Dihydromyricetin inhibits oxidative stress and apoptosis in oxygen and glucose deprivation/reoxygenation-induced HT22 cells by activating the Nrf2/HO-1 pathway

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Abstract. Cerebral ischemia-reperfusion injury (CIRI) refers to the phenomenon that ischemic injury of the brain leads to the injury of brain cells, which is further aggravated after the recovery of blood reperfusion. Dihydromyricetin (DHM) has an effective therapeutic effect on vascular diseases; however, its role in CIRI has not been investigated. The oxygen and glucose deprivation/reoxygenation (OGD/R) cell model was used on HT22 hippocampal neurons in mice, by oxygen and sugar deprivation. DHM was found to increase the cell viability of HT22 cells following OGD/R induction. The levels of malondialdehyde (MDA) decreased, superoxide dismutase (SOD) and glutathione (GSH) in the OGD/R-induced HT22 cells increased following DHM treatment, accompanied by the decreased protein expression levels of NOX2 and NOX4. DHM also inhibited cell apoptosis induced by OGD/R, and decreased the protein expression levels of Bax and caspase-3, and increased the expression levels of Bcl-2. Moreover, the expression levels of the NF-E2-related factor 2 (Nrf2)/heme oxygenase (HO-1) signaling pathway-associated proteins in OGD/R-induced HT22 were increased following DHM treatment, and the effect of DHM on oxidative stress and apoptosis was reversed after the addition of the Nrf2/HO-1 pathway inhibitor, brusatol. In conclusion, DHM inhibited oxidative stress and apoptosis in OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway.

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Key words: dihydromyricetin, cerebral ischemia reperfusion injury, oxidative stress, apoptosis, oxygen and glucose deprivation/reoxygenation, Nrf2/HO-1 pathway

Introduction

Cerebrovascular disease is a common and frequently-occurring disease in clinical practice. It has a high mortality rate, and its morbidity, mortality and probability of disability caused by cerebrovascular disease have increased year on year (1). Global cerebrovascular disease had a national mortality rate and a prevalence ratio of 28 and 142 per 100,000 persons, respectively in 2015 (2). Ischemic cerebrovascular diseases account for the vast majority of cerebrovascular diseases, among which, cerebral ischemia-reperfusion injury (CIRI) is the most serious type. CIRI refers to the phenomenon in which metabolic disorders and structural damage of some cells are aggravated, following the reduction in the amount of blood perfusion, and then the ischemic damaged tissues resume blood reperfusion (3). Thus, there is an urgent requirement to treat CIRI.

Traditional Chinese medicine (TCM) has been gradually used in the clinical treatment of cerebrovascular diseases, as there are fewer side effects, and improved efficacy and safety (4). Dihydromyricetin (DHM) is the key element of the Chinese traditional herb, ginkgo. Previous studies have confirmed that DHM has a variety of functions, including antitumor and anti-oxidative effects, and lowering blood sugar and lipid levels (5-7). Moreover, compared with western medicine for the treatment of cerebrovascular disease, DHM has fewer side effects (8). The results from *in vitro* studies has revealed that DHM decreased not only the myocardial infarction area and improved heart function, but also alleviated oxidative stress in hypoxic/reoxygenation (H/R)-induced primary cardiomyocytes (9). DHM is a novel hepatoprotective small molecule, which stimulates the expression of autophagy-associated genes and inhibits hepatic ischemia-reperfusion (I/R)-induced apoptosis by increasing the expression levels of FOXO3a and nuclear translocation (10). In addition, DHM prevents Alzheimer's disease by inhibiting the activation of NLRP3 inflammasomes in APP/PS1 transgenic mice to inhibit neuroinflammation (11). However, the role of DHM in cerebral ischemia-reperfusion cell model has not been investigated.

NF-E2-related factor 2 (Nrf2) is one of the key regulators of endogenous antioxidant defense and promotes the

transcription of a variety of antioxidant genes, including heme oxygenase (HO-1) and NAD (P) H:quinone oxidoreductase 1 (NQO1) (12). The activation of Nrf2/HO-1 signaling pathway by targeting Brg1 improved H/R-induced HT22 neuron cell apoptosis and oxidative stress (13). The results indicated that Nrf2/HO-1 plays an important role in the pathological process of CIRI. In addition, a variety of drugs targeting the Nrf2/HO-1 signaling pathway to improve neuronal injury caused by CIRI have been developed. Inactivated *Pseudomonas aeruginosa* protects against myocardial I/R injury through Nrf2 and HO-1 (14). Rutaecarpine improved neuronal injury and inhibited apoptosis, inflammation and oxidative stress by regulating the expression of the Nrf2/HO-1 signaling pathway in rats with CIRI (15). Moreover, DHM protects umbilical vein endothelial cells from injury through the Nrf2/HO-1 signaling pathway mediated by ERK and AKT (16). DHM was also found to decrease lipopolysaccharide (LPS)-induced oxidative stress and promoted the activity of the antioxidant system by activating superoxide dismutase (SOD) and the Nrf2/HO-1 signaling pathway (17). Therefore, the present study investigated whether DHM could influence the oxidative stress and apoptosis of cells in CIRI by regulating the Nrf2/HO-1 signaling pathway.

In the present study, HT22 hippocampal neurons were induced to create an oxygen and glucose deprivation/reoxygenation (OGD/R) model and the effect of DHM on oxidative stress and apoptosis of HT22-induced cells was investigated, as well as the mechanism.

Materials and methods

Cell culture. Mouse HT-22 hippocampal were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (both Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics, at 37˚C in a humidified incubator with 5% CO_2. Brusatol (BR, Sigma-Aldrich; Merck KGaA) at 0.3 μg/ml was used as an Nrf2/HO-1 pathway inhibitor.

OGD/R model. The OGD/R model is widely recognized as a model for studying cerebral ischemia at present. By injecting N_2, the concentration of CO_2 and O_2 in the incubator can be precisely controlled. The glucose in the culture medium of neurons can be deprived, thus creating the hypoxic and glucose-deficient environment for culturing neurons and simulating cerebral ischemia *in vivo*. The treatment of HT-22 cells by ODG/R was conducted as previously described (9). The mouse hippocampal HT22 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) in a normoxic 5% CO_2 cell culture incubator at 37˚C. OGD/R HT22 cells were used as an *in vitro* model of CIRI. Briefly, HT22 cells were cultured in glucose-free DMEM and then placed in hypoxic conditions (1% O_2, 94% N_2, 5% CO_2) at 37˚C for 2 h. Thereafter, the medium was discarded, normal DMEM with glucose was added and the culture was continued for 24 h of reoxygenation under normoxic condition (95% air, 5% CO_2) to produce OGD/R. HT22 cells cultured in growth culture medium under normoxic condition served as a control.

Cell Counting Kit-8 (CCK-8). The CCK-8 kit (Dojindo Molecular Technologies, Inc.) was used to detect cell proliferation. Briefly, HT-22 cells were seeded in 96-well plates ($2x10^4$ cells/ml) and after the corresponding treatment, CCK-8 solution (10 μl) was added to each well. Subsequently, the plates were incubated for 2 h at 37˚C and the absorbance of each well was detected at 450 nm using a microplate reader (BioTek Instruments; Agilent Technologies, Inc.).

ELISA. The amount of malondialdehyde (MDA, cat. no. BMS222), superoxide dismutase (SOD, cat. no. BMS222) and glutathione (GSH, cat. no. PA5-18651) was evaluated using ELISA kits (BioSource International; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. The cell supernatant was centrifuged for 5-10 min at 4˚C, at 5,000 x g.

Western blot analysis. The cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) and incubated for 30 min on ice. Total protein was quantified using a BCA protein assay kit. Following extraction of the total protein from the cells, 10% SDS-PAGE was used to separate the proteins (30 μg) and transferred to PVDF membranes, which were blocked with 10% skimmed milk for 1 h at room temperature. Following overnight incubation at 4˚C with specific primary antibodies, the membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat no. AA24142, Cell Signaling Technology, Inc.) for 2 h at room temperature. The protein levels were visualized using the Super signal West Pico Chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.). Protein expression levels were semi-quantified using ImageJ software (version 146; National Institutes of Health) with GAPDH as the loading control. The following primary antibodies from Cell Signaling Technology, Inc. were used: Anti-Bax (1:1,000; cat. no. 14796S), anti-caspase3 (1:1,000; cat no. 700128), anti-Bcl-2 (1:1,000; cat. no. 14-1028-82), anti-Nrf2 (1:1,000; cat. no. 12721T), anti-HO-1 (1:1,000; cat. no. 86806S), anti-cleaved caspase3 (1:1,000; cat. no. PA5-38438), anti-NOX2 (1:1,000; cat. no. MA5-18052), anti-NOX4 (1:1,000; cat. no. PA5-53304) and anti-GAPDH (1:1,000; cat. no. MA5-15738).

TUNEL. After fixing 4% paraformaldehyde at room temperature for 20 min, the cells were washed twice with PBS. Then, 0.2% Triton X-100 was added to the cells and treated at room temperature for 5 min. The cells were collected and washed with PBS three times, and treated with 50 μl TUNEL assay solution (Roche Diagnostics GmbH) at 37˚C in dark for 60 min and added with stop solution, followed by incubation with DAB solution and staining with hematoxylin and eosin for 5 min at room temperature. The cells were randomly selected for field observation under a light microscope (Carl Zeiss AG, magnification, x200). The experiment was repeated three times.

Statistical analysis. All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS v22.0 statistical software (IBM Corp.) and one-way ANOVA with Tukey's multiple comparison post hoc test was employed for comparison among groups.

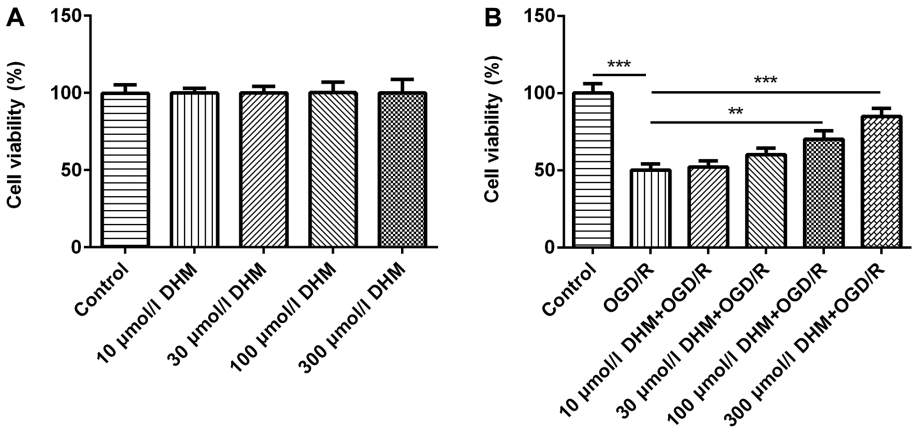

Figure 1. DHM promotes the viability of OGD/R-induced HT22 cells. (A and B) Cell Counting Kit-8 was used to detected the viability of cells. **P<0.01. ***P<0.001. DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation.

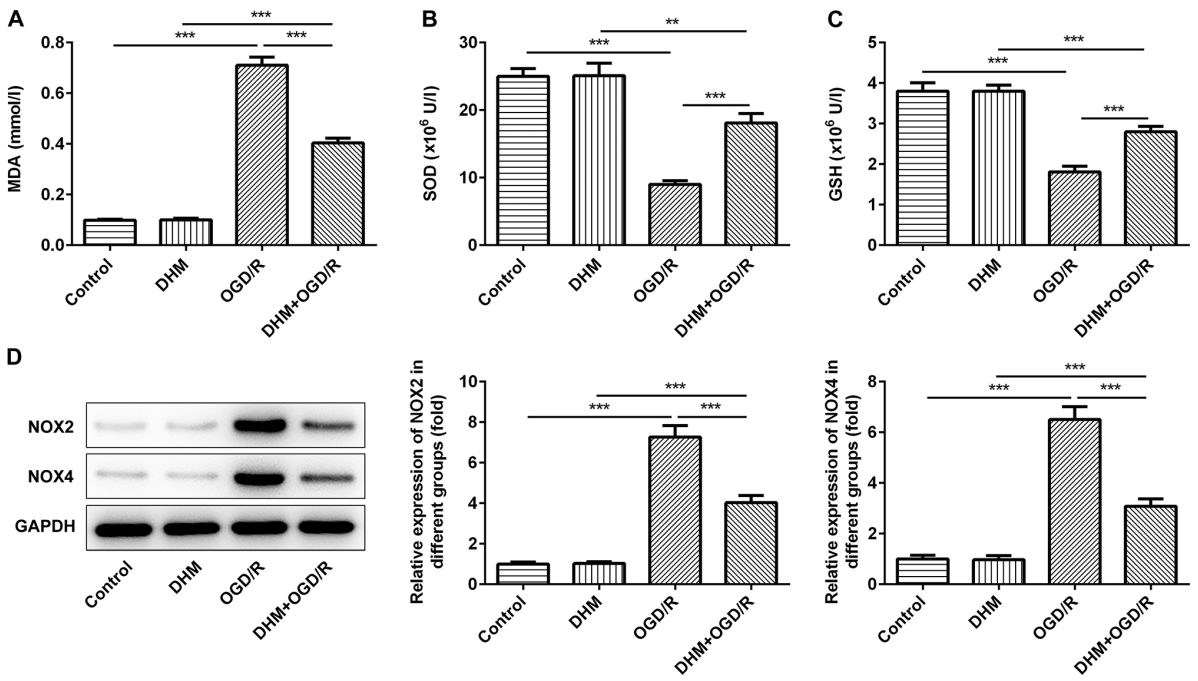

Figure 2. DHM inhibited the oxidative stress of OGD/R-induced HT22 cells. MDA (A) SOD (B) and GSH (C) were detected by ELISA assay. (D) Western blotting was used to detect the expression levels of NOX2 and NOX4. **P<0.01. ***P<0.001. DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; NOX, NADPH oxidase.

P<0.05 was considered to indicate a statistically significant difference.

Results

DHM promotes the viability of OGD/R-induced HT22 cells. CCK-8 was used to detect the effects of different concentrations of DHM (0, 10, 30, 100 and 300 μmol/l) on cell viability and no differences were found (18,19) (Fig. 1A). Subsequently, the different aforementioned concentrations of DHM were used in OGD/R-induced HT22 cells and the CCK-8 results revealed that the survival rate of OGD/R-induced HT22 cells was significantly decreased compared with that of the control group. Furthermore, the cell survival rate of the DHM+OGD/R group was increased in a dose-dependent manner, compared with the OGD/R group (Fig. 1B). It was found that 300 μmol/l DHM could significantly promote cell proliferation of OGD/R-induced HT22 cells, and DHM had no cytotoxic effect on cells at this concentration. Therefore, 300 μmol/l DHM was used for the subsequent experimentation.

DHM inhibits the oxidative stress of OGD/R-induced HT22 cells. The level of cellular oxidative stress was then investigated. The cells were grouped into either control, DHM, OGD/R, DHM+OGD/R groups, and compared with the control group. No differences were observed in the amount of MDA (Fig. 2A), SOD (Fig. 2B), GSH (Fig. 2C) and the expression levels of the oxidative stress proteins, NOX2 and NOX4 (Fig. 2D) in cells treated with DHM alone, while there was a significant increase in the OGD/R group. This indicated that

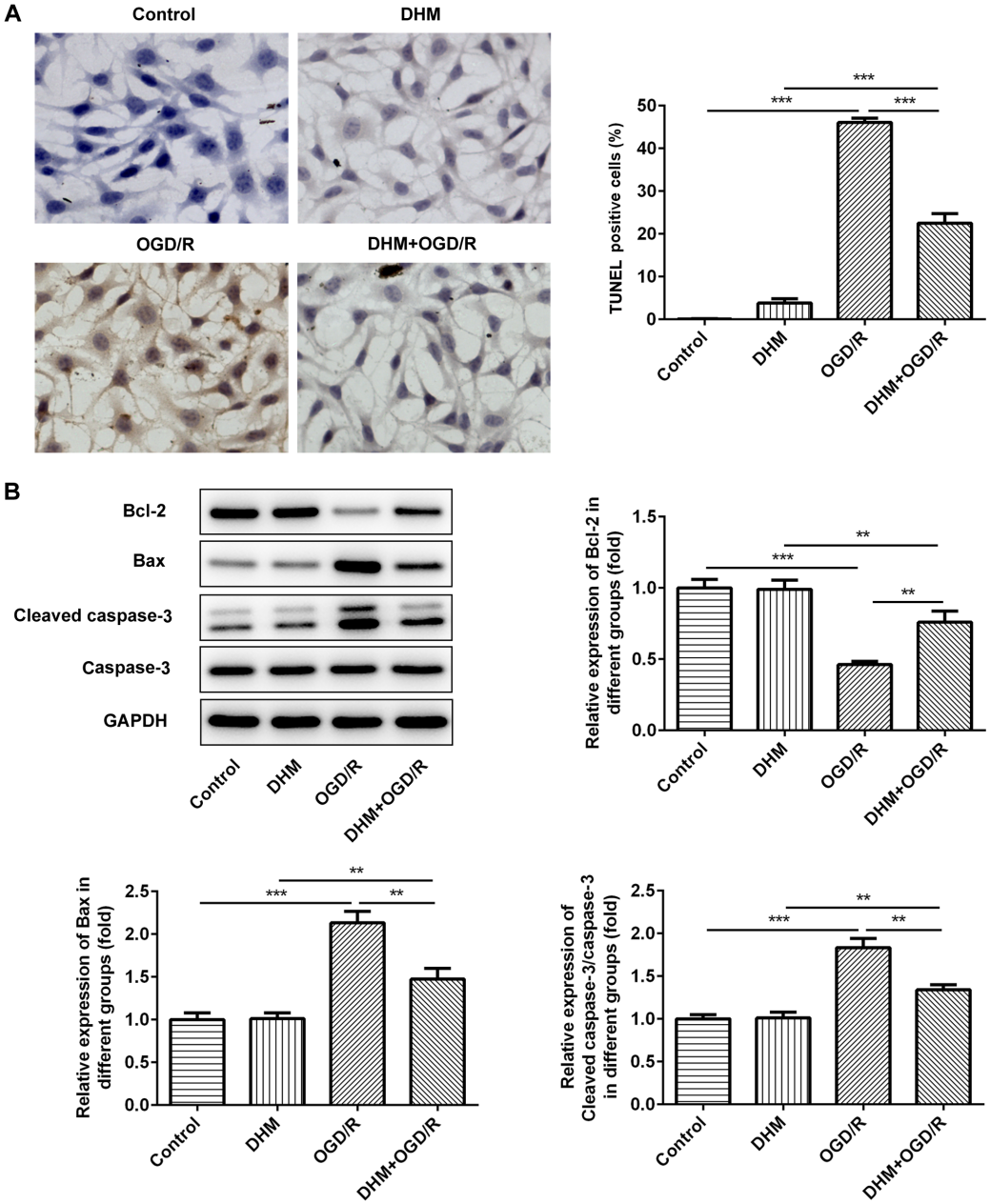

Figure 3. DHM inhibits apoptosis in OGD/R-induced HT22 cells. (A) Apoptosis of cells was detected by TUNEL (magnification, x200). (B) Western blotting was used to detect the expression levels of Bcl-2, Bax, cleaved caspase-3 and caspase-3. **P<0.01, ***P<0.001. DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation.

OGD/R successfully induced oxidative stress in HT22 cells. Compared with that in the OGD/R group, the levels of MDA, and the expression levels of the oxidative stress proteins, NOX2 and NOX4 in the DHM+OGD/R group were significantly decreased and the expression of SOD and GSH were increased, indicating that DHM could inhibit the oxidative stress of OGD/R-induced HT22 cells.

DHM inhibits apoptosis in OGD/R-induced HT22 cells. TUNEL staining was used to detect the rate of apoptosis in cells. Compared with that in the control group, there was no change in the rate of apoptosis following DHM treatment in HT22 cells alone, while there was a significant increase in that of the OGD/R group (Fig. 3A), an increase in the expression levels of the pro-apoptotic proteins, Bax and cleaved caspase-3, and a decrease in the anti-apoptotic protein, Bcl-2 (Fig. 3B). This indicates that OGD/R successfully induced apoptosis of HT22 cells. Compared with that in the OGD/R group, cell apoptosis in the DHM+OGD/R group was decreased, accompanied by the decrease in the expression levels of Bax and cleaved caspase-3, and the increase in Bcl-2; indicating that DHM inhibited the apoptosis of OGD/R-induced HT22 cells.

DHM inhibits oxidative stress and apoptosis in OGD/R-induced HT22 cells by activating Nrf2/ HO-1 signaling pathway. The expression of Nrf2 and HO-1 in the OGD/R group was significantly decreased compared with the control group, while the expression of Nrf2 and Ho-1 in the DHM+OGD/R group was reversed compared with the OGD/R group. In addition, compared with the control group, the expression of Nrf2 and

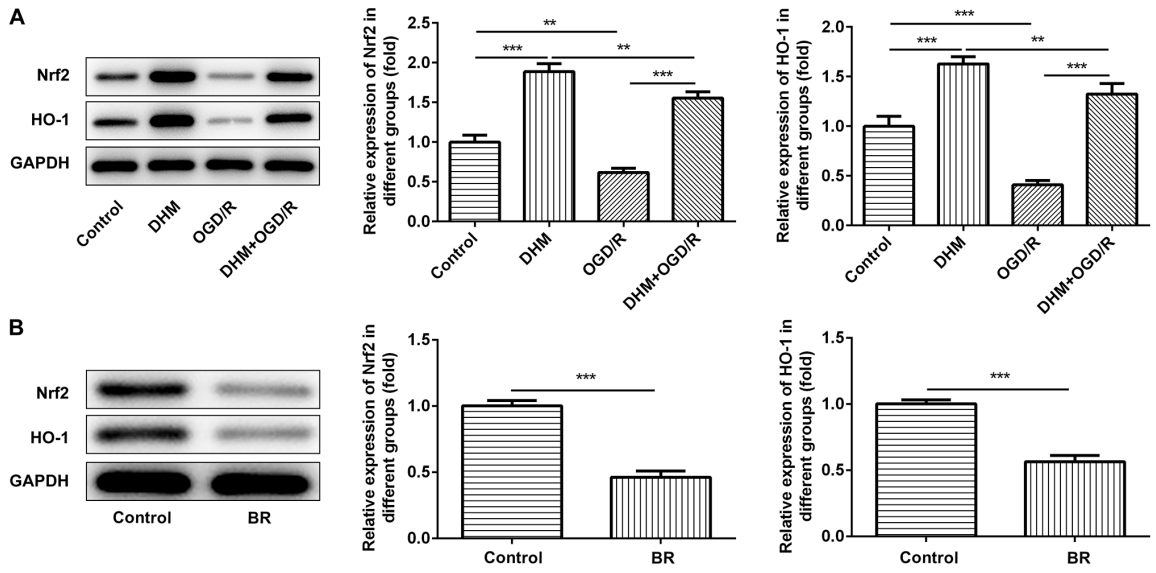

Figure 4. Nrf2/HO-1 signaling pathway is activated by DHM. (A and B) Western blotting was used to detect the expression levels of Nrf2 and HO-1. **P<0.01, ***P<0.001. DHM, dihydromyricetin; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase.

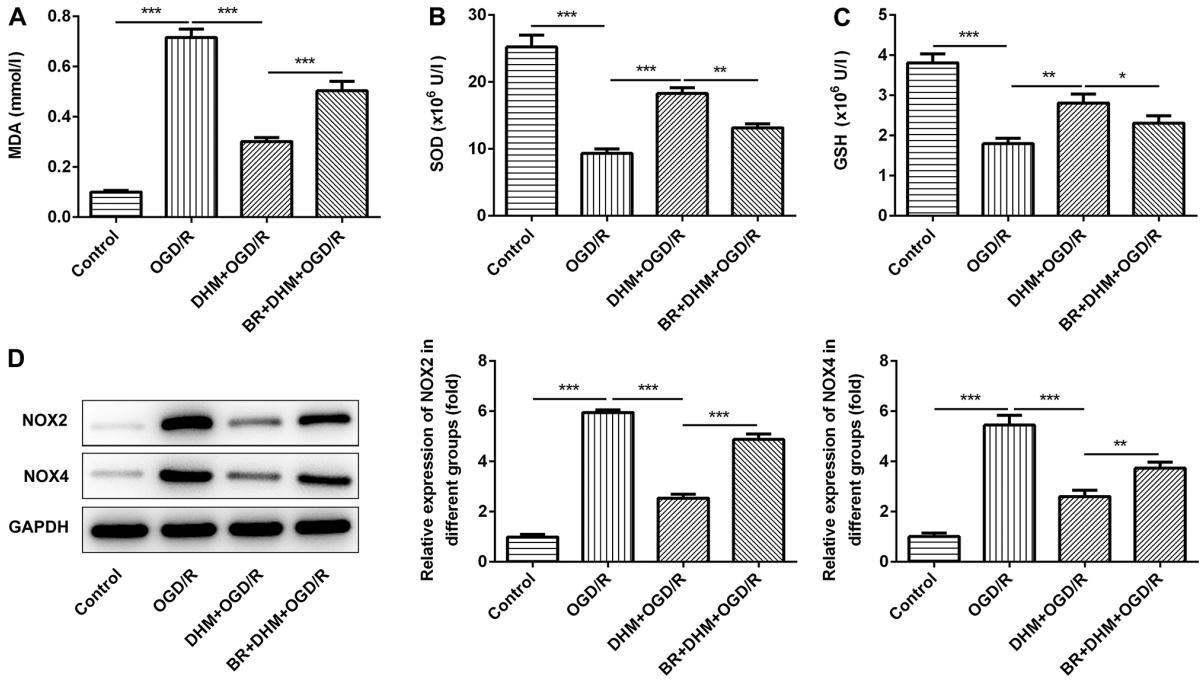

Figure 5. DHM inhibits oxidative stress of OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway. MDA (A) SOD (B) and GSH (C) were detected by ELISA assay. (D) Western blotting was used to detect the expressions of NOX2 and NOX4. *P<0.05, **P<0.01, ***P<0.001. DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; NOX, NADPH oxidase.

Ho-1 in the DHM group was significantly increased. It showed that Nrf2/HO-1 signaling pathway was inhibited after OGD/R induction, and that DHM could reverse the inhibitory effect of OGD/R on signaling pathway (Fig. 4A), indicating that the Nrf2/HO-1 signaling pathway was activated following DHM treatment. Subsequently, the Nrf2/HO-1 signaling pathway inhibitor, BR was added to the cells, and the protein expression levels of Nrf2 and HO-1 were detected using western blot analysis (Fig. 4B). Subsequently, the cells were divided into control, DHM, OGD/R, DHM+OGD/R, and BR+DHM+OGD/R groups. Compared with that in the DHM+OGD/R group, the levels of MDA (Fig. 5A), and the protein expression levels of NOX2 and NOX4 (Fig. 5D) in the BR+DHM+OGD/R group were significantly increased, the expression of SOD (Fig. 5B) and GSH (Fig. 5C) were decreased, indicating that BR could reverse the effect of DHM on OGD/R-induced oxidative stress in HT22 cells. In addition, compared with that in the DHM+OGD/R group, the rate of apoptosis in the BR+DHM+OGD/R was also increased (Fig. 6A), which was accompanied by an increase in the protein expression levels

Table I. Values of relative protein expression of cleaved caspase-3 and caspase-3 in HT22 cells (mean ± standard deviation).

Group	Cleaved caspase-3/caspase-3	Caspase-3	Cleaved caspase-3
Control	1.00±0.02	1.00±0.07	1.00±0.07
OGD/R	2.50±0.14	1.00±0.07	2.50±0.07
DHM+OGD/R	1.42±0.08	1.00±0.08	1.43±0.07
BR+DHM+OGD/R	1.94±0.02	1.02±0.06	1.98±0.11

DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation; BR, brusatol.

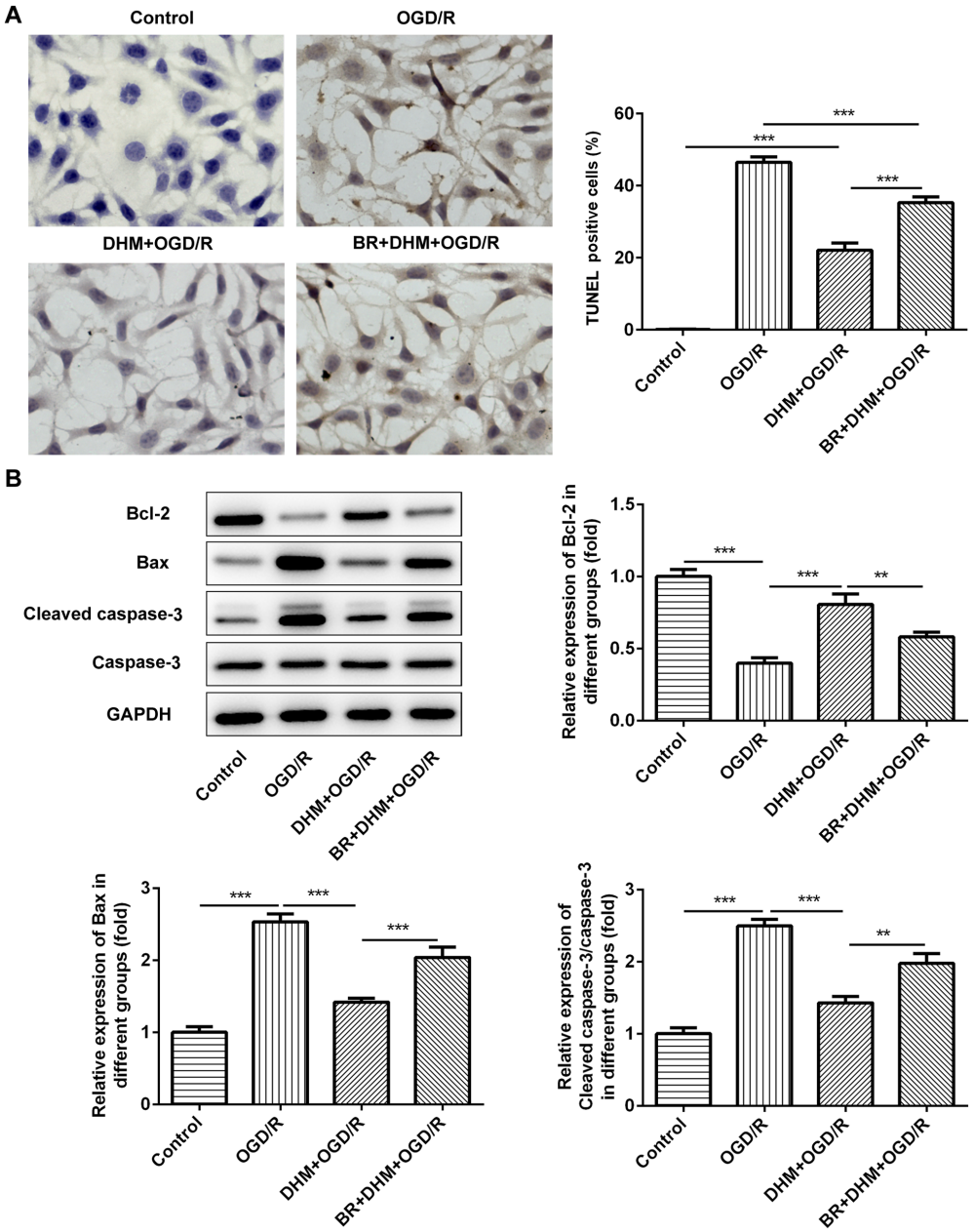

Figure 6. DHM inhibits apoptosis of OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway. (A) Apoptosis of cells were detected by TUNEL (magnification, x200). (B) Western blotting was used to detect the expression levels of Bcl-2, Bax, cleaved caspase-3 and caspase-3. **P<0.01. ***P<0.001. DHM, dihydromyricetin; OGD/R, oxygen and glucose deprivation/reoxygenation; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase; BR, brusatol.

of Bax and cleaved caspase-3 (Fig. 6B and Table I), and the decrease in Bcl-2 (Fig. 6B); indicating that BR could also reverse the effect of DHM on OGD/R-induced apoptosis of HT22 cells. The results indicate that DHM inhibited oxidative

stress and apoptosis of OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway.

Discussion

At present, the clinical treatment of ischemic cerebrovascular disease is still limited, and there is a lack of effective and safe treatment measures. Therefore, there is an urgent requirement to develop stable and safe drugs to treat CIRI.

In the present study, mouse hippocampal neuron HT22 cells were induced using OGD/R to create an *in vitro* model of CIRI. It was found that following OGR/D induction, the cell survival rate decreased, oxidative stress was activated, and apoptosis was also significantly increased, indicating the cell model was successfully established.

DHM, which is a type of active hydrogenated flavonol, has been investigated widely in pharmacology in recent years, and has been found to be effective in treating a variety of different diseases. DHM has been found to increase endothelial nitric oxide production and inhibited atherosclerosis through microRNA-21 in apolipoprotein E-deficient mice (18). DHM also ameliorated chronic social defeat stress-induced cognitive and affective disorder in mice (20). In addition, modulation of SIRT1-mediated signaling cascades in the liver contributed to the amelioration of non-alcoholic steatohepatitis in middle-aged LDL receptor knockout mice, that were fed a high-fat diet by DHM (21). Therefore, DHM has been shown to have a variety of beneficial functions in the current study and has become a potential source of treatment of CIRI. A previous study investigating ischemia reperfusion injury found that DHM enhances protection during ischemia, decreases myocardial dysfunction by enhancing anti-inflammatory activities, attenuates myocardial oxidative injury and prevents apoptosis during ischemia/reperfusion (22). In a study investigating cerebral ischemia, DHM was found to effectively prevent cerebral edema caused by whole brain I/R injury in rats caused by the ligation of the bilateral common carotid artery (23). DHM has also been found to have a neuronal protective effect on PC12 cells induced by H_2O_2 (24). The effect of DHM in CIRI is currently unclear, therefore, in the present study, DHM was used to treat OGD/R-induced HT22 cells, and it was found that DHM promoted the proliferation of OGD/R-induced HT22 cells in a dose-dependent manner. Moreover, DHM inhibited the oxidative stress response and inhibited cell apoptosis in OGD/R-induced HT22 cells. Thus, DHM has a protective effect on neurons, which is consistent with the therapeutic effect of DHM on I/R injury.

It was found that the Nrf2/HO-1 signaling pathway was inhibited following OGD/R induction and the Nrf2/HO-1 signaling pathway plays an important role in I/R injury (25,26). Different drugs can inhibit cell damage and apoptosis caused by ischemia and hypoxia by activating the Nrf2/HO-1 signaling pathway (27,28). Therefore, the present study investigated whether DHM plays a protective role on OGD/R-induced HT22 cells through the Nrf2/HO-1 signaling pathway. It was found that the Nrf2/HO-1 signaling pathway in HT22 cells was activated following treatment with DHM in HT22 cells alone or in OGD/R-induced HT22 cells. Furthermore, the Nrf2/HO-1 signaling pathway inhibitor, BR, was used and it was found that following inhibition of the Nrf2/HO-1 signaling pathway, the inhibitory effect of DHM on OGD/R-induced oxidative stress and apoptosis was reduced. Thus, the findings suggest that DHM inhibited oxidative stress and apoptosis in OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway.

In conclusion, the present study demonstrated that DHM inhibited oxidative stress and apoptosis of OGD/R-induced HT22 cells by activating the Nrf2/HO-1 signaling pathway, which indicates that DHM has the potential to be developed as a stable and safe treatment option for CIRI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and TZ wrote the manuscript and analyzed the data. JW and HZ carried out the experiments and TZ supervised the present study. QZ searched the literature and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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