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Isoflurane preconditioning protects hepatocytes from oxygen glucose deprivation injury by regulating FoxO6

YONGHUI ZHONG, XUEFANG HU and LIANGSHENG MIAO*

Department of Anesthesiology, Weinan Central Hospital, Weinan 714000, China

**Corresponding author (Email, liangshengmo@163.com)*

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The forkhead protein (FoxO) family plays a crucial role in regulating oxidative stress, cell proliferation, and apoptosis. FoxO6, a member of the FoxO family, helps regulate oxidative stress in gastric cancer and hepatocellular carcinoma. However, it is unclear whether FoxO6 participates in the protective effect of isoflurane preconditioning in liver injury caused by oxidative stress in ischemia. In this study, we explored the role and mechanism of FoxO6 in the protective effect of isoflurane preconditioning during hepatocyte injury caused by oxygen-glucose deprivation (OGD). Cells from the human fetal hepatocyte (LO2) line were incubated with 0%, 1%, 2%, 2.5%, 3%, 3.5%, 4%, or 5% isoflurane for 3 h and then exposed to OGD. Data showed that 3% isoflurane preconditioning inhibited FoxO6 expression, caspase-3 activity, and reactive oxygen species production and promoted cell viability. FoxO6 overexpression abolished the effects of 3% isoflurane preconditioning on caspase-3 activity, reactive oxygen species production, and cell viability in these cells. Moreover, FoxO6 regulated nuclear factor erythroid 2-related factor (Nrf2) expression via c-Myc after 3% isoflurane preconditioning and OGD exposure. Thus, isoflurane preconditioning prevented OGD-induced injury in LO2 cells by modulating FoxO6, c-Myc, and Nrf2 signaling.

Keywords. c-Myc; FoxO6; human fetal hepatocyte line (LO2); isoflurane preconditioning; oxygen-glucose deprivation

Abbreviations: FoxO, forkhead protein; OGD, oxygen-glucose deprivation; Nrf2, nuclear factor erythroid 2-related factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

1. Introduction

Hepatic ischemia is common after liver transplantation in which there are no effective strategies to avoid this occurrence in the clinic (Jochmans *et al.* 2017; Quillin *et al.* 2018; Ginsberg *et al.* 2016). Isoflurane is an inhaled general anesthetic. During oxidative stress, isoflurane preconditioning reportedly inhibits nitric oxide synthase production, heat shock protein expression, and reactive oxygen species (ROS) production (Olson *et al.* 2015). Isoflurane preconditioning also protects the liver against damage induced by hepatic ischemia (Zhang *et al.* 2011). However, the molecular mechanism of isoflurane during oxidative stress in the liver is unclear.

The forkhead box protein (FoxO) family of transcription factors (FoxO1, FoxO3, FoxO4, and FoxO6) has highly conserved DNA binding domains (Chen *et al.* 2018; Lin *et al.* 2018; Li *et al.* 2018). FoxO proteins help regulate antioxidative stress, cell proliferation, apoptosis, and cell differentiation (Yu *et al.* 2018; Yamaguchi *et al.* 2016; Guo *et al.* 2018). FoxO1 reportedly plays a critical role in granulosa cell apoptosis induced by oxidative stress (Zhang *et al.* 2016). FoxO3 protects resveratrol in hyperglycemia-induced renal oxidative stress injury (Wang *et al.* 2017b). FoxO6 is highly expressed in hepatocellular carcinoma tissue and is associated with oxidative stress (Chen *et al.* 2016). FoxO6 also plays an important role in oxidative stress during cell proliferation (Wang *et al.* 2017a). However, the effects of FoxO6 on cell viability, caspase-3 activity and ROS level in hepatocytes with isoflurane preconditioning during ischemia-induced liver injury remains unclear and needs to be defined. Liver ischemia is resulting in oxygen and nutrient deprivation (Xu *et al.* 2019). Thus, we exposed hepatocytes to oxygen glucose deprivation (OGD) and investigated the effects and possible molecular mechanism of isoflurane preconditioning in protecting against oxidative stress.

FoxO6 can positively regulate expression of c-Myc in gastric carcinoma (Qinyu *et al.* 2013). The oncogene c-Myc is a transcription factor that regulates cell growth and apoptosis and helps to regulate oxidative stress (Bachmann and Geerts 2018; Xiao *et al.* 2018; Deol *et al.* 2018). The upregulated expression of c-Myc leads to intracellular ROS generation in fibroblasts (Finkel 2003). Elevated expression of c-Myc in hepatocytes promotes oxidative stress in alcoholic liver disease (Nevzorova *et al.* 2016). Moreover,

c-Myc mediates the expression of nuclear factor erythroid 2-related factor (Nrf2) in fibroblasts (Kansanen *et al.* 2013). Nrf2 is the most dynamic gene in the cap-n-collar transcription factor family (Nakamura *et al.* 2018). Nrf2-mediated antioxidation is a major cellular defense mechanism, and its deletion can aggravate cytotoxicity during oxidative stress, leading to cell dysfunction, apoptosis, and even death (Liu *et al.* 2016). Nrf2 has been shown to protect the liver in different oxidative stress models (Li *et al.* 2015). Thus, we hypothesized that FoxO6 facilitates the protective effect of isoflurane preconditioning during ischemia-induced liver injury by regulating c-Myc and Nrf2 signaling.

In this study, we focused on identifying the role and molecular mechanism of FoxO6 in the protective effect of isoflurane preconditioning on cell viability, apoptosis, and ROS levels in human fetal hepatocyte (LO2) cells injured by OGD. We also tested the effects of FoxO6 overexpression on cell viability, apoptosis, and ROS levels in these cells. The data indicate that the protective effects of FoxO6 were realized via regulation of c-Myc and Nrf2 signaling.

2. Materials and methods

2.1 *Cell culture*

Cells from the immortal human liver LO2 cell line were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco), gentamycin (0.01 mg/mL), and 0.1 mM of nonessential amino acids in humid conditions (5% CO_2) at 37°C. Isoflurane preconditioning of the LO2 cells was achieved by culturing with 0%, 1%, 2%, 2.5%, 3%, 3.5%, 4%, or 5% isoflurane dissolved in DMEM for 3 hours. We chose 3hr time point on the basis of the reference (Zhang *et al.* 2012).

2.2 *Oxygen-glucose deprivation*

The LO2 cells exposed to OGD were cultured with Earle's balanced salt solution in a humid atmosphere (95% N_2 and 5% CO_2). Cells that were not exposed to OGD were preconditioned with 0%, 1%, 2%, 2.5%, 3%, 4%, or 5% isoflurane for 3 h and then cultured with Earle's balanced salt solution supplemented with sugar in a non-hypoxic atmosphere (95% air and 5% CO_2) at 37°C for 9 h. Next, all media of the LO2 cells were changed to serum-free DMEM and cultured in 5% CO_2 at 37 °C for 15 h.

2.3 *Detection of reactive oxygen species*

In LO2 cells that were preconditioned with isoflurane preconditioning and exposed to OGD, ROS generation was detected via 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes Inc., Eugene, OR, USA) and an ROS assay kit. Briefly, 70 μM of DCFH-DA was added and incubated with the cells at 37°C for 50 min in the dark. DCF fluorescence was determined using a flow cytometer (BD Labware, Franklin Lakes, NJ, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.4 *LO2 cell viability*

Viability of LO2 cells was detected via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after isoflurane preconditioning and OGD incubation. Briefly, the culture medium was abandoned, then 20 μL of MTT solution (0.5 g/L) was added. After 4 h of reaction at 37°C, dimethyl sulfoxide (150 μL/well) was added to dissolve the formazan. The results were quantified using a microplate reader (Infinite 200 Pro, Tecan, Austria) at a wavelength of 490 nm.

2.5 *Caspase-3 activity*

Caspase-3 activity was measured using a Caspase-3 Activity Assay kit (Beyotime, Nantong, China). Cells were homogenized in 100 mL of reaction buffer with caspase-3 substrate (9 mL) and incubated at 37°C for 3 h. Results were measured using an ELISA reader at 405 nm (Thermo Fisher Scientific, Waltham, MA, USA).

2.6 *Protein concentrations*

Proteins were extracted, and the protein concentration for each sample was measured with a BCA kit (Beyotime). Then, 25 μg of protein from each sample was loaded and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto a nitrocellulose membrane (Amersham, Little Chalfont, UK), followed by incubation with the primary antibodies anti-FoxO6, anti-GAPDH, anti-c-Myc, or anti-Nrf2 (Abcam Inc., Cambridge, MA, USA) overnight at 4°C. Then, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies (Boster Corporation, Wuhan, China) for 1 h. The proteins were visualized using a Bio-Rad ChemiDoc apparatus (Bio-Rad, Hercules, CA, USA). GAPDH was the reference protein.

2.7 *Recombinant plasmid construction and cell transfection*

The full-length FoxO6 (accession number XM_002346379.1) The cDNA was amplified and subcloned into the pcDNA.3.1 plasmid (Invitrogen, Carlsbad, CA,

USA) with *EcoR*Iand *BamH*I restriction sites. Subsequently, the recombinant plasmid was transfected into DH5 *Escherichia coli*–competent cells (Beyotime) to amplify overnight at 37°C. The recombinant vectors were extracted from DH5α using a TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 (Takara Biotechnology, Dalian, China) and sequenced. The correct ones were named as pcDNA.3.1-FoxO6.

LO2 cells were plated in a 96-well culture plate, followed by incubation with isoflurane preconditioning and OGD. Then, transfection was performed per manufacturer's instructions. Briefly, pcDNA.3.1-FoxO6 (0.3 μg), pcDNA.3.1 (0.3 μg), c-Myc siRNA (5′-CTT CTA CCA GCA GCA GCA G-3′; 0.5 μg), or nonspecific siRNA (0.3 μg) was mixed with 0.5 μL of TurboFect (Thermo Fisher Scientific) per well. The mixture was then diluted in fetal-bovine-serum-free DMEM (200 μL), added to the wells, and incubated for 24 hours. Transfection efficiencies were measured by qRT-PCR and western blot.

2.8 *Statistical analyses*

Statistical analyses were processed using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance followed by LSD and Bonferroni test. Data are expressed as the mean ± standard deviation. A p-value of $<.05$ was considered statistically significant.

3. Results

3.1 *Isoflurane preconditioning increased cell viability and decreased apoptosis in LO2 cells exposed to oxygen-glucose deprivation*

To investigate the effects of isoflurane preconditioning on LO2 cell viability and apoptosis after OGD, we cultured the cells in 0%, 1%, 2%, 2.5%, 3%, 4%, or 5% isoflurane dissolved in DMEM for 3 hours and then exposed them to OGD. Cell viability and apoptosis then were measured by MTT assay and a Caspase-3 Activity Assay kit, respectively. The results showed increased cell viability (figure 1A) and constrained caspase-3 activity (figure 1B) with 3%, 4% and 5% isoflurane preconditioning, compared to the LO2 cells with 0% isoflurane preconditioning.

3.2 *Isoflurane preconditioning reduced reactive oxygen species production in LO2 cells exposed to oxygen-glucose deprivation*

LO2 cells were exposed to OGD and then 3 hours of preconditioning with 0%, 1%, 2%, 2.5%, 3%, 4%, or 5% isoflurane. The results demonstrated that 3%, 4% and 5% isoflurane preconditioning significantly downregulated ROS levels (figure 2), compared to cells without isoflurane preconditioning. *Isoflurane preconditioning suppressed FoxO6 expression in LO2 cells exposed to oxygen-glucose deprivation* to assess protein expression of FoxO6 in these cells. We found that protein expression (figure 3) of FoxO6 decreased, compared to LO2 cells without isoflurane preconditioning.

3.3 *Isoflurane preconditioning and FoxO6 overexpression promoted injury in LO2 cells exposed to oxygen-glucose deprivation*

Preconditioning with 3% isoflurane markedly inhibited cell apoptosis, ROS levels and FoxO6 expression and promoted cell viability in LO2 cells exposed to OGD. Thus, we chose 3% isoflurane preconditioning in the followed experiments. To investigate how FoxO6 influences the protective effect of isoflurane preconditioning on LO2 cells exposed to OGD, we overexpressed FoxO6 via transfection with pcDNA.3.1-FoxO6 after 3% isoflurane preconditioning and OGD. Results showed that protein expression of

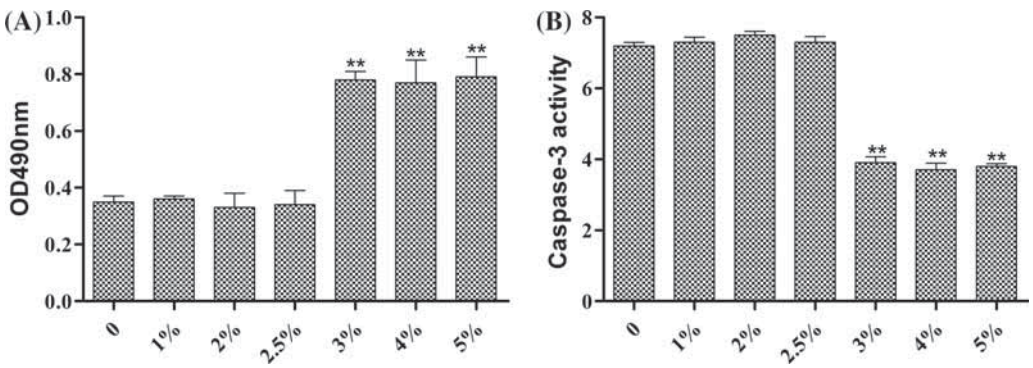

Figure 1. Effects of isoflurane preconditioning on cell viability and apoptosis in LO2 cells exposed to OGD. (**A**) The cell viability of LO2 with isoflurane preconditioning and OGD treatment were measured by MTT assay. (**B**) The cell apoptosis of LO2 with isoflurane preconditioning and OGD treatment were measured by caspase-3 activity detection. 0, 1%, 2%, 2.5%, 3%, 4%, or 5%: cells were cultured in 0%, 1%, 2%, 2.5%, 3%, 4%, or 5% isoflurane dissolved in DMEM for 3 hours and then exposed to OGD. $n = 3$, $^{**}P < .01$ versus the 0 group.

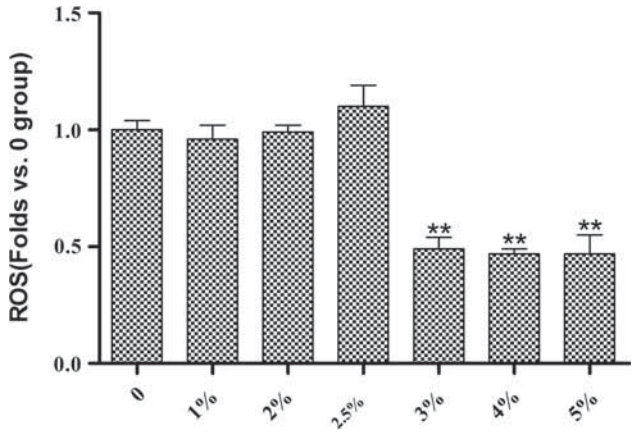

Figure 2. Effects of isoflurane preconditioning on ROS level in LO2 cells exposed to OGD. ROS level of LO2 with isoflurane preconditioning and OGD treatment were measured by ROS assay kit. $n = 3$, $^{**}P < .01$ versus the 0 group.

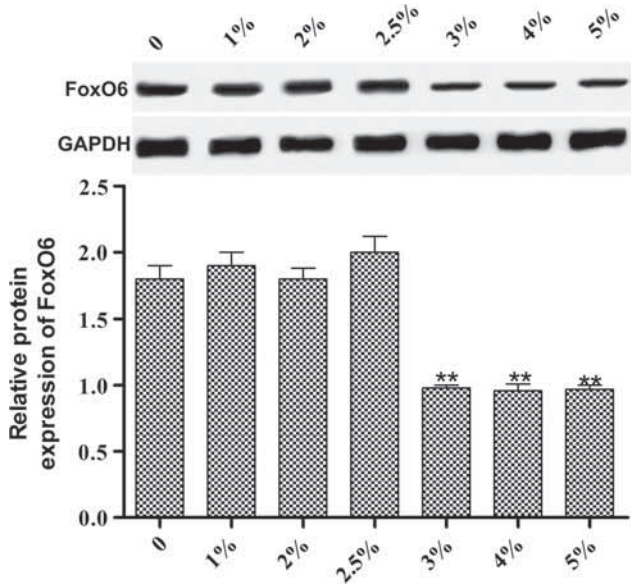

Figure 3. Effects of isoflurane preconditioning on FoxO6 expression in LO2 cells exposed to OGD. FoxO6 protein expression of LO2 with isoflurane preconditioning and OGD treatment were measured by Western blot. $n = 3$, $^{**}P < .01$ versus the 0 group.

FoxO6 (figure 4A) after transfection was highly increased in LO2 cells with isoflurane preconditioning and OGD exposure, compared to cells with isoflurane preconditioning, indicating successful transfection. Then, we measured cell viability, caspase-3 activity, and ROS levels after FoxO6 overexpression in these cells. We found that substantially restrained cell viability (figure 4B) and significantly elevated caspase-3 activity (figure 4C) and ROS levels (figure 4D).

3.4 *FoxO6 regulated Nrf2 expression by modulating c-Myc in LO2 cells that were preconditioned with isoflurane and exposed to oxygen glucose deprivation*

We measured protein expression of c-Myc and Nrf2 after FoxO6 overexpression in LO2 cells that were preconditioned with 3% isoflurane and then exposed to OGD. The data demonstrated increased expression of c-Myc and inhibited expression of Nrf2 (figure 5A). We also restrained expression of c-Myc via cell transfection with c-Myc siRNA in FoxO6-overexpressed LO2 cells. The results showed increased Nrf2 expression (figure 5B), indicating that c-Myc inhibition effectively reversed the inhibitory effect of FoxO6 overexpression on Nrf2 expression in OGD-injured cells that were preconditioned with 3% isoflurane. Thus, we conclude that FoxO6 regulated Nrf2 expression by modulating c-Myc in LO2 cells. In line with studies showing that Nrf2 may protect the liver from oxidative stress (Ramos-Tovar *et al.* 2018), isoflurane preconditioning also may protect LO2 cells from OGD injury by regulating FoxO6, c-Myc, and Nrf2 signaling.

4. Discussion

Hepatic ischemia causes an oxidative stress reaction, causing widespread damage (Ge *et al.* 2018). Oxygen-free radicals damage liver tissue mainly via non-substantial cells, such as endothelial cells and hepatic parenchymal cells (Cimmino *et al.* 2018; Seo and Jeong 2016) Isoflurane preconditioning may protect the liver against oxidative- stress-induced damage in hepatic ischemia (Zhang *et al.* 2011). In our study, we incubated LO2 cells with 0%, 1%, 2%, 2.5%, 3%, 4%, or 5% isoflurane, followed by OGD, and found that 3% isoflurane preconditioning markedly inhibited caspase-3 activity and ROS creation and increased cell viability. The 4% and 5% isoflurane amounts showed no obvious difference compared with 3% isoflurane. Probably because the isoflurane amounts of 4% and 5% are also within the effective concentration range. Thus, 3% isoflurane could effectively protect LO2 from OGD injury. However, the molecular mechanism of isoflurane preconditioning in this protective effect remains unclear.

FoxO transcription factors reportedly protect human chondrocytes against oxidative stress (Akasaki *et al.* 2014). Lee *et al.* demonstrated that FoxO is closely associated with minocycline-induced regulation of oxidative stress resistance in Drosophila (Lee *et al.* 2017). FoxO6, a member of FoxO transcription factor family, is important in oxidative stress. Chen *et al.* found that FoxO6 expression influences oxidative stress in cell proliferation among patients with stage-I tumor node metastasis (Wang *et al.* 2017a). FoxO6 virus transduction constrains ROS levels in hepatoma carcinoma HepG2 cells (Kim *et al.* 2015). However, the relationship between FoxO6, isoflurane preconditioning, and oxidative

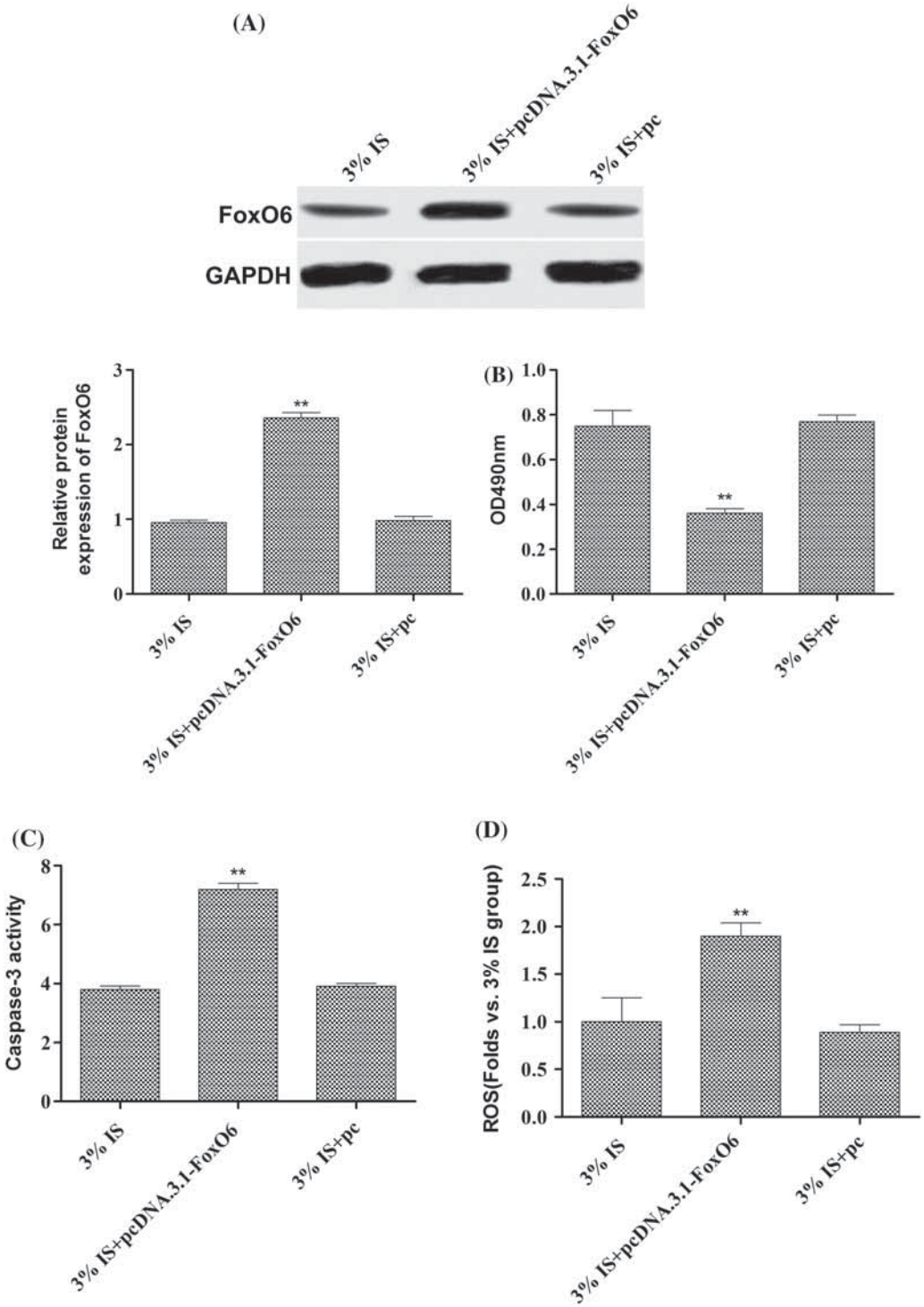

Figure 4. The effects of FoxO6 overexpression on cell viability, apoptosis and ROS level in LO2 cells with isoflurane preconditioning and OGD treatment. (**A**) FoxO6 expression after transfection in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by Western blot. (**B**) The cell viability after transfection in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by MTT assay. (**C**) The cell apoptosis after transfection in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by caspase-3 activity detection. (**D**) The ROS level after transfection in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by ROS assay kit. 3% IS: cells were cultured in 3% isoflurane dissolved in DMEM for 3 hours and then exposed to OGD. 3% IS+ pcDNA.3.1-FoxO6: Cells transfected with pcDNA.3.1-FoxO6 after 3% isoflurane preconditioning and OGD. 3% IS+ pc: Cells transfected with pcDNA.3.1 after 3% isoflurane preconditioning and OGD. $n = 3$, $^{**}P < .01$ versus the 3% IS+ pc group.

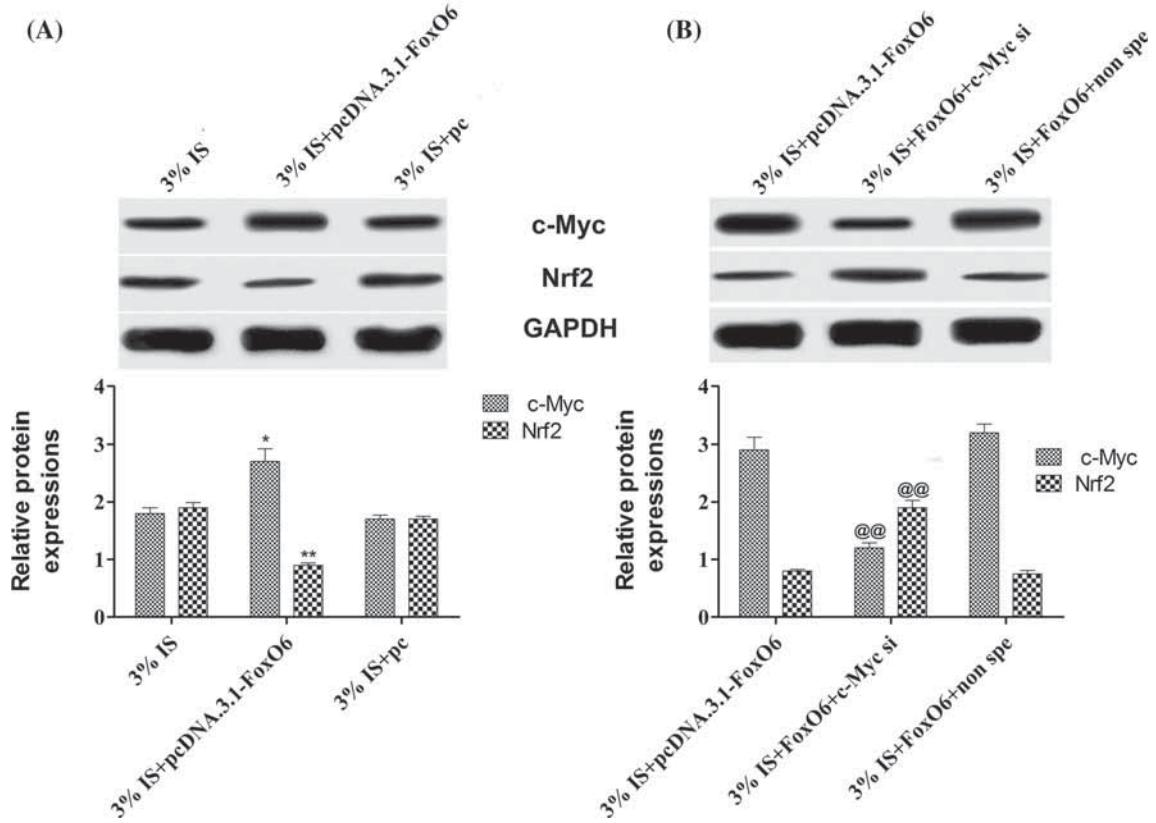

Figure 5. The regulation of c-Myc and Nrf2 by FoxO6 in LO2 cells with isoflurane preconditioning and OGD treatment. (**A**) The protein expressions of c-Myc and Nrf2 after FoxO6 overexpression in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by Western blot. (**B**) The protein expressions of c-Myc and Nrf2 after FoxO6 overexpression and c-Myc inhibition in LO2 with 3% isoflurane preconditioning and OGD treatment were measured by Western blot. 3% IS+ FoxO6+ c-Myc si: cells transfected with c-Myc siRNA after 3% isoflurane preconditioning and FoxO6 overexpression, and then OGD treatment. 3% IS+ FoxO6+non spe: cells transfected with non-specific siRNA after 3% isoflurane preconditioning and FoxO6 overexpression, and then OGD treatment. $n = 3$, $^{*}P < .05$, $^{**}P < .01$ versus the 3% IS+ pc group. $^{@@}P < .01$ versus the 3% IS+ FoxO6+non spe group.

stress in LO2 cells remains poorly defined. Our data showed that FoxO6 decreased in LO2 cells that were preconditioned with 3% isoflurane and exposed to OGD. FoxO6 overexpression effectively reversed the effects of 3% isoflurane preconditioning on cell viability, caspase-3 activity, and ROS levels in LO2 cells exposed to OGD. Thus, FoxO6 is likely involved in the protective effect of isoflurane preconditioning in these cells.

FoxO6 regulates expression of c-Myc in gastric carcinoma (Qinyu *et al.* 2013), and c-Myc mediates Nrf2 expression in fibroblasts (Kansanen *et al.* 2013). Liu *et al.* revealed that c-Myc promotes oxidative stress injury in diabetic cardiomyopathy caused by streptozotocin (Liu *et al.* 2017). Nrf2 is a key regulator of cellular oxidative stress, effectively reducing cellular ROS (Cui *et al.* 2018; Yusuf *et al.* 2018). Nrf2 upregulation reportedly blocks oxidative stress and prevents liver injury induced by carbon tetrachloride (Ramos-Tovar *et al.* 2018). We speculated that FoxO6 regulates c-Myc and Nrf2, which influences the protective effect of isoflurane preconditioning in OGD-induced injury. In this study, FoxO6 upregulation significantly elevated c-Myc expression and suppressed Nrf2 in LO2 cells that were preconditioned with isoflurane and exposed to OGD. Moreover, c-Myc inhibition by c-Myc siRNA markedly abolished the inhibitory effect of FoxO6 overexpression on Nrf2 expression in these cells. FoxO6 also regulated Nrf2 expression via c-Myc. Isoflurane preconditioning thus protected the cells from OGD injury by regulating FoxO6, c-Myc, and Nrf2 signaling.

In sum, we found that 3% isoflurane preconditioning visibly restrained FoxO6, caspase-3 activity, and ROS levels and enhanced cell viability in LO2 cells that were exposed to OGD. Overexpression of FoxO6 effectively reversed the effects of 3% isoflurane preconditioning on caspase-3 activity, ROS production, and cell viability. Meanwhile, the data indicate that FoxO6 regulated Nrf2 expression via c-Myc. Thus, the protective function of isoflurane preconditioning in LO2 cells exposed to OGD may be modulated by FoxO6, c-Myc, and Nrf2 signaling.

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