspase-9 (Fig. 10) and caspase-3 (Fig. 11). The results suggested that Z-VAD-FMK downregulated the expression levels of caspase-9, caspase-7 and caspase-3 (1, 5 and 10 μM; P<0.05).

Effects of DHL on the expression of SQSTM1 and LC3-II. To investigate the anti-autophagy effects of DHL on rat hippocampal slice injury induced by OGD/R, the present study determined the expression levels of LC3-II (Fig. 12) and SQSTM1 (Fig. 13). The expression levels of LC3-II and SQSTM1 were markedly increased in the OGD/R group compared with that of the control group. Treatment with DHL (1, 5 and 10 μM) inhibited the expression of LC3-II and SQSTM1 (P<0.05).

Discussion

In the present study, rat hippocampal slices were used to establish an OGD/R model, which is an *in vitro* model of ischemia (25,26), and ischemic stroke was simulated to investigate the protective effects and mechanisms of DHL.

Changes in the quality and quantity of LDH directly affect the energy metabolism of the body. LDH can be released into extracellular spaces through a damaged cell membrane if external stimuli, including hypoxia-ischemia, are present (27). Therefore, an LDH assay can be used to quantify the effects of OGD (28). Cechetti *et al* (29) performed an LDH assay and examined the effects of treadmill exercise on cell damage in rat hippocampal slices subjected to oxygen and glucose deprivation. The LDH assay can also be utilized to evaluate membrane integrity loss and pathological necrosis (30). In addition, TTC staining has been used to examine brain injuries quantitatively (31). In this method, TTC is oxidized only by metabolically active mitochondrial dehydrogenases and converted into red formazan. The quantity of formazan is directly proportional to cell activity (32). For example, TTC staining has been used to measure the area of cerebral infarcts in brain slices, and TTC assays have also been performed to assess cell and tissue viability (33,34). In the present study, the level of released LDH was significantly increased, and the OD_{490} of TTC staining was significantly reduced when the cells were exposed to OGD/R; this change demonstrated that the hippocampal slices were more vulnerable to OGD/R injury (26). DHL treatment markedly decreased the release of LDH and increased the OD_{490} of TTC staining.

To enhance our understanding of the mechanisms underlying the action of OGD/R, the present study elucidated the levels of apoptosis-related proteins as factors that may be involved in DHL-induced neuroprotection against rat hippocampal slice OGD/R damage, as Bcl-2 inhibits programmed cell death without affecting cellular proliferation (35). Bcl-2 was the first anti-apoptotic protein identified with a protective activity against ischemic injury (36). Furthermore, Bax, a pro-apoptotic protein found in the cytosol of healthy cells, promotes apoptosis when cells are stimulated by ischemia (37,38). In addition, Bax can combine with Bcl-2 to form polymers, increase the permeability of the mitochondrial membrane, activate the caspase-apoptosis pathway, and trigger cell apoptosis. The expression levels of Bcl-2 are

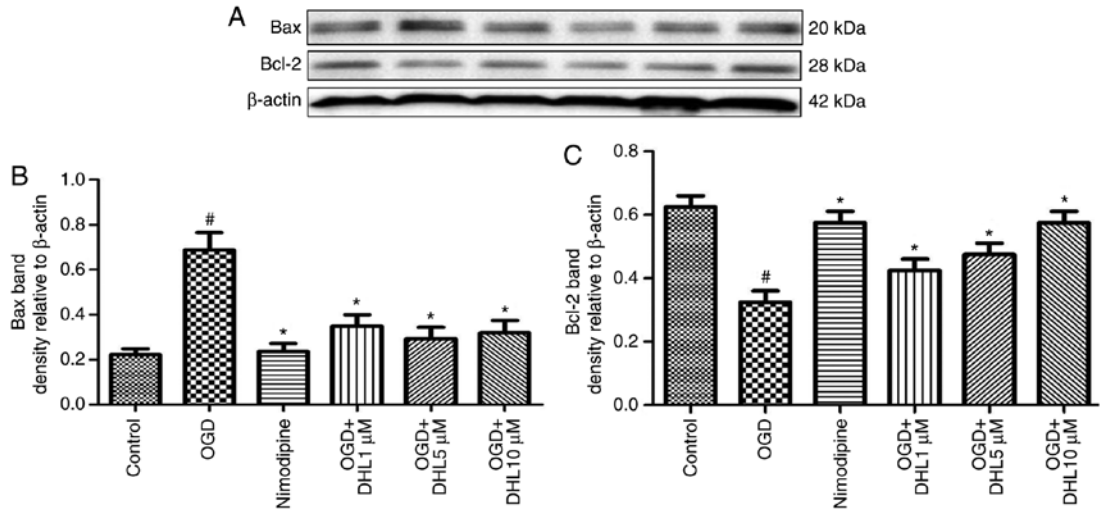

Figure 5. Effects of DHL on the expression of Bax and Bcl-2. (A) Western blot analysis of Bax and Bcl-2, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of Bax. (C) Quantitative analysis of the expression of Bcl-2. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

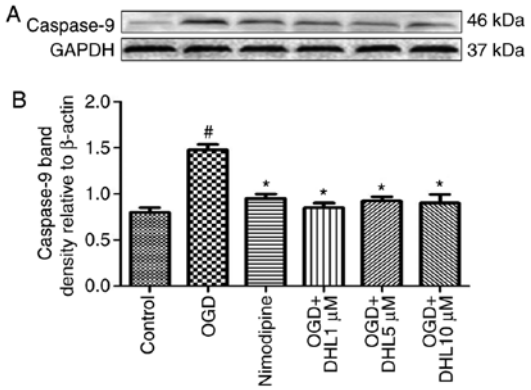

Figure 6. Effects of DHL on the expression of caspase-9. (A) Western blot analysis of caspase-9, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of caspase-9. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

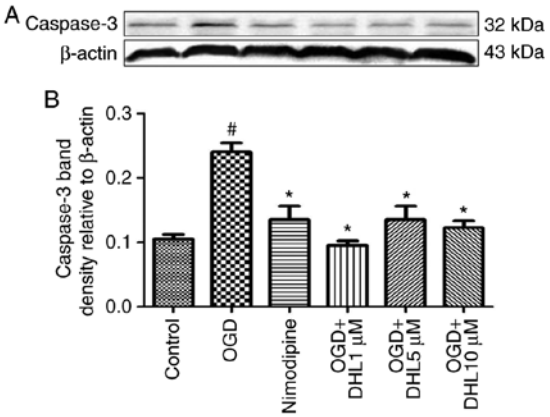

Figure 8. Effects of DHL on the expression of caspase-3. (A) Western blot analysis of caspase-3, with β-actin is shown as a loading control. (B) Quantitative analysis of the expression of caspase-3. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

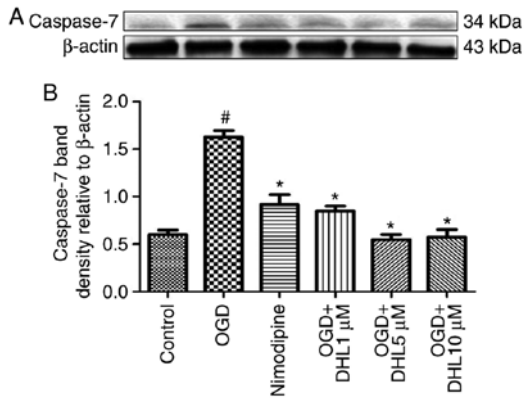

Figure 7. Effects of DHL on the expression of caspase-7. (A) Western blot analysis of caspase-7, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of caspase-7. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

decreased, and the expression levels of Bax and caspase-3 are increased in rats with cerebral ischemia injury (39,40). Cysteine-requiring aspartate proteases are a family of proteases that are involved in apoptosis *in vivo* and *in vitro* (41,42). Based on its functionality, the upstream pro-apoptotic factor caspase-9 becomes activated when a cell suffers from injury; the downstream factor caspase-7 and apoptosis of the executive factor caspase-3 are further stimulated, and various cells consequently undergo apoptosis (43,44). Secondary messengers, including Ca^{2+}, Bcl-2 and Bax, are also activated when an apoptotic signal is delivered to the mitochondria (45). As a result, cyt-c is released from the mitochondria, and apaf-1 and caspase-9 combine to form a complex (46). This activates caspase-9 and other downstream effector factors, including caspases-3, caspase-6 and caspase-7; cell apoptosis is induced as a result (45). Consistent with previous reports, the present study demonstrated that the protein expression levels of Bcl-2

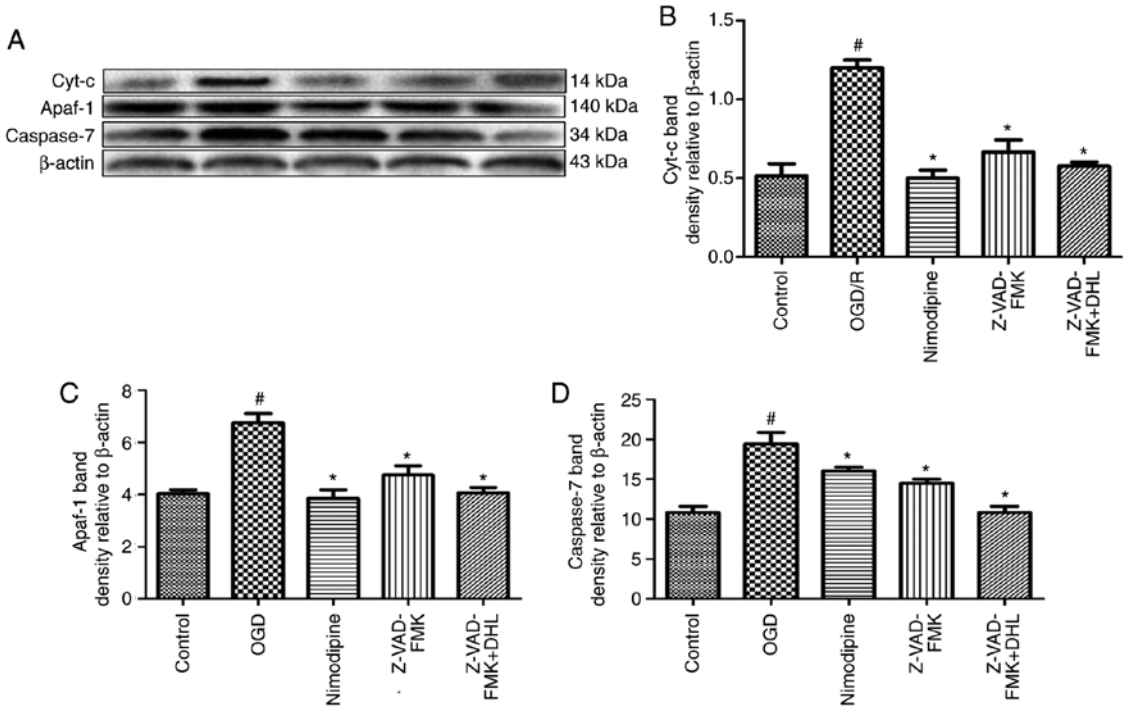

Figure 9. Effects of Z-VAD-FMK and DHL on the expression of cyt-c, apaf-1 and caspase-7. (A) Western blot analysis of f cyt-c, apaf-1 and caspase-7, with β-actin shown as a loading control. Quantitative analysis of the expression of (B) cyt-c, (C) apaf-1 and (D) caspase-7. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. cyt-c, cytochrome *c*; apaf-1, apoptotic protease activating factor 1; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

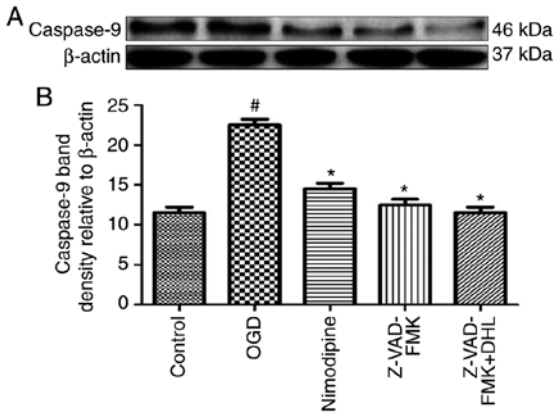

Figure 10. Effects of Z-VAD-FMK and DHL on the expression of caspase-9. (A) Western blot analysis of caspase-9, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of caspase-9. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

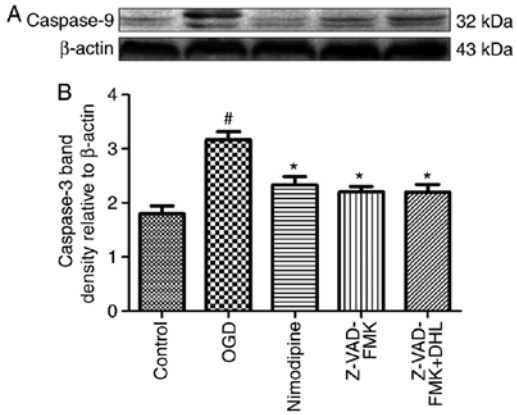

Figure 11. Effects of Z-VAD-FMK and DHL on the expression of caspase-3. (A) Western blot analysis of caspase-3, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of caspase-3. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

were markedly decreased, and the protein expression levels of Bax, cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3 were increased when the cells were exposed to OGD/R. Treatment with DHL upregulated the expression levels of Bcl-2, and downregulated the expression levels of Bax, cyt-c, apaf-1, caspase-9, caspase-7 and caspase-3. Z-VAD-FMK, a widely irreversible caspase inhibitor, was used in the present study, and the results suggested that Z-VAD-FMK downregulated the expression levels of caspase-9, caspase-7 and caspase-3. DHL treatment also downregulated caspase-9, caspase-7 and caspase-3 to a greater extent than Z-VAD-FMK treatment.

Autophagy is a programmed and physiologically conserved self-degradation process involved in focal cerebral infarction (47,48). However, whether autophagy promotes neuronal death in response to OGD/R remains to be elucidated. In the present study, LC3 and SQSTM1, which are specific markers of autophagosomes, were probed to determine whether autophagic activity is induced by DHL treatment. LC3 is used as an autophagosome marker. Upon the induction of autophagy, LC3-I becomes converted to LC3-II, which then translocates to autophagosome membranes. The ratio of LC3-II to LC3-I is increased in mice subjected to middle cerebral artery

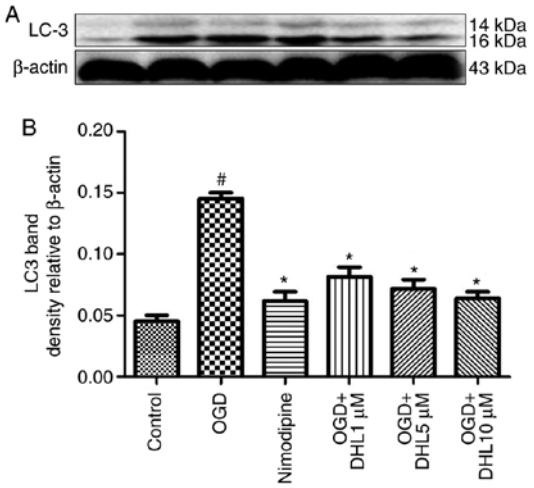

Figure 12. Effects of DHL on the expression of LC3-II. (A) Western blot analysis of LC3- II, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of LC3-II. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. LC3, microtubule-associated protein 1 light chain 3; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

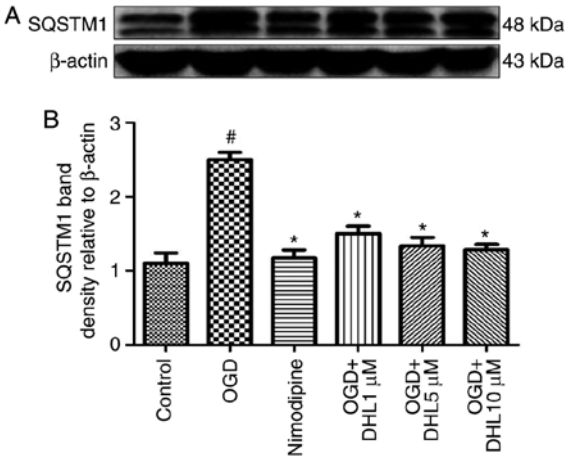

Figure 13. Effects of DHL on the expression of SQSTM1. (A) Western blot analysis of SQSTM1, with β-actin shown as a loading control. (B) Quantitative analysis of the expression of SQSTM1. Values are presented as the mean ± standard deviation (n=10). #P<0.05, compared with the control group; *P<0.05, compared with the OGD/R group. SQSTM1, sequestosome 1; DHL, dehydrocostuslactone; OGD/R, oxygen-glucose deprivation/reoxygenation.

occlusion (49). SQSTM1 (p62) is a ubiquitin-binding protein involved in autophagy. SQSTM1 binds to the autophagosomal membrane protein LC3 and carries SQSTM1-containing protein aggregates to autophagosomes. The level of SQSTM1 is used to monitor autophagic flux (46,50). In the present study, LC3-II and SQSTM1 were markedly increased in the rat hippocampal slices injured by OGD/R. This result suggested that autophagy was activated in the rat hippocampal slices exposed to OGD/R, and this change was reversed by DHL treatment.

In the present study, the desired results were not obtained in paraffinized sections, frozen sections or flow cytometry. The results in the paraffinized sections were unsatisfactory due to the smaller and thinner hippocampal sample size (400 μm), and there were more ice crystals in the frozen sections. Based on these reasons, it was not possible not evaluate the region or degree of hippocampal slice injury with hematoxylin and eosin staining, immunohistochemistry or TUNEL detection. Finally, the present study aimed to evaluate the degree of damage in the hippocampal slices using flow cytometry; the Annexin V/PI staining showed that the majority of cells were dead, and this cell death may have been induced by the preparation process of producing a single-cell suspension from the hippocampal slice.

Despite the limitations of the present study, the results revealed that DHL provided protective effects against hippocampal slice injury induced by OGD/R through anti-apoptotic and anti-autophagic effects. However, further investigations are required to determine other underlying mechanisms.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

QZ, AC and XW established the hippocampal slice injury model and performed western blotting analysis. YuZ and YH analyzed and interpreted the data. ZZ performed lactate dehdrogenase activity measurement and tetrazolium chloride staining, and wrote the manuscript. YaZ and SR designed the study, supervised the research group and revised the manuscript critically for important intellectual content. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Ningxia Medical University (Yinchuan, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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