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Dihydromyricetin ameliorates oxygen-glucose deprivation and re-oxygenation-induced injury in HT22 cells by activating the Wnt/β-catenin signaling pathway

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Abstract. Dihydromyricetin (DMY) is a natural flavonoid that possesses a wide range of pharmacological properties. The aim of the present study was to determine whether DMY could protect against nerve cell injury following ischemic stroke through antioxidant and neuroprotective effects. The effects of DMY on the viability, oxidative stress and apoptosis of HT22 cells following oxygen-glucose deprivation and re-oxygenation (OGD/R) were examined using MTT, lactate dehydrogenase (LDH), superoxide (SOD), malondialdehyde (MDA), western blot and TUNEL assays. Furthermore, Wnt/β-catenin signaling proteins in OGD/R-stimulated HT22 cells were detected in the presence or absence of DMY. In a separate experiment, the effect of DMY on OGD/R-induced HT22 cell injury was also observed in the presence of the Wnt/β-catenin inhibitor, XAV939. The results demonstrated that DMY had no impact on the survival of untreated HT22 cells, although DMY treatment significantly increased cell viability and inhibited cytotoxicity, oxidative stress and apoptosis following OGD/R. In addition, DMY upregulated the expression of Wnt/β-catenin in OGD/R-stimulated HT22 cells. In conclusion, DMY protected HT22 cells from OGD/R-induced oxidative stress and apoptosis, and its effects may be mediated by the activation of the Wnt/β-catenin signaling pathway.

Introduction

Ischemic stroke is a disorder of the blood supply in local brain tissue caused by various factors and is a major cause of death and disability in adults worldwide (1). Ischemic stroke leads to ischemic-hypoxic brain tissue necrosis, which results in neuronal dysfunction (2). A previous study has suggested that the restoration of blood flow in the ischemic hemispheric zone around the infarcted tissue constitutes the most effective therapeutic strategy for reducing the clinical symptoms of cerebral ischemia (3). However, recanalization, reperfusion and reoxygenation therapy can occasionally lead to the concomitant occurrence of brain damage, namely cerebral ischemia/reperfusion (I/R) injury (CIRI) (4). CIRI consists of various deleterious pathological changes, such as neuronal apoptosis, oxidative stress, autophagy, inflammation and necrosis, escalating to neurological dysfunction (5). Recent decades have seen an increasing incidence of ischemic stroke, and as a consequence, CIRI has become a focus of current research (6). At present, alternative treatment strategies for this disease remain lacking, hence the imperative to identify novel agents that can be used clinically (7).

Dihydromyricetin (DMY) is a natural flavonoid isolated from *Ampelopsis grossedentata* that has strong antioxidant effects (8). DMY was reported to markedly improve 1-methyl4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced behavioral impairment and dopaminergic neuronal loss in mice (9). Furthermore, in MES23.5 cells, DMY attenuated MPP^+ (a metabolite of MPTP)-induced cellular damage and reactive oxygen species (ROS) production in a dose-dependent manner (9). DMY has been shown to enhance mitochondrial function and reduce oxidative stress by upregulating sirtuin 3, thus improving the prognosis of patients with cardiac I/R injury (10). A previous study also reported that DMY significantly reduced serum transaminase activity and inhibited hepatic I/R-induced cell apoptosis (11). The aforementioned studies collectively suggest that DMY serves a neuroprotective function. It has also been revealed that DMY treatment improved mitochondrial morphology and function, inhibited the production of reactive oxygen species and reduced hippocampal lipid peroxidation in hypoxia-treated HT22 cells (12). However, whether DMY can protect HT22 from oxygen-glucose deprivation and reoxygenation (OGD/R)-induced injury is yet to be fully elucidated.

It has been reported that DMY promotes the osteogenic differentiation of human bone marrow mesenchymal stem cells (BMSCs) *in vitro*, partly through Wnt/β-catenin signaling (13).

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This suggests that DMY may also activate the Wnt/β-catenin signaling pathway in HT22 cells. Furthermore, it has been demonstrated that DMY-activated Wnt/β-catenin signaling attenuates brain injury in rats exposed to cerebral I/R (14). Thus, the aim of the present study was to investigate whether DMY could ameliorate OGD/R-induced HT22 cell injury by activating the Wnt/β-catenin signaling pathway.

Materials and methods

Cell culture and treatment. The murine hippocampal neuronal cell line, HT22, was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin. All cells were grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. DMY was purchased from Chengdu Kangbang Biological Technology, Co., Ltd. Cells ($1x10^6$ cells/well) were pretreated with Wnt signaling pathway inhibitor XAV939 (5 μM, cat. no. HY-15147, MedChemExpress) for 2 h at 37˚C.

In vitro induction of OGD/R injury in HT22 neuronal cells. The induction of OGD/R injury in HT22 neuronal cells was performed according to a previously described protocol (15). HT22 neuronal cells were seeded ($1x10^6$ cells/well) into glucose-free medium and cultured under hypoxic conditions (3% O_2 with 5% CO_2 and 92% N_2). After incubation for 8 h at 37˚C under OGD conditions, the medium was replaced with glucose-containing normal medium, and cells were grown under normoxic conditions (95% air with 5% CO_2) at 37˚C. After reoxygenation for 24 h, cells were harvested for analysis. HT22 cells that were cultured in normal medium under normoxic conditions were used as the control.

MTT assay. Cell viability was determined using the MTT Cell Viability Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions (16). HT22 cells were seeded at a density of $3x10^3$ cells/well into 96-well-plates and cultured with different concentrations of DMY (0, 10, 30, 100 or 300 μmol/l) for 24 h at 37˚C. Cells were exposed to OGD/R prior to a 4-h incubation with 20 μl MTT solution administered to each well. DMSO was then added to each well to dissolve the formazan particles. The absorbance in each sample was then measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Each assay was performed in triplicate.

Measurement of lactate dehydrogenase (LDH) concentration. An LDH assay kit (C0016, Beyotime Institute of Biotechnology) was used to detect the concentration of LDH in HT22 cells (17). According to the manufacturer's protocol, the cell supernatant was collected via centrifugation at 400 x g for 10 min at room temperature, after which 20 μl of the supernatant was mixed with 2,4-dinitrophenylhydrazine and incubated at 37˚C for 15 min. NaOH (0.4 M) was added into the mixture and incubated for a further 15 min at 37˚C. After the mixture was kept at room temperature for 5 min, the absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Measurement of malondialdehyde (MDA) and superoxide (SOD) levels. The levels of MDA and SOD were measured using a Lipid Peroxidation MDA Assay kit (S0131S) and a Cu/Zn-SOD Mn-SOD Assay kit (S0103; both Beyotime Institute of Biotechnology), respectively. To determine MDA levels, the MDA test solution was mixed with a crude enzyme solution at a ratio of 3:1, placed in a water bath at 100˚C for 30 min, then cooled on ice. After adding the mixture into the cell supernatant, absorbance values at 532 nm were measured using a microplate reader (Thermo Fisher Scientific, Inc.). The SOD test solution was also prepared using components of the commercial kit, and the absorbance was measured at 450 nm (18).

Western blotting. Following treatment, HT22 cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Beyotime Institute of Biotechnology). Protein concentrations were subsequently determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (40 μg) were separated by 10% SDS-PAGE and then transferred to PVDF membranes (MilliporeSigma). The membranes were blocked with 5% bovine serum albumin for 1.5 h at room temperature (BSA; Gibco; Thermo Fisher Scientific, Inc.) and incubated with primary antibodies overnight at 4˚C. Samples were then washed with 5% BSA in PBS/0.1% Tween-20 before incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using an ECL kit (Beyotime Institute of Biotechnology), and the gray value was analyzed using ImageJ software (version 1.4.3.67; National Institutes of Health). The antibodies used in this experiment were as follows: Anti-NADPH oxidase 2 (NOX2; rabbit; 1:1,000; cat. no. ab129068; Abcam), anti-NOX4 (rabbit; 1:1,000; cat. no. ab133303; Abcam), anti-Bcl-2 (mouse; 1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), anti-Bax (mouse; dilution 1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), anti-cleaved-caspase3 (mouse; 1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), anti-cleaved-caspase9 (mouse; 1:1,000; cat. no. 9509; Cell Signaling Technology, Inc.), anti Wnt3 (rabbit; 1:1,000; cat. no. 2721; Cell Signaling Technology, Inc.) anti-β-catenin (rabbit; 1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.), anti-GAPDH (rabbit; 1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) and HRP-conjugated anti-mouse IgG or anti-rabbit IgG (1:2,000; cat. nos. 7076 or 7074; Cell Signaling Technology, Inc.).

TUNEL assay. Cell apoptosis was assessed using a TUNEL Apoptosis Detection kit (Beyotime Institute of Biotechnology) (19). Cells were washed with PBS and then fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, cells were incubated with permeabilization solution for 5 min at room temperature followed by TUNEL solution for 1 h at 37˚C. Then, 50 μl DAB (5 mg/ml) was added for 10 min at 15˚C and hematoxylin for 10 sec at room temperature. A total of 5 fields of view were randomly selected and apoptotic cells were observed under glass coverslip with PBS under a fluorescence microscope (magnification x200; Olympus Corporation).

Statistical analysis. Quantitative data are presented as the mean ± SD and statistical analysis was performed using

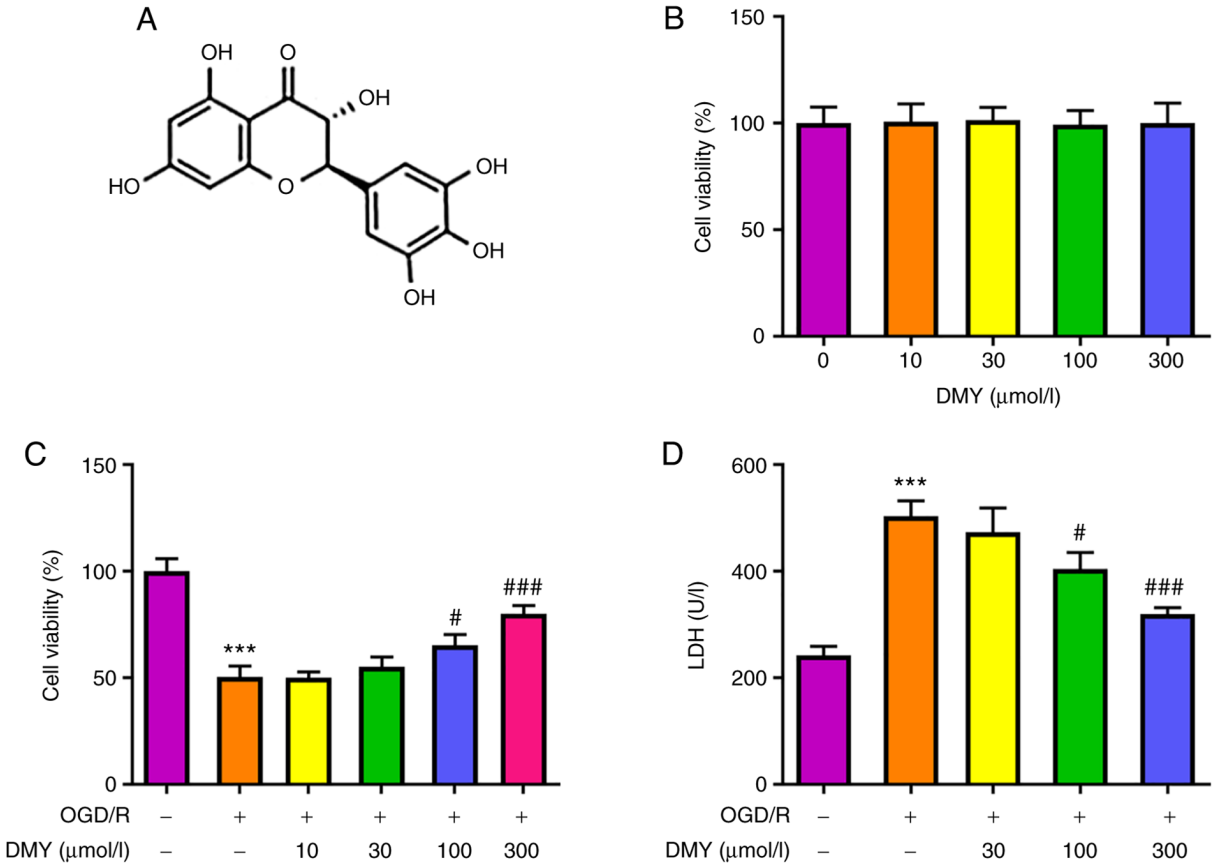

Figure 1. Effects of DMY on HT22 cells following OGD/R. (A) Chemical structure of DMY. (B) MTT assay of HT22 cell viability after treatment with DMY (0, 10, 30, 100, 300 μmol/l). (C) MTT assay of HT22 cell viability after OGD/R with or without DMY (10, 30, 100, 300 μmol/l) treatment. (D) LDH release in HT22 cells after OGD/R with or without DMY (30, 100, 300 μmol/l) treatment. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. OGD/R group. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation.

GraphPad Prism 8 (GraphPad Software, Inc.). Differences between groups were compared using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated three times.

Results

Effect of DMY treatment on the viability of HT22 cells. The chemical structure of DMY is presented in Fig. 1A. MTT assay revealed that different concentrations (10, 30, 100 and 300 μmol/l) of DMY did not affect the viability of HT22 cells (Fig. 1B). However, following OGD/R and DMY treatment (Fig. 1C), the viability of HT22 cells was reduced compared with the control group. DMY administered at 10 and 30 μmol/l had no significant effect on cell viability compared with the OGD/R group, whereas 100 and 300 μmol/l DMY significantly enhanced cell viability. Subsequently, the effect of DMY on OGD/R-induced HT22 cytotoxicity was examined using an LDH assay (Fig. 1D). Compared with the OGD/R group, DMY administered at a low concentration (30 μmol/l) did not affect LDH levels, while a higher concentration (100 or 300 μmol/l) of DMY significantly reduced LDH levels. These results indicated that DMY treatment was not cytotoxic to HT22 cells, and its propensity to improve cell viability and alleviate OGD/R-induced cytotoxicity.

DMY treatment inhibits OGD/R-induced oxidative stress. To investigate the effect of DMY treatment on OGD/R-induced HT22 cell injury, the oxidative stress of HT22 cells was examined by detecting the levels of SOD and MDA. Fig. 2A presents the changes in SOD and MDA levels detected by the corresponding kits. OGD/R led to a marked decrease in SOD levels in HT22 cells compared with the control group. Following DMY treatment, the levels of SOD increased in a concentration-dependent manner. Furthermore, the levels of MDA significantly increased in OGD/R-stimulated HT22 cells, but significantly decreased with increasing DMY concentration. Additionally, the protein expression levels of NOX2 and NOX4 increased following OGD/R, while DMY treatment decreased their expression in a concentration-dependent manner (Fig. 2B). These data suggested that DMY serves a protective effect against OGD/R-induced oxidative stress in HT22 cells.

DMY treatment inhibits OGD/R-induced cell apoptosis. The effect of DMY on OGD/R-induced cell apoptosis was assessed. As presented in Fig. 3A and B, the results of the TUNEL assay revealed the presence of significantly more apoptotic cells in the OGD/R model group. However, DMY treatment significantly reduced the number of apoptotic cells in a concentration-dependent manner. Moreover, DMY reversed the OGD/R-induced downregulation of Bcl-2 and

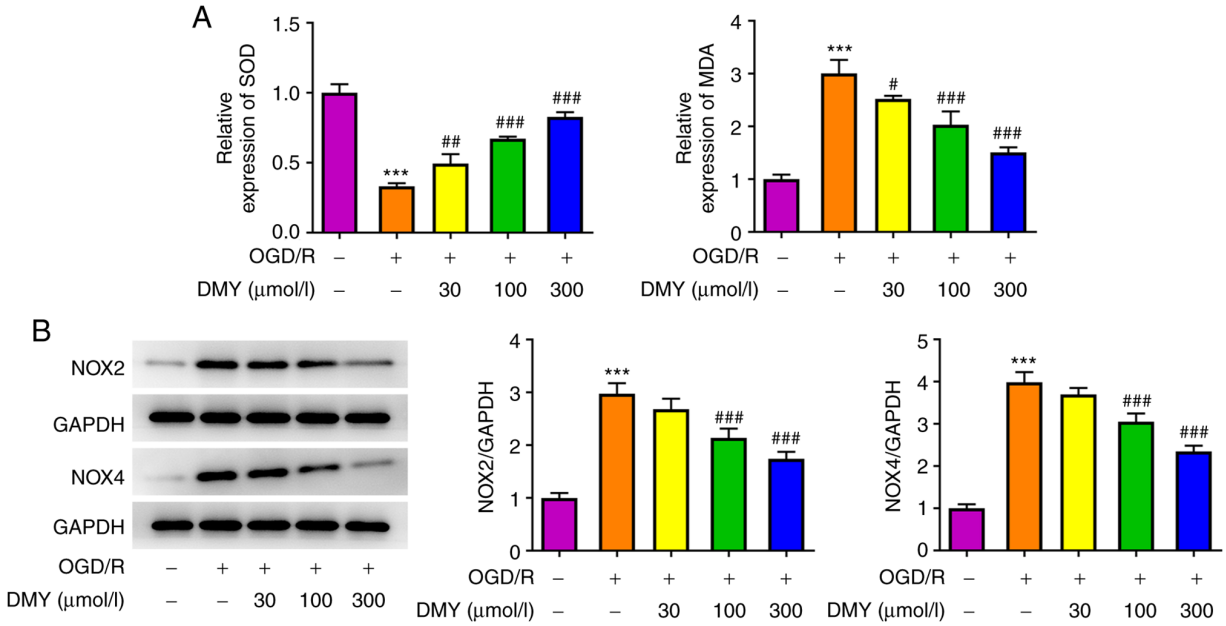

Figure 2. Effects of DMY on oxidative stress following OGD/R. Changes in (A) SOD and MDA levels and (B) protein expression of NOX2 and NOX4 following treatment with 30, 100 and 300 μmol/l DMY in OGD/R cells. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OGD/R group. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation; MDA, malondialdehyde; NOX, NADPH oxidase 1.

upregulation of cleaved caspase-3 and cleaved caspase-9 (Fig. 3C). These results indicated that DMY treatment could suppress OGD/R-induced apoptosis.

DMY activates the Wnt/β-catenin signaling pathway in HT22 cells. To determine the mechanism underlying the amelioration of OGD/R cell injury following DMY treatment, proteins related to the Wnt/β-catenin signaling pathway were analyzed. The protein expression levels of Wnt and β-catenin significantly declined in the OGD/R model group but increased after DMY treatment in a dose-dependent manner (Fig. 4). Therefore, a positive association may exist between DMY and the expression of the Wnt/β-catenin signaling pathway in HT22 cells.

DMY inhibits OGD/R-induced HT22 cell injury by activating the Wnt/β-catenin signaling pathway. The effects of DMY were investigated in OGD/R-injured HT22 cells after inhibiting the Wnt/β-catenin signaling pathway using XAV939. In the aforementioned experimental results, DMY exerted the most marked protective effects at a concentration of 300 μmol/l; therefore, this concentration was used in the following experiments. The results of western blotting revealed that XAV939 (5 μM) pretreatment inhibited the protein expression of Wnt, but not β-catenin, (Fig. 5A). In MTT assays, XAV039 did not affect cell viability following DMY treatment (Fig. 5B). By contrast, LDH assays revealed a significant LDH release in OGD/R-stimulated HT22 cells treated with both DMY and XAV939, when compared with DMY treatment alone (Fig. 5C). Similarly, MDA, NOX2 and NOX4 levels significantly increased, although SOD expression remained unchanged, in the XAV939 and DMY co-treatment group compared with the DMY group (Fig. 5D and E).

Furthermore, the XAV939 pretreatment group exhibited significantly higher apoptosis rates than the DMY group (Fig. 6A and B). Increased levels of Bax, cleaved caspase-3 and cleaved caspase-9 were also observed, although Bcl-2 expression remained unaltered (Fig. 6C). These results suggested that XAV939 pretreatment attenuated the effect of DMY on OGD/R-induced oxidative stress and apoptosis in HT22 cells.

Discussion

Ischemic stroke, also known as cerebral infarction, refers to the localized ischemic necrosis and softening of brain tissue caused by the disturbance of cerebral blood circulation, ischemia and hypoxia; it is a disease that is primarily diagnosed in middle-aged and elderly individuals (20-22). Ischemic stroke has various clinical manifestations, most of which are symptoms of focal neurological deficits, such as hemiplegia and the disturbance of consciousness (23,24). After treatment, certain patients may be left with varying degrees of nerve and limb damage seriously affecting their quality of life (25,26). Therefore, preventing nerve cell injury, restoring nerve function and suppressing cytotoxicity may be key to the treatment of this condition (27).

DMY has demonstrated promising potential in neural protection in addition to its antioxidant qualities, as evidenced by previous research. For example, in Parkinson's disease, DMY has been found to exert a neuroprotective effect by reducing the disturbance of behavior and dopaminergic neuron loss *in vivo* and ameliorating neuronal injury and ROS production *in vitro* (9,28). It has also been reported that DMY treatment alleviated adriamycin-induced cardiotoxicity, cardiomyocyte apoptosis and oxidative stress in an imprinting control region (ICR) mouse model (29). The results of the

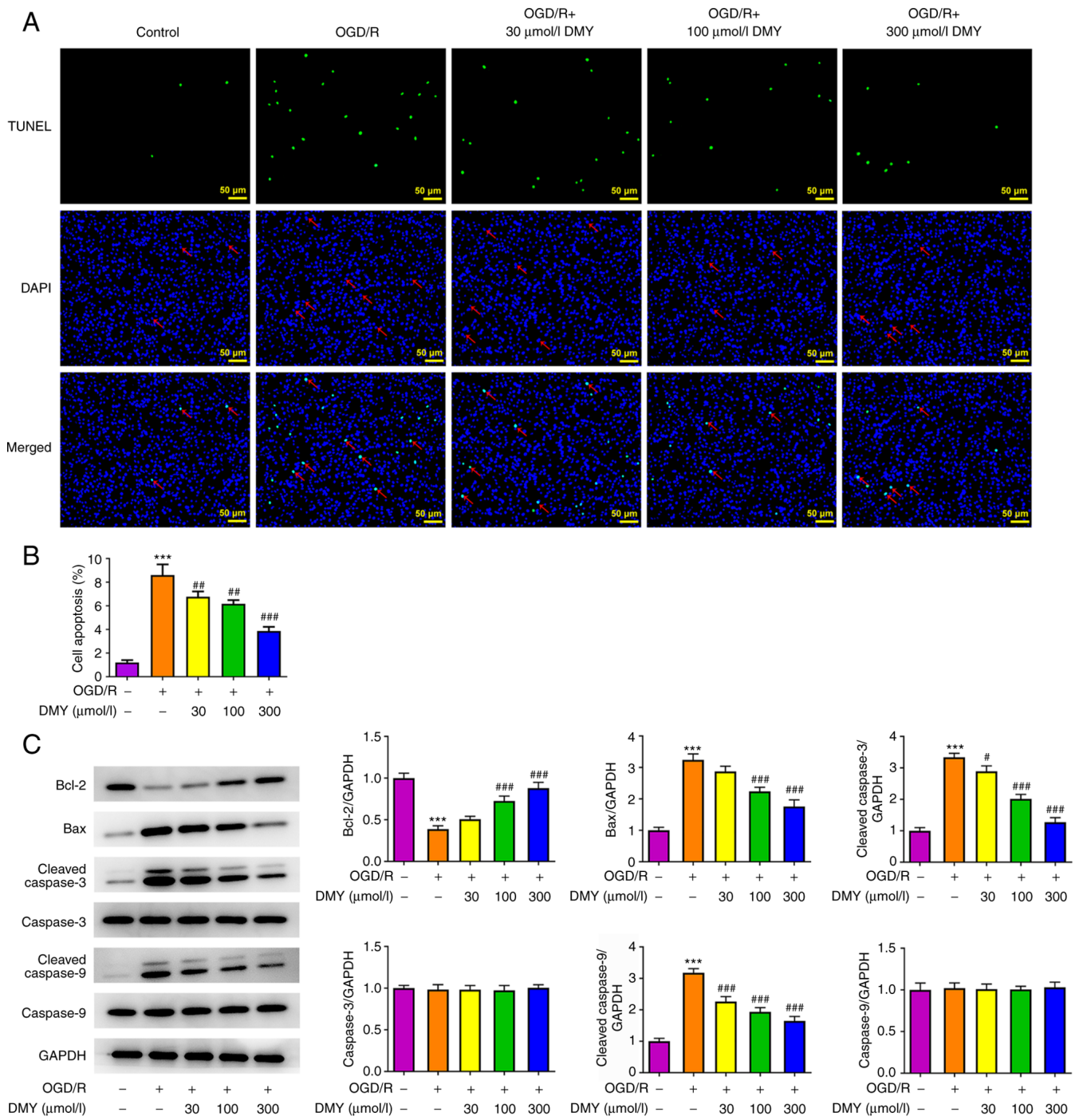

Figure 3. Effects of DMY on apoptosis following OGD/R. (A and B) Cell apoptosis rate after treatment with 30, 100 and 300 μmol/l DMY in OGD/R cells. (C) Changes in the protein expression of Bcl-2, cleaved caspase-3 and cleaved caspase-9 after treatment with 30, 100 and 300 μmol/l DMY in OGD/R cells. Red arrows indicate TUNEL-positive cells. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. OGD/R group. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation.

current study are consistent with these, in that treatment with DMY increased the survival rate of HT22 neurons under OGD/R and effectively prevented OGD/R-induced cytotoxicity in a concentration-dependent manner.

DMY is a known scavenger of ROS (which are considered as the mediators of oxidative stress) with pharmacological value in cardiovascular and other diseases (8,30,31). DMY also improves mitochondrial function and reduces oxidative stress by upregulating sirtuin 3 signaling, which may represent a promising therapeutic approach and improve the prognosis of patients with cardiac I/R injury (10). In the present study, DMY alleviated OGD/R-induced oxidative stress in HT22 cells by enhancing the activity of the antioxidant enzyme SOD and inhibiting that of the oxidant MDA, as well as the levels of oxidative stress indicators NOX2 and NOX4.

Chen *et al* (11) determined that DMY diminished the activity of serum aminotransferase and inhibited cell apoptosis in a mouse model of liver I/R. This suggested that while DMY promotes the apoptosis of cancer cells, it also prevents cell injury induced by different stimulants through

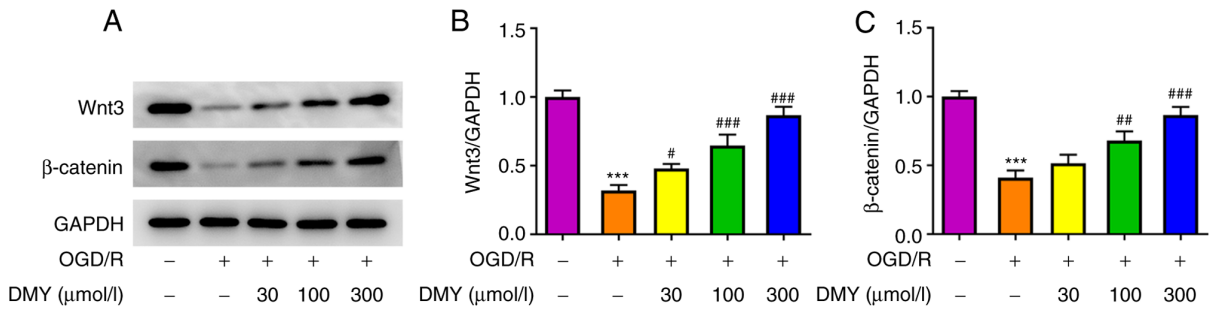

Figure 4. Effects of DMY on the Wnt/β-catenin signaling pathway in HT22 cells. (A) The protein expression of Wnt3 and β-catenin were measured in HT22 cells following OGD/R with or without DMY treatment. Changes in the relative expression of (B) Wnt3 and (C) β-catenin. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; #P<0.05, ##P<0.01, ###P<0.001 vs. OGD/R group. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation.

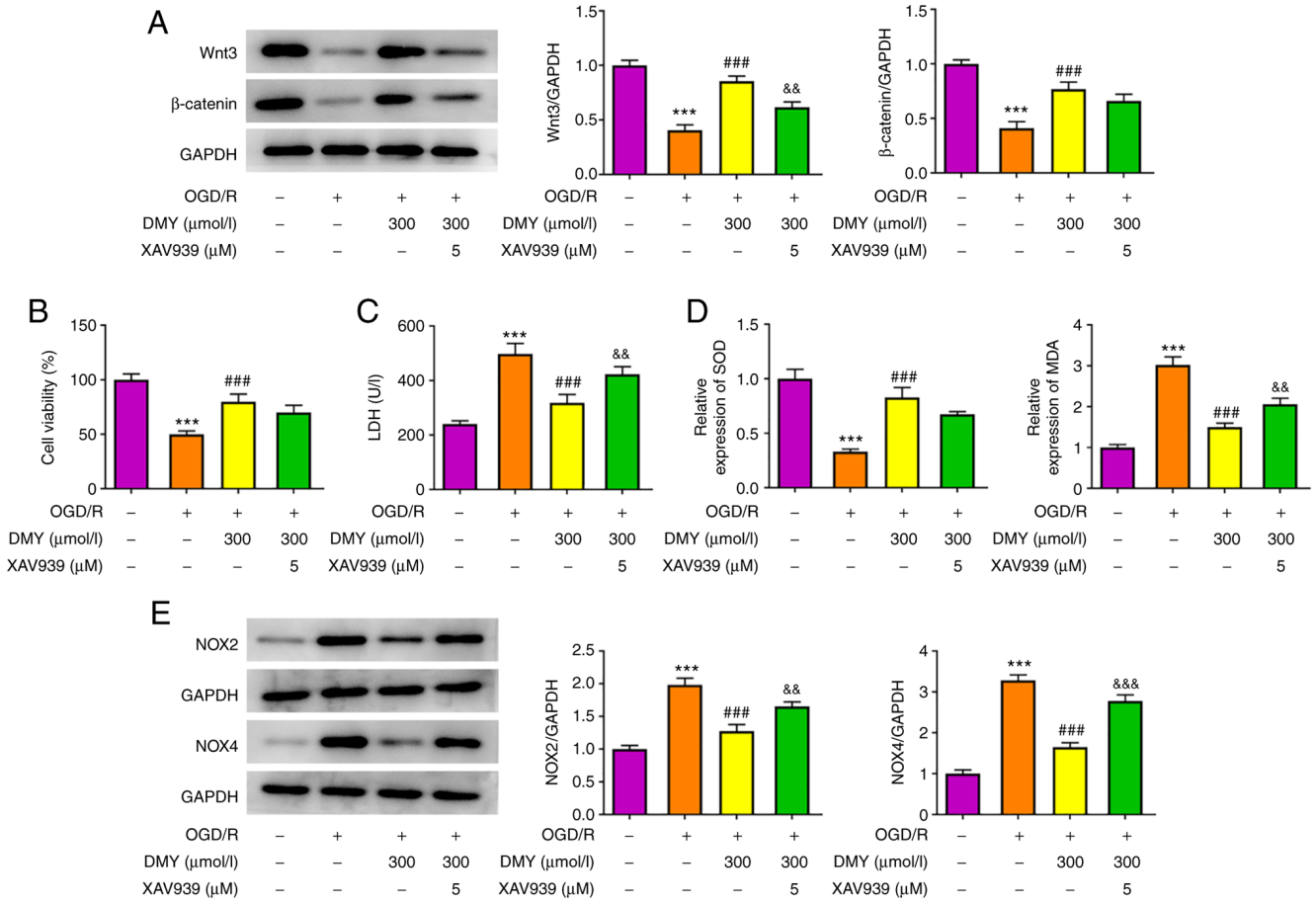

Figure 5. Role of Wnt/β-catenin signaling pathway in the effects of DMY on OGD/R-induced oxidative stress. (A) Protein expression of Wnt3 and β-catenin in OGD/R-stimulated HT22 cells treated with DMY (300 μmol/l) in the presence or absence of Wnt signaling pathway inhibitor XAV939. (B) Viability of OGD/R-stimulated HT22 cells treated with DMY in the presence or absence of XAV939. (C) Cytotoxicity in OGD/R-stimulated HT22 cells treated with DMY in the presence or absence of XAV939. (D) Changes in SOD and MDA expression in the presence or absence of XAV939. (E) Changes in NOX2 and NOX4 expression. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; ###P<0.001 vs. OGD/R group; &&P<0.01, &&&P<0.001 vs. OGD/R + DMY. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation; MDA, malondialdehyde; NOX, NADPH oxidase 1; superoxide dismutase.

the inhibition of unfavorable apoptosis. Similar results were also observed in the present study; OGD/R-induced HT22 cell apoptosis was largely improved in a concentration-dependent manner by DMY treatment. Additionally, DMY increased the expression of the anti-apoptotic protein Bcl2 and reduced that of the apoptosis markers Bax, cleaved caspase-3 and cleaved caspase-9.

The Wnt/β-catenin signal transduction pathway is a key participant in several cellular processes such as cell survival, apoptosis, cellular metabolism and the mediation of oxidative stress (32-34). It has been demonstrated that Wnt/β-catenin signaling has pleiotropic functions in the neuronal activities of patients with Alzheimer's disease, regulating neuronal survival and neurogenesis (35). Furthermore, DMY can

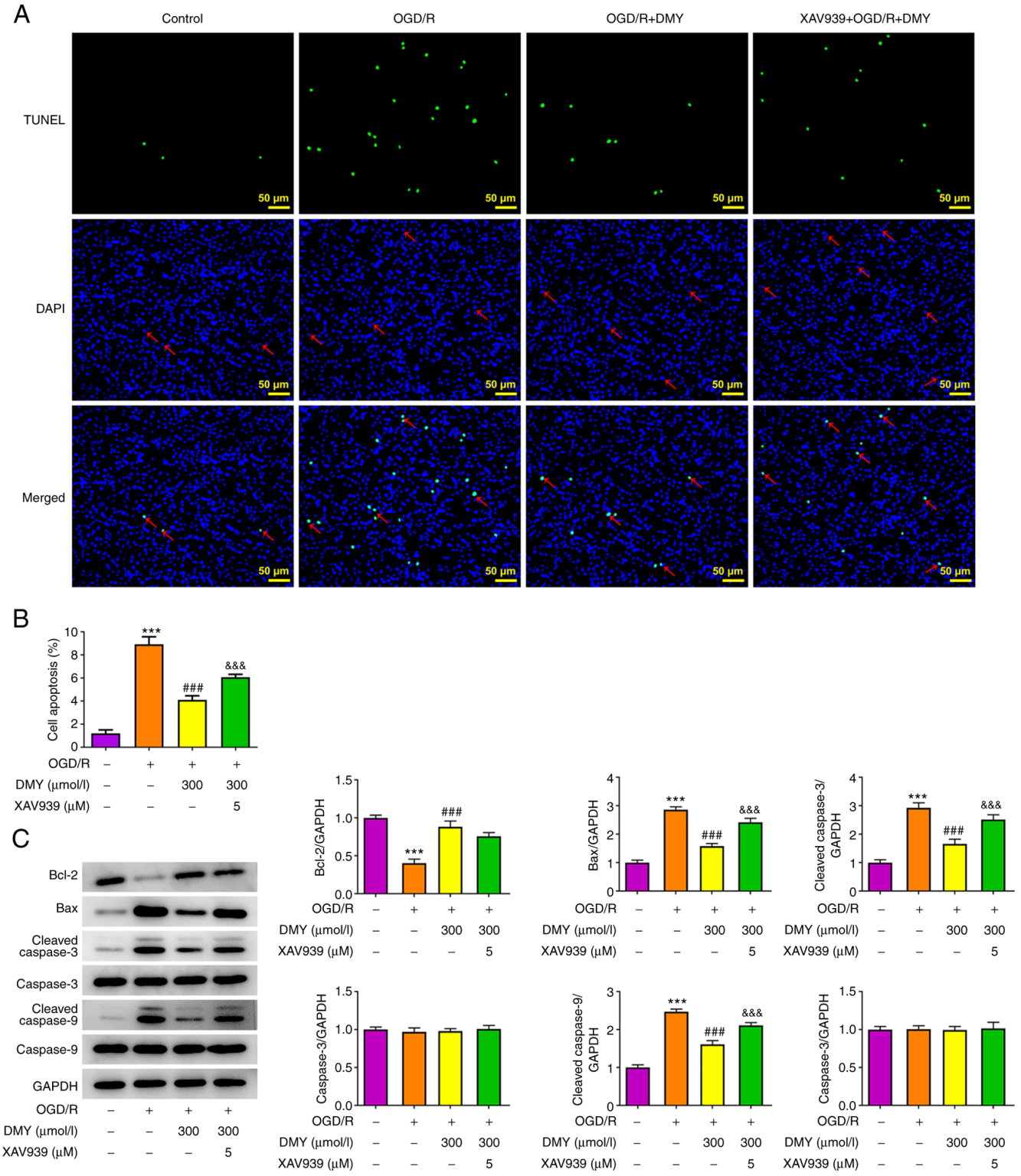

Figure 6. Role of Wnt/β-catenin signaling pathway in the effects of DMY on OGD/R-induced apoptosis. (A and B) Cell apoptosis was detected by TUNEL assay in OGD/R cells pretreated with XAV939 and then treated with 300 μmol/l DMY. (C) Changes in the protein expression of Bcl-2, cleaved caspase-3 and cleaved caspase-9 in OGD/R cells pretreated with XAV939 and then treated with 300 μmol/l DMY. Red arrows indicate TUNEL-positive cells. Data are presented as the mean ± SD. n=3 per group. ***P<0.001 vs. Control; $^{###}$P<0.001 vs. OGD/R group; $^{\&\&\&}$P<0.001 vs. OGD/R + DMY. DMY, dihydromyricetin; OGD/R, oxygen-glucose deprivation and re-oxygenation.

strengthen BMSC osteogenic differentiation *in vitro* by activating Wnt/β-catenin (13). In a rat model of cerebral I/R injury, treatment with DMY demonstrated a protective effect on brain injury by activating PI3K/AKT/GSK3β and its downstream Wnt/β-catenin signaling pathway (14). In the present study, downregulated expression of Wnt3 and β-catenin was detected in HT22 cells following OGD/R, with additional DMY treatment restoring their expression levels. Furthermore, the Wnt inhibitor XAV939 reduced the protective effects of DMY against the OGD/R-induced cytotoxicity, oxidative stress and

apoptosis of HT22 cells. Thus, these results verify the authors' hypothesis that DMY could improve OGD/R-induced HT22 cell injury by activating the Wnt/β-catenin pathway.

To the best of our knowledge, the present study was the first to elucidate the role of DMY in the treatment or prevention of ischemic stroke. The findings of the current study revealed that DMY could ameliorate OGD/R-induced injury of HT22 neurons in part by activating the Wnt/β-catenin signaling pathway. However, the neuroprotective effect of DMY should be further verified in animal models of ischemic stroke.

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Availability of data and materials

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

Authors' contributions

XT and FP wrote the manuscript and analyzed the data. YJ, XJ and XZ performed the experiments and supervised the study. XJ searched the literature and revised the manuscript for important intellectual content. XT and FP confirm the authenticity of all the raw data. 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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