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Original Article

Xanthotoxol inhibits cerebral ischemia/reperfusion injury-induced hippocampal neuronal cell apoptosis through suppressing the p38 MAPK and JNK signaling pathways

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Abstract: Xanthohumol (XN) is a prenylated chalcone derived from hops (*Humuluslupulus* L.). Several studies showed that XN exhibited potent neuroprotective activity. However, the precise mechanism and effect of XN in cerebral ischemia/reperfusion (I/R) injury needs further investigations. In the present study, we investigated the role of XN in regulating oxygen-glucose deprivation/reperfusion (OGD/R)-induced neuron apoptosis. The current study demonstrated that XN pretreatment ameliorates OGD/R-induced cell viability loss and decreased OGD/R-induced ROS generation and MDA level in hippocampal neurons. XN also dose-dependently inhibited the expression of Bax and caspase-3, and enhanced the expression of Bcl-2 in hippocampal neurons. At last, we found that XN pretreatment inhibits OGD/R-induced activation of p38 MAPK and JNK signaling pathways in hippocampal neurons. In conclusion, our present study showed that XN protected neuronal injury induced by OGD/R through suppression of p38 MAPK and JNK signaling pathways. This study provides evidence that XN may serve as a potential therapeutic agent for treatment of ischemic diseases.

Keywords: Xanthohumol (XN), oxygen-glucose deprivation/reperfusion (OGD/R), hippocampal neurons, apoptosis

Introduction

Cerebral ischemia is one of the most common causes of death with high morbidity, high disability, and high mortality in adults worldwide [1]. It is characterized by a reduction or complete blockade of regional cerebral blood flow that results in deficient glucose and oxygen supply [2]. Regular oxygen and glucose supply plays a critical role in regulating normal neuronal functions. Loss of the regular supply, even for a short period, will lead to ischemia/re-oxygenation, eventually causing neuronal cell death and brain damages [3]. Moreover, a large body of evidence suggests that oxidative stress is a fundamental mechanism of cerebral ischemia/reperfusion injury [4-6]. Therefore, inhibiting apoptosis and oxidative stress in ischemic neurons may provide an effective therapeutic intervention in cerebral ischemia.

Xanthohumol (XN), a prenylated chalcone derived from hops (*Humuluslupulus* L.), was shown to exhibit anti-tumor, anti-obesity, free radical-scavenging, and anti-inflammatory activities [7-9]. Recent research find that XN up-regulates the transcription of NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1), and increased the level of the endogenous antioxidant glutathione in lipopolysaccharide-induced stimulated BV2 cells [10]. In addition, treatment with XN 10 min before middle cerebral artery occlusion (MCAO) dose-dependently attenuates focal cerebral ischemia and improved neurobehavioral deficits in cerebral ischemic rats [11]. However, the role of XN on oxygen-glucose deprivation/reperfusion (OGD/R)-induced cell injury in the model of hippocampal neurons has not been explored. Therefore, the aim of this research was to investigate the effect of XN on OGD/R-treated primary hippocampal neuronal cells. Our results demonstrated that XN protects against OGD/R-induced apoptosis, at least inpart, by scavenging excessive ROS and inhibiting the mitochondrion-dependent apoptotic pathway.

Materials and methods

Cell isolation and cell culture

All animal research was carried out in accordance with the regulations of the Ruikang Hospital Affiliated to Guangxi University to Chinese Medicine Committee on Ethics in the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Ruikang Hospital Affiliated to Guangxi University to Chinese Medicine (China). Primary hippocampal neuronal cells were prepared from neonatal SD rats. Briefly, the hippocampi tissues were dissected and dissociated in 0.25% trypsin-EDTA, and primary hippocampal neurons were maintained in neurobasal medium supplemented with B-27, glutamine (0.5 mM), glutamate (25 μM), and 1% penicillin/streptomycin. The cultures were maintained in a humidified incubator with 5% CO_2 at 37°C for 14 days. Culture medium was changed two times a week.

OGD/R treatment

OGD/R was performed as previously described [12]. Briefly, cell culture media was removed and cells were washed twice with glucose-free DMEM. Then cells were incubated in the glucose-free medium in an oxygen-free incubator (95% N_2 and 5% CO_2) in absence or presence of various concentrations of XN (1, 5 and 10 μM) for 2 h. At the end of 3 h OGD, the cells were incubated with conditioned DMEM in absence or presence of indicated concentrations of XN for 24 h.

Cell viability assay

Cell viability was evaluated by CCK-8 assay. In brief, neurons at a density of 1×10^4 cells/well were seeded into 96-well plates. After 24 h of incubation, CCK-8 (10 μl) was added to each well. Absorbance was measured at 570 nm using an enzyme linked immunosorbent assay plate reader (Olympus, Tokyo, Japan).

Lactate dehydrogenase (LDH) assay

The cell cytotoxicity was assessed by the LDH assay using the Cytotoxicity Detection Kit. Briefly, after treatment, the media were collected and centrifuged at 6,000 rpm for 5 min, and the supernatants were harvested. One hundred microliters of the LDH reaction was added into each well and incubated in the dark at room temperature for 30 min. The colorimetric compound was measured spectrophotometrically at 490 nm (Perkin-Elmer, VICTOR3).

Measurement of intracellular ROS

The level of intracellular ROS was evaluated with DCF-DA (Molecular Probes, Beijing, China). Briefly, treated cells were loaded with 30 μM DCF-DA at 37°C for 30 min. The cells were kept on ice in the dark and at least 10,000 cells for each sample were analyzed using a Becton Dickinson FACSC alibur (BD Biosciences, San Jose, CA). Intracellular ROS levels were expressed as the average DCF fluorescence intensity of the cells.

Determination of malondialdehyde (MDA) level

The MDA level was measured with the respective commercial kits (Beyotime, Haimen, Jiangsu, China). MDA levels were measured by a method based on a reaction with thiobarbituric acid. The optical density at 532 nm was measured with a microplate reader (Molecular Device, Spectra Max 190, USA).

Cell apoptosis assays

Cell apoptosis was evaluated by Annexin V-FITC and PI staining followed by analysis with flow cytometry (Beckman-Coulter, Brea, CA, USA). Briefly, after treatment, the cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellets were washed twice with PBS followed by fixation with 70% ice-cold ethanol and stored at -20°C overnight. The pellets were washed with cold PBS and stained with propidiumiodide and Annexin V.

Cell apoptosis was also assessed by TUNEL staining. Briefly, after treatment, the cells were fixed with 1% paraformaldehyde for 10 min. After washing, incubated the cells in the DNA labeling solution for 60 min at 37°C, and then incubated the cells with anti-BrdU-FITC antibody solution in the dark for 30 min. Images were captured using a fluorescence microscope (DM4000B, Leica, Wetzlar, Germany).

Western blot

The neurons were washed with PBS and lysed with RIPA buffer (50 mM Tris-HCl pH 7.2, 150

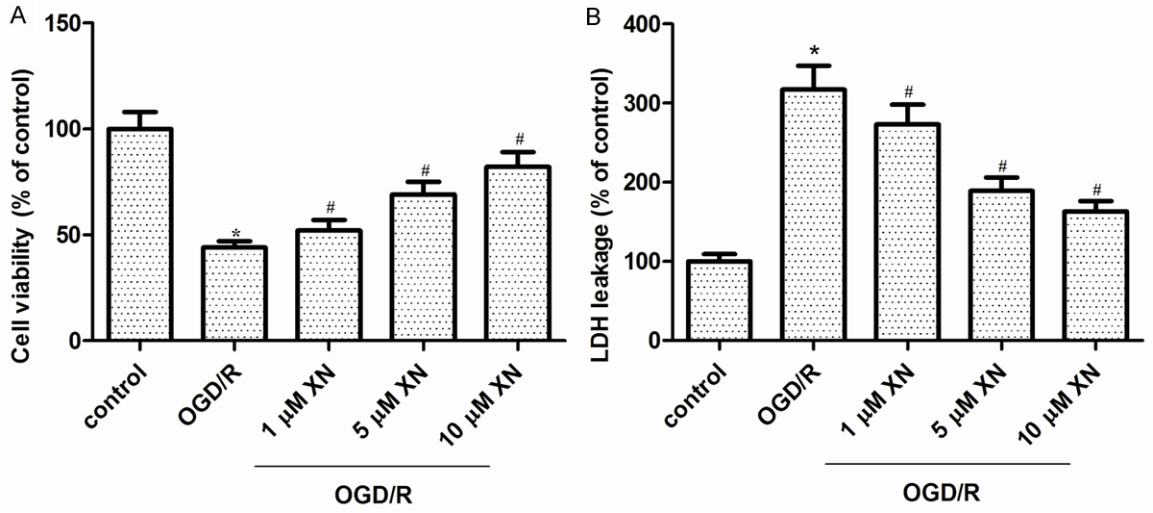

Figure 1. XN pretreatment ameliorates OGD/R-induced cell viability loss in hippocampal neurons. Primary hippocampal neurons were pretreated with indicated concentration of XN (1-10 μM, 2 h pretreatment), and were maintained under OGD for 3 h, followed by 24 h of re-oxygenation. A: Cell viability was tested by CKK-8 assay. B: Cell cytotoxicity was tested by LDH release. Data are reported as the means ± S.D. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

mM NaCl, 1% Triton X-100, and 0.1% SDS). Then cells were centrifuged at 10,000 rpm for 10 min at 4°C. Protein concentrations were determined by the BCA method. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). The membranes were blocked with 5% fat-free milk in Tris-buffer saline (TBS) for 1 h, and incubated with primary antibodies [anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK and anti-GAPDH (Santa Cruz, CA, USA)] at 4°C overnight. Next, membranes were washed three times with TBS and Tween 20 [TBST; 10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20] for 10 min at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

Results were presented as mean ± S.D. Statistical analysis was performed using One-way ANOVA analysis. P<0.05 was considered statistically significant.

Results

XN pretreatment ameliorates OGD/R-induced cell viability loss in hippocampal neurons

To explore the effect of XN on OGD/R-induced cellular viability in hippocampal neurons, the CCK-8 assay was used. Compared to the control group, the cell viability was significantly decreased after OGD/R treatment. However, pretreatment with different concentrations of XN 2 h prior to OGD/R greatly increased cell viability, as compared with the OGD/R group (**Figure 1A**).

We further analyzed whether XN pretreatment could influence the OGD/R-induced cellular cytotoxicity of hippocampal neurons. The results of LDH assay showed that LDH leakage was increased after OGD/R treatment, and SA incubation attenuated OGD/R-induced LDH release in a dose-dependent manner (**Figure 1B**).

XN pretreatment attenuates OGD/R-induced oxidative stress in hippocampal neurons

Then, we examined the effects of XN on the OGD/R-induced oxidative stress in hippocampal neurons. As shown in **Figure 2A**, OGD exposure significantly increased intracellular ROS generation inhippocampal neurons. Conversely, XN pretreatment decreased OGD/R-induced ROS generation, exhibiting a dose-dependent

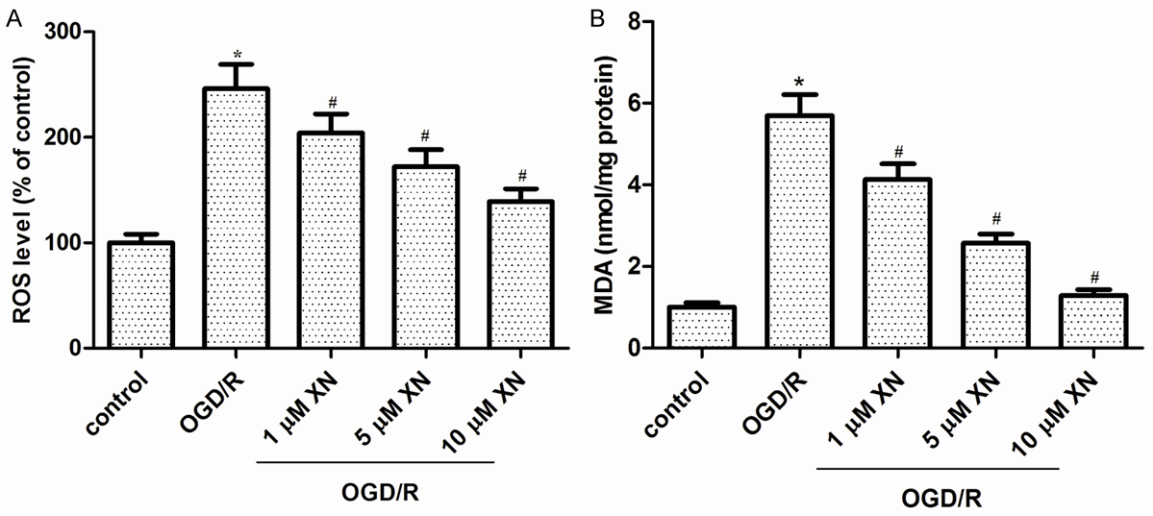

Figure 2. XN pretreatment attenuates OGD/R-induced oxidative stress in hippocampal neurons. Primary hippocampal neurons were pretreated with indicated concentration of XN (1-10 μM, 2 h pretreatment), and were maintained under OGD for 3 h, followed by 24 h of re-oxygenation. A: ROS generation was tested with DCF-DA. B: MDA level was determined by a method based on the reaction with thiobarbituric acid. Data are reported as the means ± S.D. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

manner. Similarly, XN pretreatment also inhibited OGD/R-induced MDA level in hippocampal neurons (**Figure 2B**).

XN pretreatment inhibits OGD/R-induced cell apoptosis in hippocampal neurons

We investigated the effect of XN on cell apoptosis by flow cytometry-based Annexin V-FITC/PI double staining. As shown in **Figure 3A**, OGD/R exposure sharply increased the apoptosis rate in hippocampal neurons. However, XN pretreatment reversed OGD/R-induced cell apoptosis. In TUNEL staining, OGD/R increased the apoptosis of hippocampal neurons, and this effect was apparently reserved by XN (**Figure 3B**).

Furthermore, we investigated the effects of XN on pro-apoptotic protein (Bax) and anti-apoptotic (Bcl-2) protein levels in OGD/R-treated hippocampal neurons. Western blot results in **Figure 3C** demonstrated that the expression of Bcl-2 protein was significantly decreased in OGD/R-treated hippocampal neurons compared with the control, whereas an enhanced expression of Bax protein was observed. However, pretreatment with XN dose-dependently inhibited the expression of Bax and caspase-3, and enhanced the expression of Bcl-2 in hippocampal neurons (**Figure 3C**).

XN pretreatment inhibits OGD/R-induced activation of p38 and JNK signaling pathways in hippocampal neurons

A growing body of evidence indicates that p38 MAPK and JNK signaling pathways are involved in cerebral I/R injury *in vivo* and *vitro*. Therefore, to elucidate whether these signaling pathway are involved in the neuroprotective activity of XN, we examined the expression of p38 MAPK and JNK1/2 during OGD/R. As shown in **Figure 4**, the levels of phospho-p38 MAPK and phospho-JNK1/2 expression were enhanced by OGD/R, which was remarkably inhibited by pretreatment of XN in hippocampal neuronal cells.

Discussion

The main findings of the present study can be summarized as follows: (1) XN pretreatment ameliorates OGD/R-induced cell viability loss in hippocampal neurons; (2) XN pretreatment decreased OGD/R-induced ROS generation and MDA level in hippocampal neurons; (3) pretreatment with XN dose-dependently inhibited the expression of Bax and caspase-3, and enhanced the expression of Bcl-2 in hippocampal neurons; (4) XN pretreatment inhibits OGD/R-induced activation of p38 MAPK and JNK signaling pathways in hippocampal neurons.

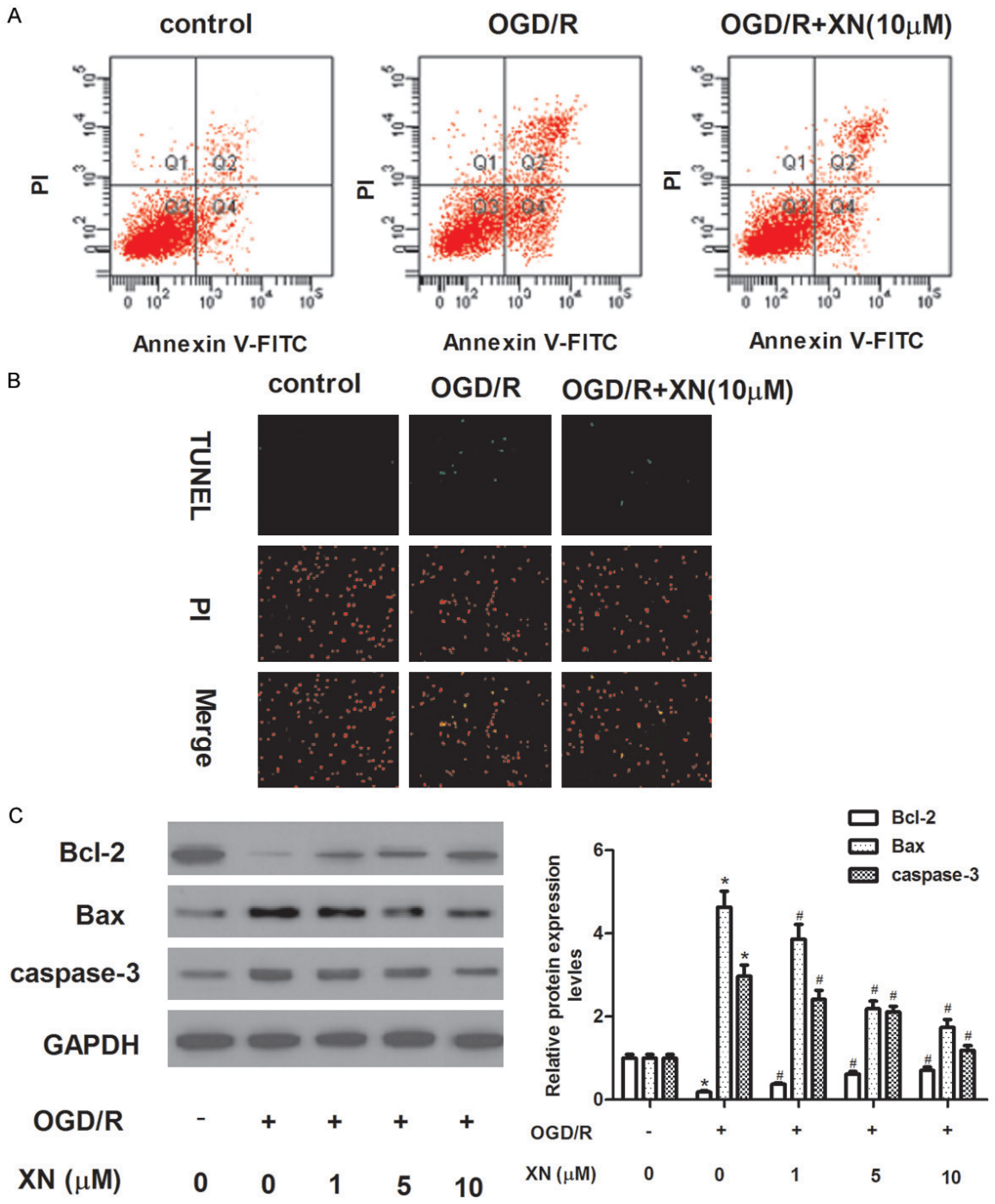

Figure 3. XN pretreatment inhibits OGD/R-induced cell apoptosis in hippocampal neurons. Primary hippocampal neurons were pretreated with XN (2 h pretreatment), and were maintained under OGD for 3 h, followed by 24 h of re-oxygenation. A: Cell apoptosis was evaluated by Annexin V-FITC and PI staining. B: Representative images of TUNEL-positive nuclei in green fluorescent color and total nuclei staining with propidium iodide (PI). C: The expression of Bcl-2, Bax and caspase-3 was detected by western blot. Quantitative analysis was performed by measuring the intensity relative to the control. Data are reported as the means ± S.D. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

Previous studies have demonstrated that OGD induces the apoptosis in neurons [13]. Thus, herein, to evaluate the neuroprotective effects of XN on ischemia/reperfusion injury, an *in vitro* OGD/R model was established hippocampal neuronal cells. The results of the current study

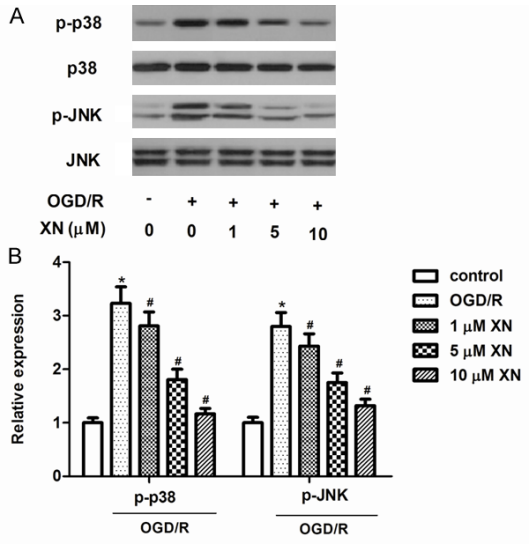

Figure 4. XN pretreatment inhibits OGD/R-induced activation of p38 and JNK signaling pathways in hippocampal neurons. Primary hippocampal neurons were pretreated with indicated concentration of XN (1-10 μM, 2 h pretreatment), and were maintained under OGD for 3 h, followed by 24 h of re-oxygenation. A: The expression of p-p38, p38, p-JNK and JNK proteins was detected by western blot. B: Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. Data are reported as the means ± S.D. **P*<0.05 *vs.* control group. #*P*<0.05 *vs.* OGD/R group.

show that OGD inhibits cell viability. These results are in agreement with the findings of previous studies [14, 15]. The neurons that were pretreated with XN prior to OGD/R exhibited increased cell viability, which suggests that XN exerts a protective effect in hippocampal neurons against OGD-induced cell viability.

OGD/R-induced neuronal cell damage is accompanied with oxidative stress. It was reported that OGD/R causes the oxidants/antioxidants imbalance via free radical-mediated oxidative stress induction [16]. Numerous clinical and experimental observations have shown increased ROS production in cerebral tissues with ischemia-reperfusion [4, 17]. In the current study, we found that OGD/R-induced ROS production and MDA level was significantly inhibited by XN, which could explain its neuroprotective activity in cerebral ischemia.

Bcl-2, an anti-apoptotic protein, prevents the accumulation of cytochrome c in the cytosol, and the subsequent caspase activation; whereas Bax was identified as the pro-apoptotic member that triggers the release of caspases. Therefore, alterations in the levels of anti- and pro-apoptotic proteins influence apoptosis [18]. Caspases play a critical role in the regulation of cell apoptosis [19]. Among the caspases, the effector caspase-3 has been involved in apoptotic cell death after ischemia [20, 21]. In this study, we found that pretreatment with XN dose-dependently inhibited the expression of Bax and caspase-3, and enhanced the expression of Bcl-2 in hippocampal neurons. These findings indicate that XN protects against OGD-induced injury by inhibiting apoptosis in hippocampal neurons by down-regulating caspase-3 activation and modulating the Bcl-2/Bax ratio.

Previous studies showed that expression of p38 MAPK changes in ischemic brain tissue and that inhibition of the p38 MAPK activation can provide protection in a variety of *in vitro* and *in vivo* models of brain injury by prevention of cell death [22-24]. Moreover, JNK signaling pathway also plays a critical role in brain injury after ischemia and reperfusion [25-27]. Consistent with the previous studies, herein, we observed that there was also a significant up-regulation of p-p38 MAPK and p-JNK in primary hippocampal neurons exposed to 2 h of OGD followed by 24 h of reoxygenation. Interestingly, the levels of p-p38 MAPK and p-JNK were also greatly down-regulated in XN-treated hippocampal neuronal cells. These results suggest that XN may our present study showed that XN inhibits OGD/R-induced cell apoptosis through activation of p38 and JNK signaling pathways in hippocampal neurons.

In summary, our present study showed that XN protected neuronal injury induced by OGD/R through suppression of p38 MAPK and JNK signaling pathways. This study provides evidence that XN may serve as a potential therapeutic agent for treatment of ischemic diseases.

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Disclosure of conflict of interest

None.

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