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ORIGINAL RESEARCH

Remimazolam Suppresses Oxidative Stress and Apoptosis in Cerebral Ischemia/Reperfusion Injury by Regulating AKT/GSK-3β/NRF2 Pathway

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Introduction: The mechanism of remimazolam, a benzodiazepine that activates γ-aminobutyric acid a (GABAa) receptors, in cerebral ischemia/reperfusion (I/R) injury is not well understood. Therefore, we explored whether remimazolam activates protein kinase B (AKT)/glycogen synthase kinase-3β (GSK-3β)/nuclear factor erythroid 2-related factor 2 (NRF2) to attenuate brain I/R injury in transcerebral I/R-injured rats and transoxygenic glucose deprivation/reperfusion (OGD/R)-injured SY5Y cells.

Material and Methods: Remimazolam was added at the beginning of cell and rat reperfusion, and the PI3K/AKT inhibitor LY294002 was added to inhibit the AKT/GSK-3β/NRF2 pathway 24 h before cellular OGD/R treatment and 30 min before rat brain I/R treatment. The viability and apoptosis rate of SY5Y cells, neurological deficit score, cerebral infarction volume and morphological changes of rat brain cells as well as the protein expression of Bax, Bcl2, Caspase 3, Cleaved-Caspase 3 and the number of TdT-mediated dUTP Nick-End Labeling (TUNEL)-positive cells in the penumbral region were detected. Reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), NRF2, heme oxygenase 1 (HO-1), AKT, P-AKT, GSK-3β, P-GSK-3β protein expression, and nuclear translocation of NRF2 were measured in cell and animal assays.

Results: Reduced SY5Y cell viability and increased apoptosis caused by OGD/R injury, elevated neurological deficit scores and cerebral infarct volume induced by brain I/R injury in rats, cerebral cell injury, as well as elevated Bax, Cleaved-Caspase 3, decreased Bcl2, and increased number of TUNEL-positive cells in rat brain tissue were all moderated by remimazolam. Decreased GSH-Px, SOD and Elevated MDA, ROS induced by OGD/R-injured SY5Y cells and brain I/R-injured rats were moderated by remimazolam. Meanwhile, remimazolam increased NRF2, HO-1, P-AKT, P-GSK-3β, and the nuclear accumulation of NRF2. The PI3K/AKT inhibitor LY294002 reversed the role of remimazolam in brain I/R injury.

Conclusion: This study demonstrates that remimazolam activates the AKT/GSK-3β/NRF2 pathway, thereby attenuating oxidative stress and apoptosis to protect against brain I/R injury.

Keywords: remimazolam, AKT/GSK-3β/NRF2 signaling pathway, ischemia-reperfusion injury, apoptosis, oxidative stress

Introduction

Stroke is the leading cause of disability and death worldwide[1] and most are caused by ischemia of brain tissue due to vascular obstruction.[2] The optimal treatment to avoid the severity of ischemic stroke is that the removal of vascular obstruction allows reperfusion of ischemic brain tissue.[3] However, reperfusion exacerbates the neurological damage and neurological dysfunction caused by ischemia in the perfused area, bringing about cerebral ischemia-reperfusion (I/R) injury.[4] Among the many complex mechanisms affecting cerebral ischemia-reperfusion injury, the important pathophysiological pathways are oxidative stress and apoptosis.[5] Therefore, exploring the regulatory mechanisms of oxidative stress and apoptosis may provide a mechanistic basis for mitigating cerebral I/R injury.

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After brain I/R, excess reactive oxygen species (ROS) triggers oxidative stress, resulting in apoptosis.[6] Important proteins involved in the apoptotic process are Bax, Bcl2 and Cleaved-Caspase 3.[7,8] Nuclear factor erythroid 2-related factor 2 (NRF2) regulates the endogenous antioxidant system.[9] Carboxypropyl acetate increased NRF2 expression and caused a decrease in ROS and malondialdehyde (MDA) levels, thereby attenuating cerebral I/R injury.[10] Pretreatment with the natural phytoestrogen Biochanin A promotes NRF2 nuclear translocation, leading to an increase in the endogenous antioxidants glutathione peroxidase (GSH-Px), heme oxygenase 1 (HO-1) and superoxide dismutase (SOD), and a decrease in the level of MDA, which attenuates cerebral I/R injury.[11] Protein kinase B (AKT) and glycogen synthase kinase-3β (GSK-3β) are both serine/threonine protein kinases that are widely expressed in the CNS.[12,13] AKT down-regulates GSK-3β through Ser9 phosphorylation, which has been reported to reduce neuronal apoptosis and promote neoplastic cell proliferation.[14] GSK-3β negatively regulates NRF2 and is involved in the nuclear or extra-nuclear NRF2 distribution.[15] Natural lignans Lyoniresinol activates the AKT/GSK-3β/NRF2 pathway to reduce oxidative stress and shows beneficial effects on cerebral ischemia-reperfusion injury.[16]

Remimazolam is a benzodiazepine that activates the γ-aminobutyric acid a (GABAa) receptor, a compound with a structure similar to imipramine.[17,18] It has been shown that midazolam activates the NRF2 pathway, thereby attenuating liver tissue injury and oxidative stress induced by CCL4-treated mice.[19] The GABAa receptor antagonist, bromantanine (BIC), inhibits topiramate (TPM)-induced neuroprotection by modulating the AKT/GSK3 pathway and increasing oxidative stress, neuronal apoptosis and neurodegeneration.[20] Recent studies have shown that remimazolam is effective in improving neurological dysfunction, reducing infarct volume, and attenuating cortical neuronal damage after cerebral I/R injury.[21]

Therefore, we investigated the neuroprotective effects of remimazolam against oxidative stress and apoptosis in cerebral I/R injury and the role of AKT/GSK-3β/NRF2 signaling pathway in remimazolam-induced neuroprotection.

Materials and Methods

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Animal

Rats (Sprague-Dawley, Male, 230 g-260 g) purchased from Jinan Pengyue Experimental Animal Breeding Co. in China. The Ethics Committee of Medicine Faculty of Qingdao University approved the rat experiment (Approval No. AHQU-MAL20211105). All animal experiments were conducted in strict compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Qingdao University Animal Experimentation Guidelines. The rats were divided into 5 rats/cage and placed in a room with a constant temperature of 24°C and 12/12 h day/night conditions for one week of adaptive rearing.

Cerebral I/R Injury Model of the Rat (I/R)

Isoflurane was used to anesthetize rats (Dose: 5% induction and 1.5-2% maintenance anesthesia). Silicone-coated sutures (Beijing Genetimes Technology, Inc, China) were inserted from the right external carotid artery of the rat, passed through the right internal carotid artery, and terminated at the beginning of the right middle cerebral artery. The depth of suture insertion was 18.5±0.5 mm. The suture was removed after 2 h and then perfused for another 24 h.

Lateral Ventricular Injection

A brain stereotaxic localizer (Beijing ZS DICHUANG Technology Limited, China) was used to localize the right ventricle of the rat (Coordinates: 1.5 mm on the right side, 0.8 mm behind the cerebellar angle, 4.0 mm from the cranial surface). A 1 mm burr hole was drilled in the skull at the localization coordinates, and LY294002 (HY-10108, MCE, USA) dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mmol/L or DMSO was injected into the right ventricle using a microsyringe (injection volume of 10 μL, rate of 2 μL/min). After the injection was completed for 5 min, the needle was withdrawn and the burr hole was closed.

Grouping of Animal Experiments

Part I

(1) Sham group: Other operations were identical to the I/R treatment except that no silicone-coated sutures were inserted into the rat brain vessels.
(2) RE group: rats were treated identically to the Sham group and 16 mg/kg of remimazolam was injected intraperitoneally two hours postoperatively.
(3) I/R group: rats were I/R treated.
(4) I/R+RE group: rats were I/R treated and 16 mg/kg of remimazolam was injected intraperitoneally immediately at the beginning of reperfusion.

Part II

Rats were divided into the Sham group, the I/R group, the I/R+RE group, the I/R+RE+LY group and the I/R+LY group, and LY stands for LY294002. Rats in the first three groups were treated in the same way as the corresponding groups in Part I, except that DMSO (10 μL/each rat) was injected via the lateral ventricle 30 min before surgery. Two inhibitor groups of rats were injected with LY294002 (50 mmol/L, 10 μL/each rat) via the lateral ventricle 30 min before surgery. Meanwhile, the I/R+RE+LY group were injected with 16 mg/kg of remimazolam via intraperitoneal injection immediately at the beginning of reperfusion.

Neurological Deficit Score

Neurologic deficits were assessed rats in each group after 24 h reperfusion using the Longa 5-point scale.[22]

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

After removing the rat brain and placing it in the refrigerator at -20°C for 30 min, it was made into five 2 mm-thick coronal slices. 2% 2,3,5-triphenyltetrazolium chloride (S19026, Yuanye, China) solution was used to soak the brain sections at 37°C for 30 min, and turned over every 10 min, avoiding light throughout. After stained tissues were photographed, ischemic (white) and normal (red) areas were measured by ImageJ software (National Institutes of Health) with the formula: percentage of infarct volume = total volume of cerebral infarction/total volume of brain tissue × 100%.

Hematoxylin-Eosin (HE) Staining

Rat brains were fixed using 4% paraformaldehyde. The brains were then dehydrated, embedded in paraffin, and made into 4 μm coronal slices, sectioned in striatum, and sectioned using hematoxylin-eosin staining (C0105S, Beyotime, China). Finally, the cellular morphology of the sectioned tissue was observed by a light microscope.

Cell Culture

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, D6501, Solarbio Life Sciences, China) containing 1% penicillin-streptomycin (P1400, Solarbio Life Sciences, China) and 10% fetal bovine serum (164210, Procell, China) was used to culture the SY5Y cells (Chinese Academy of Sciences).

Oxygen-Glucose Deprivation Reperfusion Model for SY5Y Cells (OGD/R)

Glucose-free Earle's balanced salt solution (EBSS, H2045, Solarbio Life Sciences, China) was used to replace the culture medium. An anoxic seal box was used to contain the cells. The box was placed in a cell incubator for a continuous period of 6 h. Then, the cells were rapidly re-inhaled with oxygen and glucose, and the cells were collected after a further perfusion for 24 h.

Cellular Experiments Grouping

Part I

(1) Control group: SY5Y cells were untreated.
(2) RE group: The medium was spiked with 100 μg/mL of remimazolam.
(3) OGD/R group: SY5Y cells were OGD/R treated.
(4) OGD/R+RE group: SY5Y Cells were treated identically to the OGD/R group and the medium was spiked with 100 μg/mL of remimazolam at the start of reperfusion.

Part II

SY5Y Cells were divided into the Control group, the OGD/R group, the OGD/R+RE group, the OGD/R+RE+LY group, and the OGD/R+LY group, and LY represents LY294002. The first three groups of cells were treated in the same way as the corresponding groups in Part I. For both inhibitor groups, the culture medium was supplemented with 10 μmol/L of LY294002 24 h before OGD/R-treated cells. At the same time, the OGD/R+RE+LY group was supplemented with 100 μg/mL remimazolam at the beginning of reperfusion.

Cell Viability

96-well plates were used to inoculate SY5Y cells. Cells were treated according to grouping, followed by the addition of 10 μL/well of CCK-8 reaction solution (C0038, Beyotime, China). After reacting at 37°C for 2 h under light protection, the absorbance of the different grouped cells at 450 nm was measured using a multifunctional enzyme labeling instrument (TECAN, Switzerland).

Apoptosis Rate

SY5Y cells in six-well plates were processed according to grouping. Cells were collected and centrifuged at 4°C (300 g/min for 5 min). An apoptosis kit (40302ES60, Yeasen, China) was used to detect apoptosis.

Reactive Oxygen Species Levels

SY5Y cells in six-well plates were treated according to different groupings. Tissues from the ischemic penumbra of rats in each treatment group and the corresponding area of the brain in the sham-operated group were cut and digested, filtered through a strainer and centrifuged to obtain the cell precipitates. Reactive oxygen kits (CA1410, Solarbio Life Sciences, China) were used to detect ROS levels.

Levels of Oxidants and Antioxidant Enzymes

Cell and animal samples were assayed in strict accordance with the instructions for the Malondialdehyde (MDA, BC0025, Solarbio Life Sciences, China) Level Measurement Kit, Glutathione Peroxidase (GSH-Px, BC1195, Solarbio Life Sciences, China) Activity Detection Kit and Superoxide Dismutase (SOD, BC0175, Solarbio Life Sciences, China) Activity Detection Kit.

Immunofluorescence

After fixation and embedding of the brain tissue, 4 μm coronal sections of the striatum were made. Coverslips with groups of SY5Y cells were fixed. Sections or coverslips were permeabilized and closed. For TdT-mediated dUTP Nick-End Labeling (TUNEL) and Neuronal Nuclei (NeuN) double-labeling staining, sections were dressed sequentially using NeuN antibody (26975-1-AP, Proteintech, Wuhan, China, 1:50), secondary antibody (E-AB-1145, Elabscience, Wuhan, China, 1:150) and TUNEL Detection Solution (C1090, Beyotime, Shanghai, China). For NRF2 and NeuN double-labeling staining, sections were sequentially incubated with NeuN antibody (26975-1-AP, Proteintech, China, 1:50), secondary antibody (E-AB-1145, Elabscience, China, 1:150), NRF2 antibody (16396-1-AP, Proteintech, China, 1:50), and secondary antibody (E-AB-1146 Elabscience, China, 1:150). While coverslips with groups of SY5Y cells were incubated with NRF2 antibody (16396-1-AP, Proteintech, China, 1:50) and secondary antibody (E-AB-1146, Elabscience, China, 1:150). Finally, cell nuclei were stained using DAPI, and images were obtained by immunofluorescence microscopy.

Western Blot

Tissue of the cerebral ischemic penumbra region or SY5Y cells from each group were collected. Total proteins were extracted by lysing penumbra tissue or SY5Y cells by tissue cell lysate (R0010, Solarbio Life Sciences, China) containing phosphatase inhibitor (PR20015, Proteintech, China) and protease inhibitor (P6730, Solarbio Life Sciences, China). Cytoplasmic and cytosolic proteins from brain tissue or SY5Y cells was isolated by The Cytoplasmic and Cytosolic Protein Extraction Kit (P0028, Beyotime, China). Next, Cytoplasmic and cytosolic protein concentrations or total protein concentration were determined. After sequential operations of electrophoresis, membrane transfer and closure, the PVDF membrane containing protein was obtained. After that, P-GSK-3β (5558T, CST, USA, 1:1000), GSK-3β (12456T, CST, USA, 1:1000), P-AKT (4060T, CST, USA, 1:2000), AKT (9272S, CST, USA, 1:1000), HO-1 (43966S, CST, USA, 1:1000), NRF2 (16396-1-AP, Proteintech, China, 1:5000), Bax (2772T, CST, USA, 1:1000), Bcl2 (ab196495, Abcam, UK, 1:1000), Cleaved-Caspase 3 (9661T, CST, USA, 1:1000), Caspase 3 (19677-1-AP, Proteintech, China, 1:1000), Histone H3 (17168-1-AP, Proteintech, China, 1:2000), β-actin (GB15003-100, Servicebio, China, 1:5000) antibodies were incubated with PVDF membrane. After incubating the membrane with secondary antibody (E-AB-10476, Elabscience, China, 1:1000), enhanced chemiluminescence reagent (ECL) was added dropwise, and a developer (Vilber Lourmat Fx, France) exposed each protein band. Finally, ImageJ software (National Institutes of Health) was used to analyze Protein bands.

Statistical Analysis

The results were calculated by GraphPad Prism 8.0 (GraphPad Software, Chicago, United States) and are reported as mean±standard deviation ($\bar{x} \pm s$). The normality of the data was verified by Normality and Lognormality Tests. Analyses of all experimental data were conducted using one-way ANOVA with Tukey or Dunnett post hoc tests. If $P<0.05$, a statistically significant result was reached.

Results

Effects of Remimazolam on Brain I/R Injury

Twenty-four hours after reperfusion, we assessed the effects of remimazolam on brain I/R injury by performing neurological deficit scores, TTC staining, and HE staining in the experimental rats. The results showed that rats with I/R injury had significantly higher neurologic deficit scores and cerebral infarct volume, and a large number of neuronal cells died (Figure 1A–D). However, rats post-treated with remimazolam had significantly lower neurologic deficit scores, cerebral infarct volume, and injured neuronal cells (Figure 1A–D).

Cell viability of untreated SY5Y cells and OGD/R-treated SY5Y cells placed at different concentrations of remimazolam were assayed. We found that untreated SY5Y cell viability was not affected by different concentrations of remimazolam, however, remimazolam concentration of 100 μg/mL reduced the decrease in SY5Y cell viability caused by OGD/R (Figure 1E). OGD/R induced SY5Y cell damage and cell viability was significantly reduced, while remimazolam attenuated SY5Y cell damage and cell viability was elevated (Figure 1F).

Effects of Remimazolam on Apoptosis in Brain I/R Injury

Apoptosis is a key feature of cerebral I/R injury. The effect of remimazolam on the apoptosis rate of OGD/R-treated SY5Y cells was examined using flow cytometry. We found that OGD/R-injured SY5Y cells with a significantly higher apoptosis rate, but the percentage of apoptosis was significantly reduced after post-treatment of SY5Y cells with remimazolam (Figure 2A and B).

In each group of rats, we examined Cleaved-Caspase 3, Bax and Bcl2. After ischemic stroke in rats, Bcl2 was reduced, Cleaved-Caspase 3 and Bax was elevated, and the Cleaved-Caspase 3/Caspase 3 and Bax/Bcl2 ratios were elevated (Figure 2C–G). However, rats post-treated with remimazolam elevated Bcl2, decreased Cleaved-Caspase 3 and Bax, and decreased Cleaved-Caspase 3/Caspase 3 and Bax/Bcl2 ratios (Figure 2C–G).

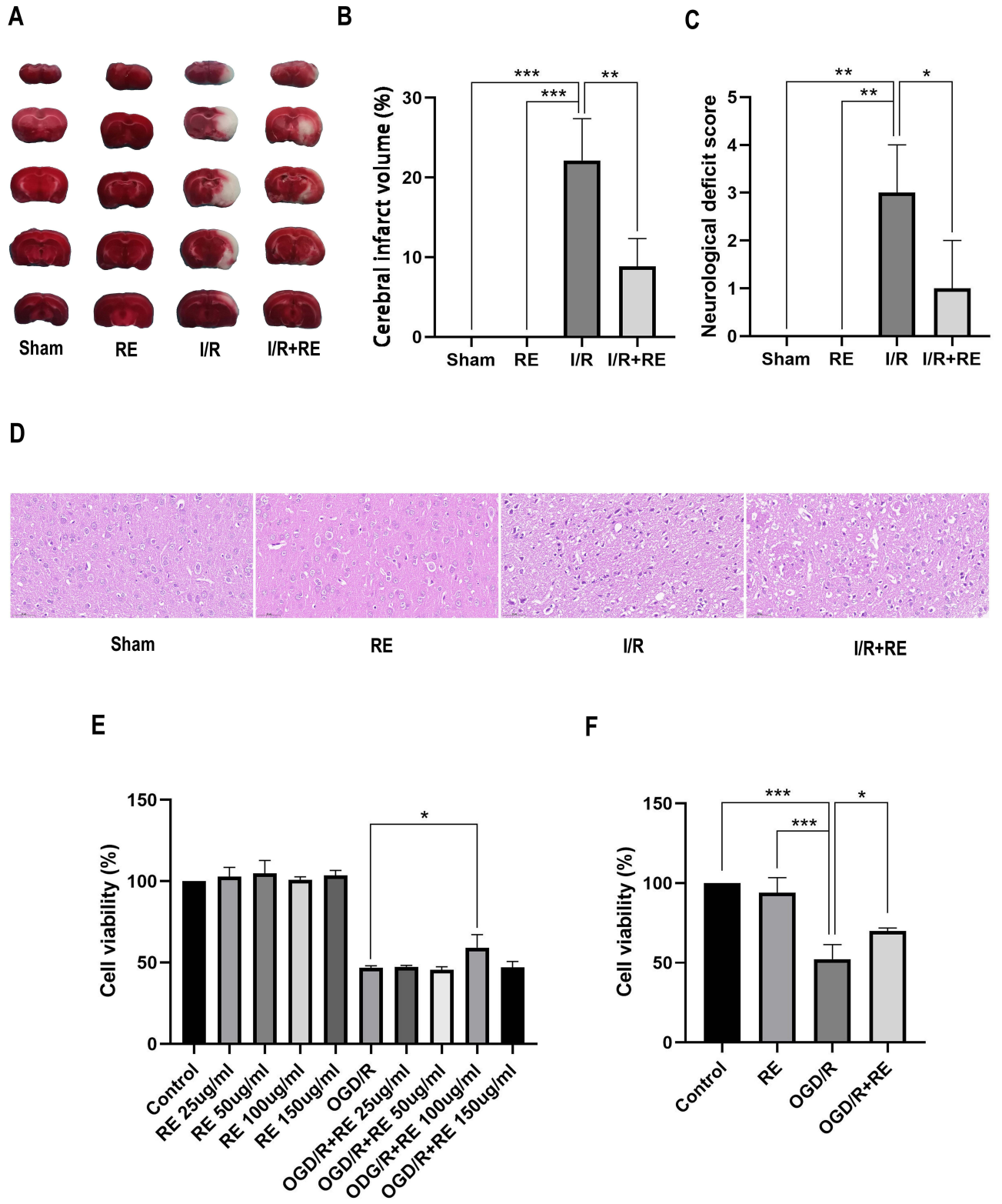

Figure 1 Neuroprotective effects of remimazolam in brain ischemia/reperfusion (I/R) injury. (**A**) 2,3,5-triphenyltetrazolium chloride (TTC) staining of rat brain tissue. (**B**) Infarct volume in rat brain sections. (**C**) Scoring of neurological deficits in rats. (**D**) Hematoxylin-eosin (HE) staining of rat brain coronal sections. n=3. (**E**) SY5Y cell viability in normal and hypoxia-reoxygenation states after post-treatment (0-150 μg/mL) with remimazolam. (**F**) SY5Y cell viability in groups post-treated (100 μg/mL) with remimazolam. n=3. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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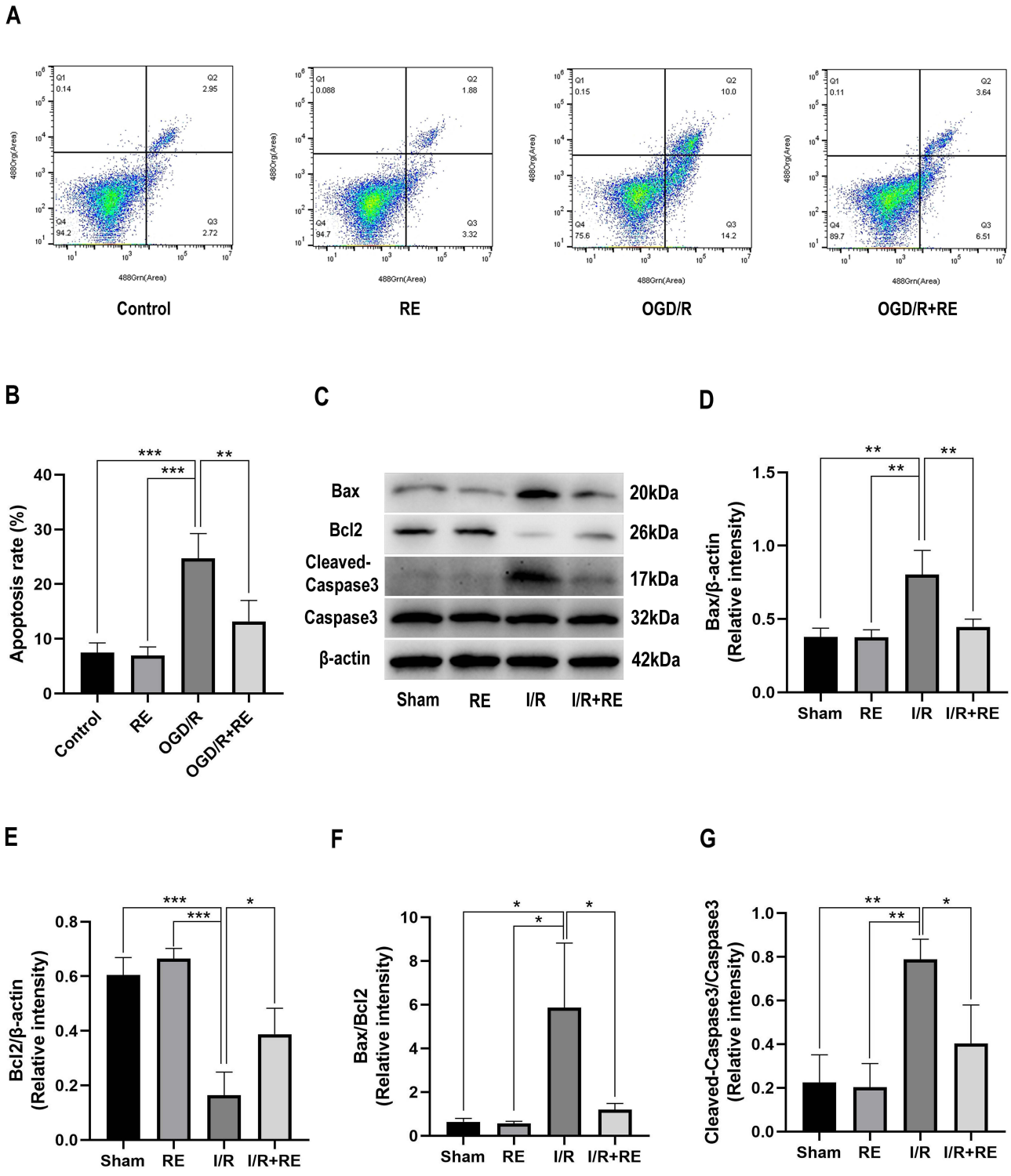

Figure 2 Anti-apoptotic effect of remimazolam in brain ischemia/reperfusion (I/R) injury. (**A**) Flow cytometry apoptosis results of SY5Y cells in each group. (**B**) Cell apoptosis rate. n=3. (**C**) Bcl2, Bax, Cleaved-Caspase 3 and Caspase 3 protein bands in the tissue of the ischemic penumbra region of rat brain in each group. Protein relative expression: (**D**) Bax, (**E**) Bcl2. Protein ratio: (**F**) Bax/Bcl2, (**G**) Cleaved-Caspase 3/Caspase 3. n=3. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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Effects of Remimazolam on Oxidative Stress in Brain I/R Injury

Remimazolam reduced oxidative stress in cerebral I/R injury. We assayed ROS, MDA, SOD, GSH-Px levels to detect the effect of remimazolam on oxidative stress. In OGD/R-treated SY5Y cells and brain I/R-treated rat tissues in the cerebral ischemic penumbra region, MDA and ROS levels were increased, and SOD and GSH-Px levels were reduced (Figure 3A–J). However, remimazolam reduced MDA and ROS, and increased GSH-Px and SOD (Figure 3A–J).

Effects of Remimazolam on the AKT/GSK-3β/NRF2 Pathway

Among the factors that regulate redox homeostasis, NRF2 is among the important ones. Activation of AKT/GSK-3β can regulate HO-1 levels by affecting NRF2 expression. Therefore, we further examined the effects of remimazolam on NRF2, HO-1, P-AKT and P-GSK-3β proteins. Decreased P-AKT, P-GSK-3β and NRF2, and increased HO-1 in OGD/R-treated SY5Y cells and I/R-treated rats (Figure 4A–J). Interestingly, remimazolam significantly upregulated NRF2, HO-1, P-AKT and P-GSK-3β (Figure 4A–J).

PI3K/AKT Inhibitor Reverses Activation of AKT/GSK-3β/NRF2 Pathway by Remimazolam in Brain I/R Injury

Consecutively, to investigate whether AKT/GSK-3β is involved in the neuroprotective effects of remimazolam by modulating the NRF2 pathway, SY5Y cells and SD rats were pretreated with the PI3K/AKT inhibitor LY294002 and protein expression was subsequently assessed. In OGD/R-treated SY5Y cells and I/R-treated rats, remimazolam elevated NRF2, HO-1, P-AKT and P-GSK-3β (Figure 5A–J). However, the inhibitor LY294002 significantly down-regulated NRF2, HO-1, P-AKT and P-GSK-3β (Figure 5A–J).

In OGD/R-treated SY5Y cells and I/R-treated rat brain neuronal cells, cytoplasmic and nuclear protein expression as well as immunofluorescence staining showed that NRF2 was predominantly present in the cytoplasm, but its nuclear accumulation increased significantly after remimazolam treatment (Figure 6A–H). Therefore, in cerebral ischemia-reperfusion injury, remimazolam can activate NRF2 translocation to the nucleus. In contrast, NRF2 nuclear translocation was significantly attenuated after using the inhibitor LY294002 (Figure 6A–H).

PI3K/AKT Inhibitors Reverse the Antioxidant Effects of Remimazolam in Brain I/R Injury

In cellular experiments, the OGD/R+RE+LY and OGD/R+LY groups significantly elevated MDA and ROS, and decreased GSH-Px and SOD compared with the OGD/R+RE group (Figure 7A–E). Similarly, in animal experiments, the I/R+RE+LY and I/R+LY groups significantly increased MDA and ROS, and decreased GSH-Px and SOD compared to the I/R+RE group (Figure 7F–J).

PI3K/AKT Inhibitor Reverses the Anti-Apoptotic Effect of Remimazolam in Brain I/R Injury

In cellular experiments, the apoptosis rate was elevated in the OGD/R+RE+LY group and the OGD/R+LY group compared to the OGD/R+RE group (Figure 8A and B). In animal experiments, the I/R+RE+LY group and the I/R+LY group decreased Bcl2, increased Cleaved-Caspase 3 and Bax, and increased Cleaved-Caspase 3/Caspase 3 and Bax/Bcl2 ratios compared to the I/R+RE group (Figure 8C–G). Similarly, TUNEL staining showed that the number of TUNEL-positive cells was elevated in the I/R-treated rats compared to the Sham group rats, and remimazolam decreased the number of TUNEL-positive cells in the I/R-treated rats. Moreover, the number of TUNEL-positive cells was elevated in rats in the two inhibitor LY294002 groups compared to rats in the I/R+RE group (Figure 8H and I).

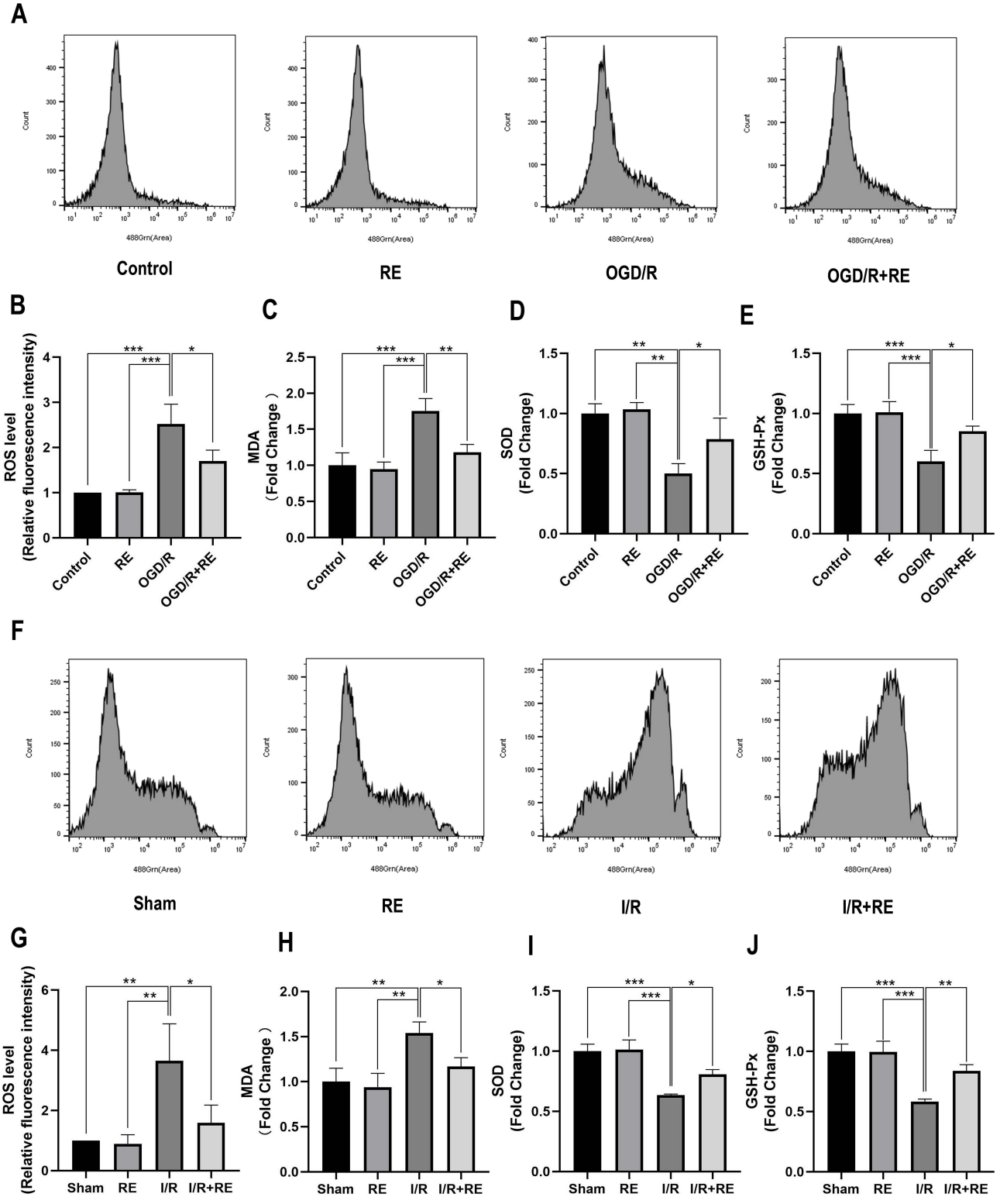

Figure 3 Antioxidant effects of remimazolam in brain ischemia/reperfusion (I/R) injury. (**A**) Flow cytometry detection of reactive oxygen species (ROS) levels in SY5Y cells. (**B**) Relative intensity of ROS fluorescence. Relative contents of malondialdehyde (MDA) (**C**), superoxide dismutase (SOD) (**D**) and glutathione peroxidase (GSH-Px) (**E**) of SY5Y cells in each group. n=3. (**F**) Flow cytometry detection of ROS content in the brain tissues. (**G**) Relative intensity of ROS fluorescence. Relative contents of MDA (**H**), SOD (**I**) and GSH-Px (**J**) of rats. n=3. **P<0.05*, ***P<0.01*, ****P<0.001*.

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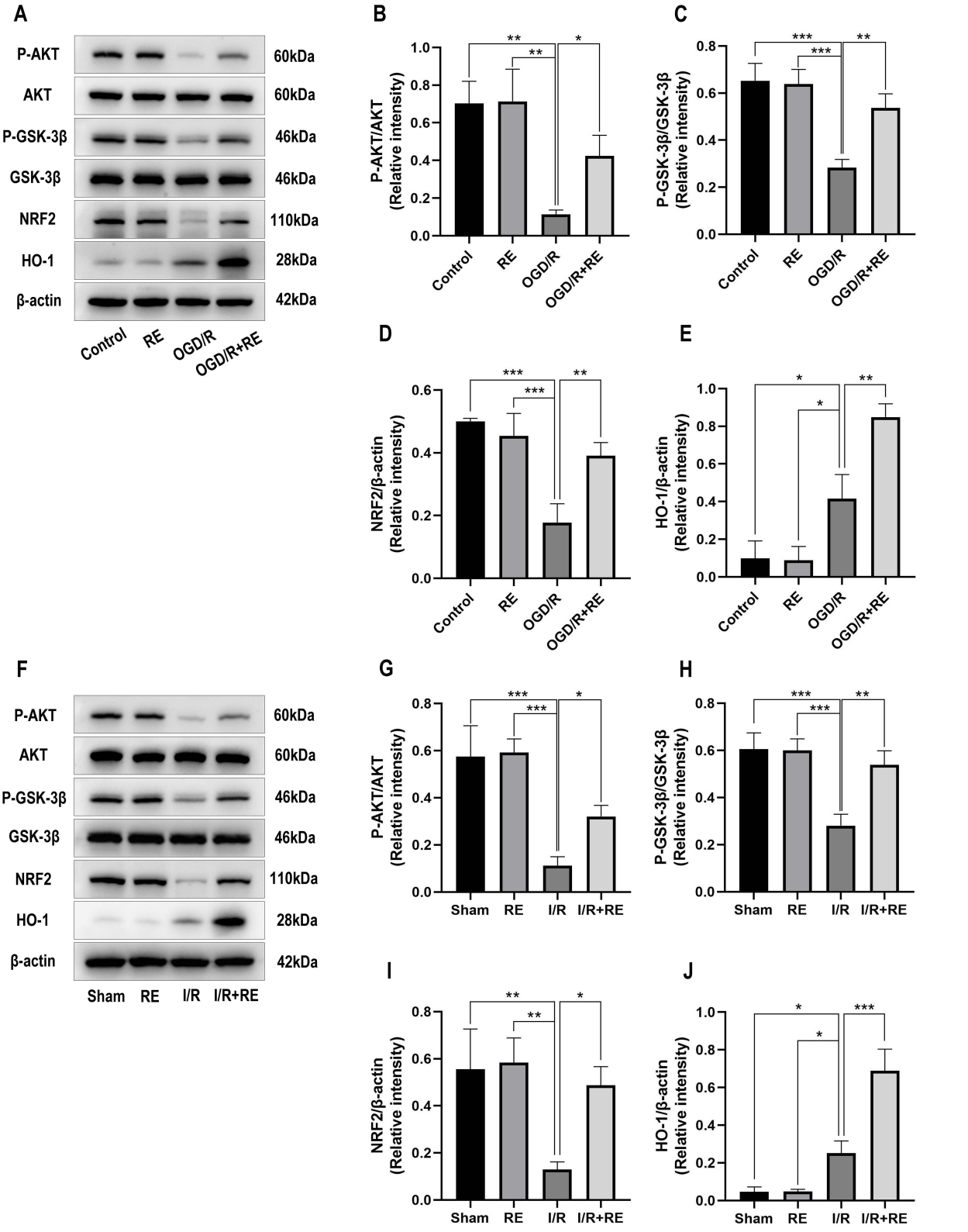

Figure 4 Remimazolam activates the protein kinase B (AKT)/glycogen synthase kinase-3β (GSK-3β)/nuclear factor erythroid 2-related factor 2 (NRF2) pathway in brain ischemia/reperfusion (I/R) injury. (**A**) AKT/GSK-3β/NRF2 pathway protein bands in SY5Y cells of each group. Protein ratio: (**B**) P-AKT/AKT, (**C**) P-GSK-3β/GSK-3β. Protein relative expression: (**D**) NRF2, (**E**) Heme oxygenase 1 (HO-1). n=3. (**F**) AKT/GSK-3β/NRF2 pathway protein bands in tissues of the ischemic penumbra region of the rat brain in each group. Protein ratio: (**G**) P-AKT/AKT, (**H**) P-GSK-3β/GSK-3β. Protein relative expression: (**I**) NRF2, (**J**) HO-1. n=3. **P<0.05*, ***P<0.01*, ****P<0.001*.

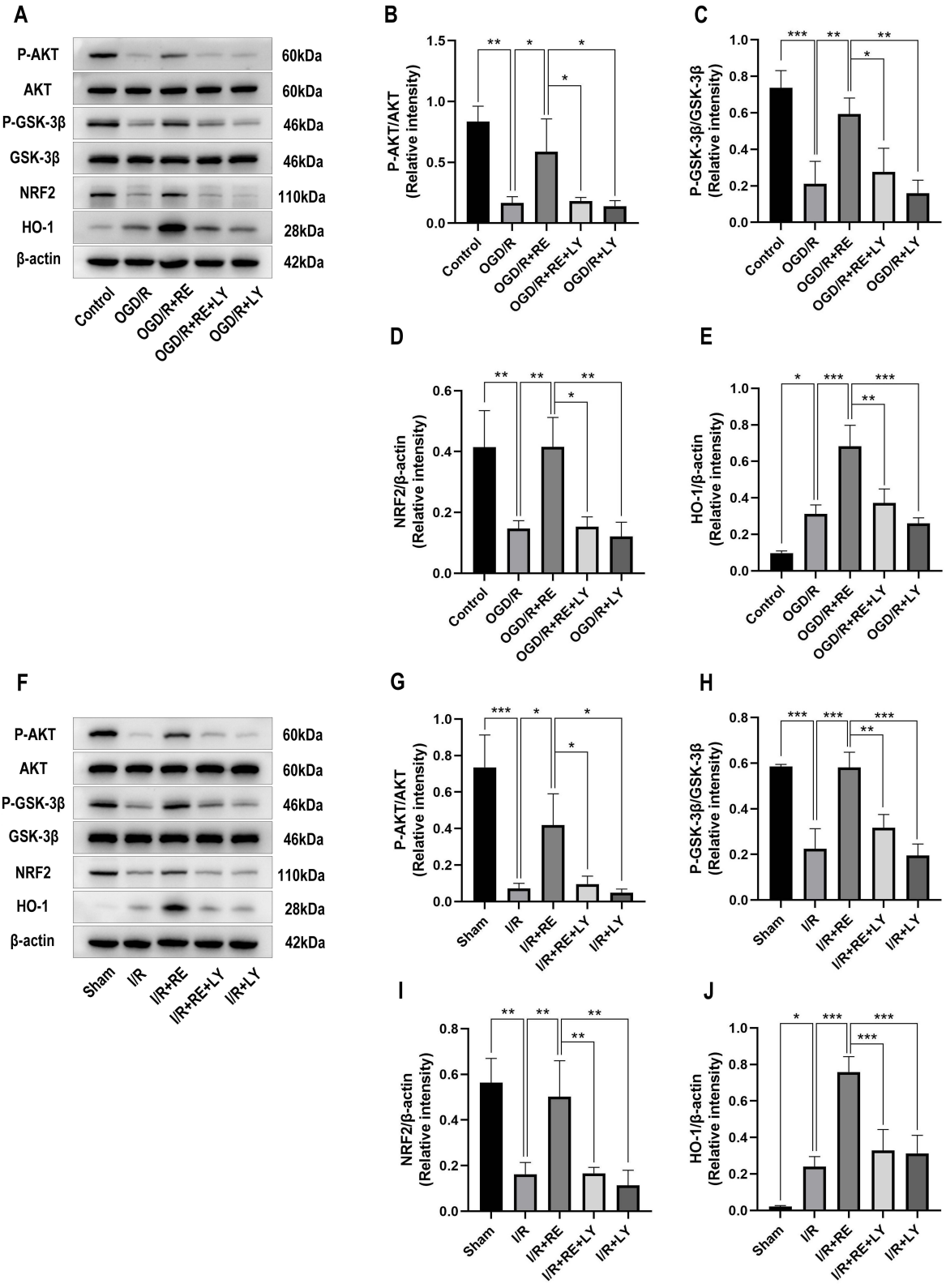

Figure 5 Effect of LY294002 reversal of remimazolam on protein kinase B (AKT)/glycogen synthase kinase-3β (GSK-3β)/nuclear factor erythroid 2-related factor 2 (NRF2) pathway in brain ischemia/reperfusion (I/R) injury. (**A**) AKT/GSK-3β/NRF2 pathway protein bands in SY5Y cells of each group. Protein ratio: (**B**) P-AKT/AKT, (**C**) P-GSK-3β/GSK-3β. Protein relative expression: (**D**) NRF2, (**E**) Heme oxygenase 1 (HO-1). n=3. (**F**) AKT/GSK-3β/NRF2 pathway protein bands in tissues of the ischemic penumbra region of the rat brain in each group. Protein ratio: (**G**) P-AKT/AKT, (**H**) P-GSK-3β/GSK-3β. Protein relative expression: (**I**) NRF2, (**J**) HO-1. n=3. **P<0.05*, ***P<0.01*, ****P < 0.001*.

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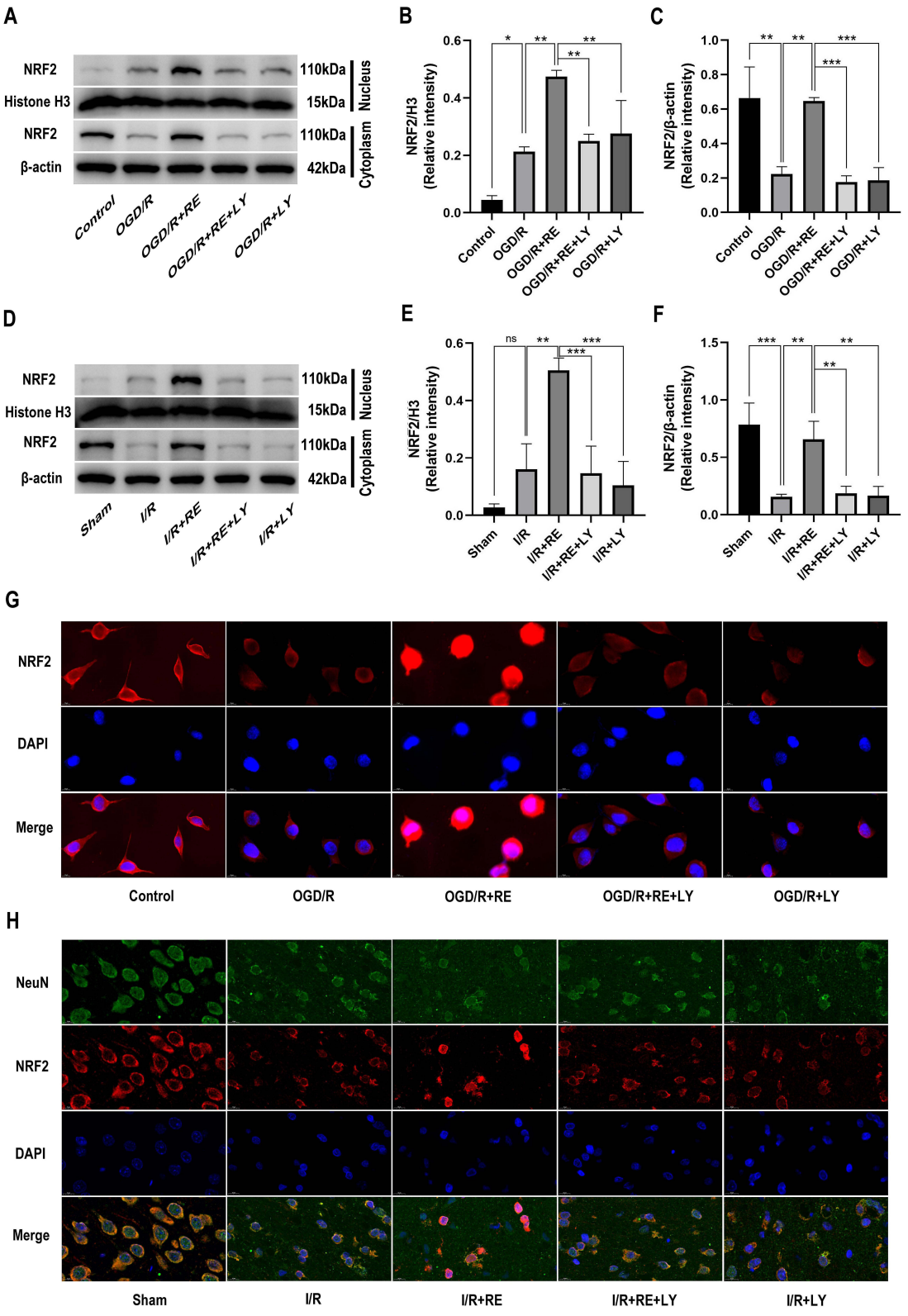

Figure 6 Nuclear factor erythroid 2-related factor 2 (NRF2) nuclear translocation. (**A**) Cytosolic and cytoplasmic NRF2 protein bands in groups of SY5Y cells. Relative expression of NRF2 cytosolic (**B**) and cytoplasmic (**C**) proteins in SY5Y cells of each group. n=3. (**D**) Cytosolic and cytoplasmic NRF2 protein bands of histiocytes in the penumbral region of cerebral ischemia in rats of all groups. Relative expression of nuclear (**E**) and cytoplasmic (**F**) NRF2 proteins in rats of each group. n=3. (**G**) Immunofluorescence results of NRF2 nuclear translocation of SY5Y cells in each group. (**H**) Immunofluorescence results of NRF2 nuclear translocation in rat ischemic cerebral cortical neuronal cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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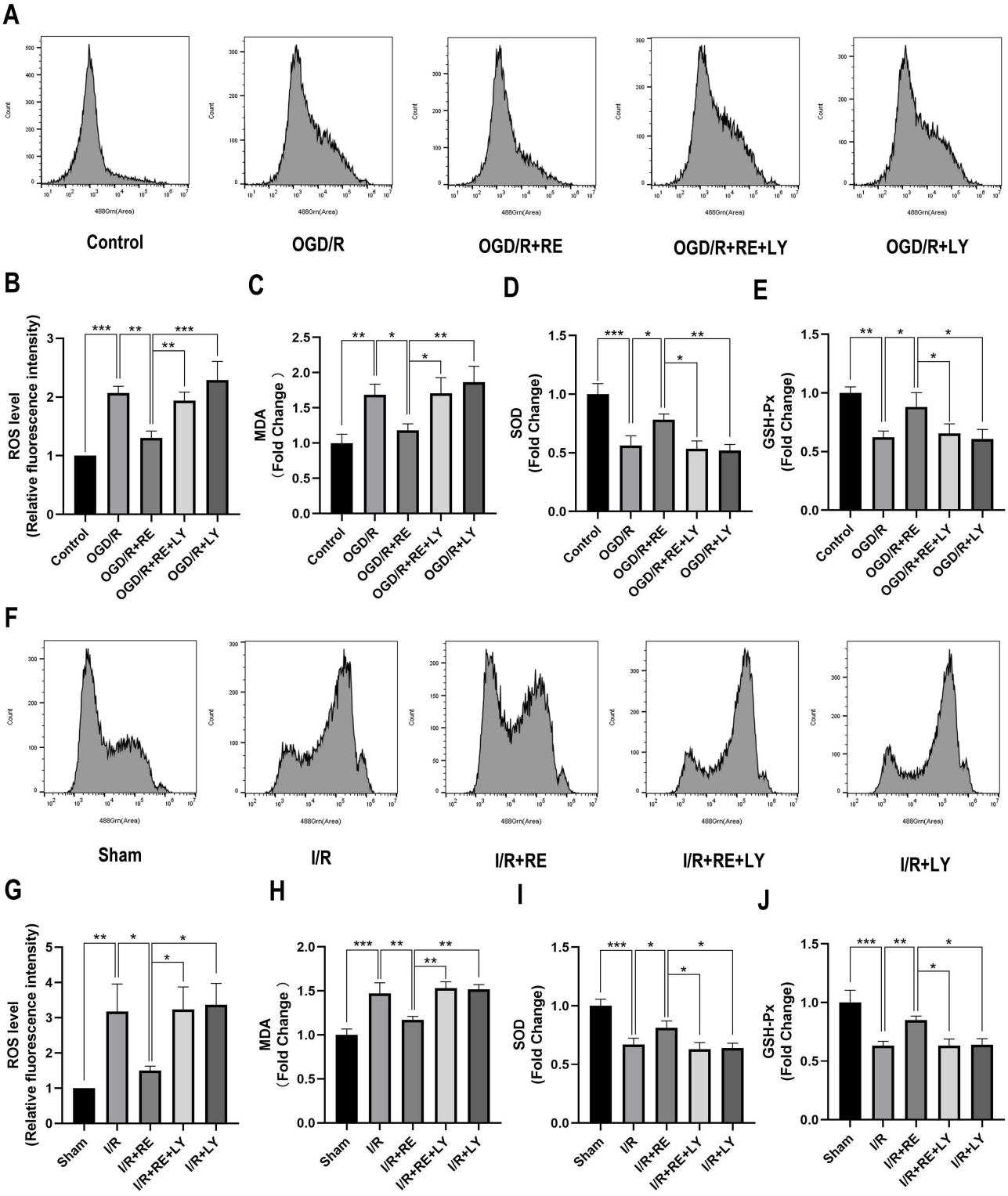

Figure 7 Effect of LY294002 on the antioxidant effect of remimazolam in brain ischemia/reperfusion (I/R) injury. (**A**) Flow cytometry to detect the reactive oxygen species (ROS) content of SY5Y cells in each group. (**B**) Relative intensity of ROS fluorescence. Relative contents of malondialdehyde (MDA) (**C**), superoxide dismutase (SOD) (**D**) and glutathione peroxidase (GSH-Px) (**E**) of SY5Y cells in each group. n=3. (**F**) Flow cytometry detection of ROS content in the brain tissues. (**G**) Relative intensity of ROS fluorescence. Relative contents of MDA (**H**), SOD (**I**) and GSH-Px (**J**) in the cerebral ischemic penumbra region of rats. n=3. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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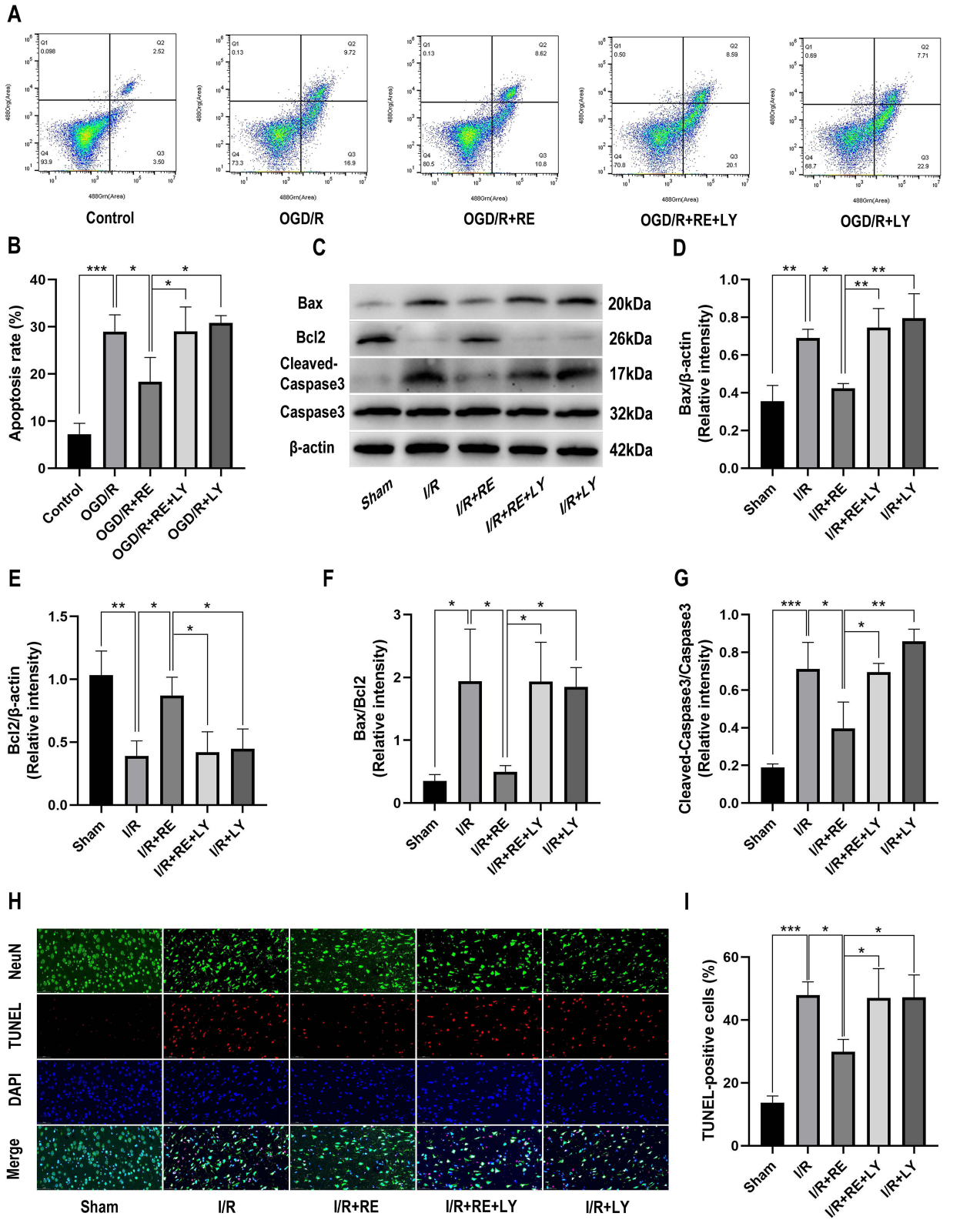

Figure 8 LY294002 reverses the anti-apoptotic effect of remimazolam in brain ischemia/reperfusion (I/R) injury. (**A**) Flow cytometry apoptosis results of SY5Y cells in each group. (**B**) Cell apoptosis rate. n=3. (**C**) Bcl2, Bax, Cleaved-Caspase 3 and Caspase 3 protein bands in the tissue of the ischemic penumbra region of rat brain in each group. Protein relative expression: (**D**) Bax, (**E**) Bcl2. Protein ratio: (**F**) Bax/Bcl2, (**G**) Cleaved-Caspase 3/Caspase 3. (**H**) Results of TdT-mediated dUTP Nick-End Labeling (TUNEL) immunofluorescence staining of neuronal cells in the ischemic cerebral cortex of rats in each group. (**I**) Percentage of TUNEL-positive neuronal cell counts. n=3. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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Discussion

In this study, the neuroprotective effect of remimazolam in brain I/R injury had been demonstrated by cellular and animal experiments. A rat brain I/R injury model simulating ischemic stroke was used to assess the neuroprotective effect of remimazolam. Brain histopathologic damage, infarct volume and neurological deficit score are common indicators to assess brain injury.[23] We observed a reduction in neurological function score, infarct volume and injured neuronal cells in remimazolam-treated rats, suggesting that remimazolam can attenuate stroke-induced brain injury. Meanwhile, the neuroprotective effect of remimazolam was confirmed by a cellular cerebral I/R injury model. Remimazolam post-treatment had no effect on normal SY5Y cells. Post-treatment of SY5Y cells with remimazolam attenuated OGD/R-induced reduction in cell viability.

Apoptosis is considered to be a important mechanism of neuronal death in ischemic stroke,[24] as well as a process of active cell death in which Bcl2, Caspase 3 and Bax genes are involved in key regulation.[25] When Bcl2 is upregulated, Bcl2 binds Bax to generate heterodimers and inhibit apoptosis. When Bax is upregulated, Bax becomes a homodimer and accelerates apoptosis.[26] Therefore, the Bax/Bcl2 ratio is considered an important predictor of apoptosis.[27] In addition, Bax homodimers as apoptosis executors can activate Caspase 3,[28] which stimulates cleavage of Caspase 3 protein to generate activated Cleaved-Caspase 3 and induce apoptosis.[29] Therefore, Cleaved-Caspase 3 can directly reflect apoptosis.[30] The current results showed that cerebral ischemia-reperfusion injury induced an increase in Cleaved-Caspase 3 and Bax, and a decrease in Bcl2, and an increase in Cleaved-Caspase 3/Caspase 3 and Bax/Bcl2 ratios, and an increase in the number of TUNEL-positive cells in rat brain tissues. However, remimazolam effectively decreased apoptosis-associated proteins Bax and Cleaved-Caspase 3, increased Bcl2, decreased Bax/Bcl2 and Cleaved-Caspase 3/Caspase 3 ratios, and decreased the number of TUNEL-positive cells. Meanwhile, remimazolam decreased the number of apoptotic deaths in OGD/R-treated SY5Y. These results suggest that remimazolam attenuates apoptosis in I/R-treated rats and OGD/R-treated SY5Y cells.

Among the many factors that cause cerebral I/R injury, the most relevant one is oxidative stress.[31] In brain I/R injury, as a result of excess ROS production, NRF2 is translocated from the cytoplasm to the nucleus, which triggers the expression of HO-1.[32] The endogenous antioxidant bilirubin, which can protect cells, is produced by HO-1 catalyzing the conversion of the oxidant heme.[33] In addition, excess of reactive oxygen species generates MDA.[34] NRF2 regulates SOD and GSH-Px, thereby counteracting antioxidant production.[35] The levels of GSH-Px, SOD, MDA and ROS have been suggested to be indices of oxidative stress.[36,37] In this study, remimazolam inhibited ROS, MDA production, elevated SOD, GSH-Px, upregulated NRF2, HO-1, and promoted nuclear accumulation of NRF2 in cerebral I/R injury. The results above suggest that the beneficial effects shown by remimazolam on brain I/R injury correlate with the activation of NRF2 resulting in a reduction of oxidative stress.

Oxidative stress and apoptotic responses in brain I/R injury have been reported to involve the AKT/GSK-3β pathway.[38,39] Our results showed that remimazolam increased protein expression of phosphorylated AKT and phosphorylated GSK-3β in cerebral ischemia-reperfusion injury. GSK-3β is a key protein involved in non-Kelch-like oxidized chloropropane-associated protein 1 (Keap1)-dependent degradation of NRF2. GSK-3β activation promotes the degradation of NRF2, and phosphorylated GSK-3β promotes NRF2 expression in the nucleus and antioxidant enzyme production.[40] Stimulated by oxidative stress, NRF2 achieves separation from Keap1 and increases nuclear accumulation, initiating antioxidant enzyme genes transcription, thereby exerting a protective effect.[41] An effective strategy to activate NRF2 was once thought to be inhibition of Keap1.[42] However, direct inhibition of Keap1 was later found to lead to severe cardiovascular events.[43] Therefore, we explored the role of the non-Keap1-dependent NRF2 activation pathway AKT/GSK-3β/NRF2 in remimazolam-induced cerebral ischemic tolerance. We observed that LY294002 pretreatment reversed the remimazolam-induced increase in P-AKT, P-GSK-3β, NRF2 and HO-1, decreased NRF2 nuclear translocation, upregulated MDA and ROS, and downregulated GSH-Px and SOD in brain I/R. The results suggest that antioxidant effects of remimazolam on brain I/R injury are related to the AKT/GSK-3β/NRF2 pathway. Moreover, after brain I/R injury, active GSK-3β aggravated neuronal damage by activating pro-apoptotic Caspase 3.[44,45] Oxidative stress induces apoptosis,[46] and NRF2 inhibits apoptosis by increasing Bcl2 or decreasing activated Caspase 3.[47] Our results showed that LY294002 reversed the remimazolam-induced decrease in Cleaved-Caspase 3/Caspase 3 and Bax/Bcl2 ratios as well as in the number of TUNEL-positive cells in I/R-treated rats and increased apoptotic rate in OGD/

R-injured SY5Y cells. In conclusion, our study suggests that remimazolam attenuates oxidative stress and apoptosis in cerebral I/R injury by activating the AKT/GSK-3β/NRF2 signaling pathway.

There are limitations in this study. The conclusions of our study were obtained from basic in vivo and in vitro experiments; however, whether remimazolam exerts a protective effect on patients with cerebral ischemia-reperfusion injury needs to be verified by clinical trials. In addition, we did not explore the changes in the protective effect of remimazolam on cerebral ischemia-reperfusion injury over time. Therefore, we can further explore the molecular regulatory mechanism of remimazolam on cerebral ischemia-reperfusion injury in the future.

Conclusion

Taken together, remimazolam attenuates oxidative stress and apoptosis in cerebral I/R injury, resulting in neuroprotective effects, and its neuroprotective mechanism may involve the AKT/GSK-3β/NRF2 pathway.

Animal Ethics

The Ethics Committee of Medicine Faculty of Qingdao University approved the rat experiment, and the ethical review form number is AHQU-MAL20211105.

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Disclosure

All authors report that this study does not involve any conflict of interest.

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