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Protective effect of 2-deoxy-D-glucose on the brain tissue in rat cerebral ischemia-reperfusion models by inhibiting caspase-apoptotic pathway

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Summary. We observed the effect of 2-deoxy-D-glucose (2-DG) on the brain tissue in rat cerebral ischemia-reperfusion (I/R) and explored its mechanism. After observing the effect of 2-DG on endoplasmic reticulum stress (ERS), rats were randomly divided into sham-operation group, I/R group and I/R+2-DG group (each group with 60 rats). I/R models were prepared by middle cerebral artery occlusion. In I/R+2-DG group, each rat was given intraperitoneal 2-DG of 100 mg/kg once a day for 7 days before brain ischemia. According to different time points (3 h, 6 h, 12 h, 24 h and 48 h) after I/R, each group was divided into 5 subgroups (each subgroup with 12 rats). Nerve cell apoptosis, and the expressions of mRNA and protein of glucose regulated protein 78 (GRP78), cleaved-caspase-9 and cleaved-caspase-3 were determined with TUNEL, Western blotting and RT-PCR, respectively, in rat cerebral hippocampal CA1 area at each time point. TUNEL-positive cells were significantly less in I/R+2-DG group than in I/R group at each time point (all $P<0.01$). In I/R and I/R+2-DG groups, the expressions of mRNA and protein of GRP78 reached the maximum 12 h after I/R, and cleaved-caspase-9 and cleaved-caspase-3 reached the maximum 24 h after I/R. Compared with sham-operation group, the expressions of mRNA and protein of GRP78, cleaved-caspase-9 and cleaved-caspase-3 were all significantly increased (all $P<0.01$) in I/R and I/R+2-DG groups. However, the expressions of mRNA and protein of GRP78 were significantly higher in I/R+2-DG group than in I/R group (all $P<0.05$), but the expressions of mRNA and protein of cleaved-caspase-9 and cleaved-caspase-3 were all significantly lower in I/R+2-DG group than in I/R group (all $P<0.05$). We conclude that 2-DG has a neuroprotective effect on the brain tissue in rat cerebral ischemia-reperfusion models. The mechanism may be that 2-DG starts ERS followed by up-regulation of mRNA and protein of GRP78 and down-regulation of mRNA and protein of cleaved-caspase-9 and cleaved-caspase-3, which blocks the apoptotic pathway.

Key words: 2-deoxy-D-glucose, Endoplasmic reticulum stress, Cerebral ischemia-reperfusion injury, Glucose regulated protein 78, Cleaved-caspase-9, Cleaved-caspase-3

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Introduction

Ischemia-reperfusion (I/R) injury, a complex pathophysiological process, is associated with many factors. It is caused by stress reaction induced by blood supply insufficiency. After restoring blood flow, neurological damage may be more severe than that before restoring blood flow, and it is irreversible. I/R injury may occur in many organs, such as brain, heart, liver, kidney, etc. Cerebral I/R injury can lead to brain dysfunction and/or neuronal death (Ashafaq et al., 2012). It is reported that cerebral I/R can induce endoplasmic reticulum stress (ERS) which is closely related to nerve

cell apoptosis (Paschen et al., 2003; DeGracia and Montie, 2004; Shen et al., 2004). I/R can induce ischemic hypoxia, energy depletion, loss of nutrient substance, accumulation of free radicals and Ca^{2+}-functional disorder. These may lead to the disorder of physiological function of endoplasmic reticulum, triggering ERS (Shen et al., 2004; Nakka et al., 2010; Raghubir et al., 2011). Mild ERS shows a protective effect on cells by up-regulation of glucose regulated protein 78 (GRP78) (Lehotsky et al., 2009), because GRP78 can trigger unfolded protein response (UPR) and can strengthen Ca^{2+} regulation and correction for protein mis-folding or non-folding (Bertolotti et al., 2000). However, excessively long ERS may irritate endoplasmic reticulum apoptosis-signal pathways and induce nerve cell apoptosis, aggravating cerebral I/R injury (Rasheva and Domingos, 2009; Hetz, 2012).

2-deoxy-D-glucose (2-DG), an inhibitor of Glucose-6-phosphokinase, can inhibit the generation of induced enzyme. It is used as a bacteriostatic agent because it can readily enter cells, and is not easily decomposed (Hasegawa et al., 2001). It also is a natural antibiotic because it has physiological and pharmacological effects (Azema et al., 2004). Animal experiments have displayed that 2-DG can inhibit epileptic seizures by up-regulation of GRP78 without any adverse reactions (Guo and Matison, 2000; Garriga-Canut et al., 2006). It can quickly enter the brain and is accumulated in a relatively stable form of 6-phosphate-2-DG which can inhibit intracellular glucose oxidation (Rejdak et al., 2001). At the same time, 6-phosphate-2-DG is difficult to be metabolized, so it can persistently inhibit glucose oxidation and interfere with protein glycosylation (Yu and Mattson, 1999). Glycosylation is a key step for synthesis and secretion of proteins within the endoplasmic reticulum, and abnormal glycosylation may lead to accumulation of protein mis-folding or non-folding in the endoplasmic reticulum (Yu and Mattson, 1999; Kang and Hwang, 2006). The purpose of the study was to explore the neuroprotective effect of 2-DG on the brain tissue in rat cerebral I/R injury models and its mechanism, providing a basis for finding new drugs treating ischemic cerebrovascular disease.

Materials and methods

All study methods were approved by ethics committee of the First Affiliated Hospital, Liaoning Medical University.

Animals and reagents

Two hundred SD rats weighing between 280 g and 320 g were provided by Liaoning Changsheng Biotechnology Co. Ltd [Jinzhou, China, License number: SCXX (Liao) 2003-0007]. 2-DG (batch number: 154176, purity: 99.0%) was purchased from Zhiyuan Chemical Reagent Co., Ltd (Tianjin, China). Polyclonal antibodies of GRP78, immunohistochemical kits and color reagent kits were purchased from Bioss Company (Beijing, China). Antibodies of cleaved-caspase-9 and cleaved-caspase-3 were purchased from Cell Signaling Technology (Manchester, Indiana, USA). NeuN antibody was purchased from Abcam Company (Massachusetts, USA). 2,3,5-triphenyl tetrazolium chloride was purchased from Sigma (San Francisco, California, USA). RT-PCR reverse transcription kits, Trizol, sepharose and peptide synthase were purchased from Tkara Biotechnology Co., Ltd (Dalian, China). TUNEL kit was purchased from Beyotime Biotechnology Co. Ltd (Shanghai, China). FITC-labeled sheep-anti rat IgG was purchased from Jinqiao Biological Technology Co., Ltd (Beijing, China).

Grouping

First, 20 rats were randomly divided into sham-operation group, 2-DG group, I/R group and I/R+2-DG group. In I/R+2-DG group, each rat was given intraperitoneal 2-DG of 100 mg/kg (2-DG was diluted to 50 mg/ml with double distilled water) once a day for 7 days before I/R. In 2-DG group, rates did not undergo I/R, but administration of 2-DG was the same as that in I/R+2-DG group. In sham-operation and I/R groups, each rat was given intraperitoneal double distilled water instead of 2-DG once a day for 7 days before I/R. The effect of 2-DG on ERS was observed by cresyl violet staining, TUNEL and Western blotting 24h after I/R (preliminary experiment). Secondly, 180 rats were randomly divided into sham-operation group, I/R group and I/R+2-DG group (each group with 60 rats). Administration was the same as that in preliminary experiment. In all rats, brain ischemia lasted 30 min, and then blood supply was restored. In each group, rats were respectively killed at 3 h, 6 h, 12 h, 24 h and 48 h after I/R,

I/R model preparation

I/R models were prepared by the right middle cerebral artery occlusion (Longa et al., 1989). In sham-operation group, the thread was immediately pulled out after it was inserted into the right middle cerebral artery, and other procedures were the same as that in I/R and I/R+2-DG groups. Successful models were that rats had Horner's sign and marked hemiplegia of the left limbs with 1-3 scores of neurological behavior (Longa et al., 1989).

Preliminary experiment

Cresyl violet staining: Rat brain tissue was fixed with 4% of paraformaldehyde 24 h after I/R, and then was subjected to paraffin embedding and slicing. The sections were stained with 1% of cresyl violet for 20-30 minutes. After dehydration, transparency and mounting, the number of normal neurons was counted under an optical microscope. After cresyl violet staining, neuronic

nissl bodies were lyons blue.

The procedures of TUNEL and Western blotting are described below.

Nerve cell apoptosis detected with TUNEL/NeuN method

Rats were fixed after they were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mg/kg). A cut was made on the neck to expose the right common carotid artery. The right common carotid artery was filled with 4% of paraformaldehyde at 4°C after douche with 50 ml of physiological saline. After decapitation, the brain tissue at the coronal view and 6-11 mm away from the olfactory bulb was collected. The brain tissue was fixed with 4% of paraformaldehyde for 24 h followed by gradient dehydration, transparency and paraffin embedding. The tissue was sectioned at a thickness of 5 μm. Nerve cells were first labeled with NeuN immunohistochemisty using the following steps: Sections were dewaxed, incubated with DNase-free proteinase K for 15-30 min followed by addition of 0.3% TritonX-100 and 3% calf serum albumin at room temperature for one hour. Monoclonal antibody of NeuN (abcam, 1:100) was added at 4°C overnight followed by addition of FITC-labeled sheep-anti rat IgG (1:200) in the dark for one hour. PBS was used as negative control instead of primary antibody. According to the instructions of TUNEL apoptosis kit, TUNEL reaction liquid (red fluorescence) was added at 37°C in the dark for one hour followed by nuclear counterstaining with Hoechst33258. After washing with PBS, sections were subjected to fluorescence quenching. In each group, the numbers of both TUNEL and NeuN positive cells were counted in 5 non-overlapping high power fields.

Protein expressions of GRP78, cleaved-caspase-9 and cleaved-caspase-3 in rat cerebral hippocampal CA1 area detected with immunohistochemistry

Sections underwent SABC procedures according to the instructions. PBS was used as negative control instead of primary antibody. One slice from rat cerebral hippocampal CA1 area was selected from each rat, and then 5 non-overlapping high power fields (×400) were observed under a light microscope. The number of positive cells was counted.

Protein expressions of GRP78, cleaved-caspase-9 and cleaved-caspase-3 in rat cerebral hippocampal CA1 area detected with Western blotting

Cell lysis solution was added into the sample, and then the sample was centrifuged at 4°C for one hour at 17000 r/min. The supernatant fluid was collected and the sediment was removed. The sample was placed in boiling water for 5 min, underwent electrophoresis, and then was transferred on PVDF membrane. After sealing with TTBS buffer solution containing 5 % of dried milk for 3 h, primary antibodies of GRP78, cleaved-caspase-9 and cleaved-caspase-3 were added at 4°C overnight. After rinsing, horseradish peroxidase-labeled goat anti rabbit IgG was added at room temperature for one hour. After rinsing, ECL reaction was performed. The integrated density value of the target band was determined with image analysis system, and then was compared with that of β-actin.

mRNA expressions of GRP, caspase-9 and caspase-3 in rat cerebral hippocampal CA1 area detected with RT-PCR

Rat cerebral hippocampal tissue and liquid nitrogen were ground up, total RNA was extracted with Trizol reagent. The purity and concentration of total RNA were determined using nucleic acid protein quantitative analyzer. The RNA with OD260/OD280 values of 1.8-2.0 was used for this experiment. Reverse transcription was performed according to the instructions of kit. Primers of GRP79, caspase-9, caspase-3 and β–actin were designed based on GenBank using Primer 5.0 software (Table 1) and synthesized by Tkara Biotechnology Co., Ltd (Dalian, China). PCR was performed in a volume of 20 μl using 1 μl of reverse transcription product as templates. PCR conditions were as follows: 94°C for 2 min, 94°C for 30 s, 55°C for 45 s, 72°C for 30 s, 30 cycles; 72°C for 10 min. PCR products underwent 1.5 % gel electrophoresis followed by image scanning and analysis using Gene Tools software. Rat housekeeping gene β-actin was used as an internal reference. The relative values of GRP78, caspase-9 and caspase-3 mRNA expression were calculated according to the formula: gray value f target gene/ gray value of β-actin.

Table 1. mRNA primer sequences of GRP78, caspase-9 and caspase-3.

	Upstream primers	Downstream primers	Pre-amplified fragments
GRP78	5′-GAAGACAAAGGGACAGGAAACAA-3′	5′-AGCAAACTTCTCGGCGTCA-3′	108bp
caspase-9	5′-CTGAGCCAGATGCTGTCCCATA- 3′	5′-GACACCATCCAAGGTCTCGATGTA-3′	176bp
caspase-3	5′-GAGACAGACAGTGGAACTGACGATG-3′	5′-GGCGCAAAGTGACTGGATGA-3′	147bp
β–actin	5′-CCTAAGGCCAACCGTGAA-3′	5′- AGCCAGGGCAGTAATCTC-3′	630bp

Note: GRP78: glucose regulated protein 78.

Statistical analysis

Statistical treatment was performed with SPSS17.0 software. Data were expressed as mean standard deviation ($\bar{\chi}\pm s$). One-factor analysis of variance was used in the data with homogeneity of variance and rank sum test was used in the data with heterogeneity of variance. Statistical significance was established at $P<0.05$.

Results

Results of the preliminary experiment

Cresyl violet staining indicated that cell arrangement was regular, cytoplasm was lilaceous, nuclei were large and round, and nucleoli were clear in sham-operation and 2-DG groups. In I/R group, the number of normal neurons decreased and some cells were triangular with small size and color depth. Compared with I/R group, the number of normal neurons increased in I/R+2-DG group (Fig. 1A,B). TUNEL staining displayed that a few apoptotic cells (pink cells) were seen in sham-operation and 2-DG groups; lots of apoptotic cells were present in I/R group; and the number of apoptotic cells decreased in I/R+2-DG group as compared to I/R group (Fig. 1C,D). Western blot showed that although GRP78, a key factor in ERS, significantly increased in both 2-DG group and I/R group as compared to sham-operation group, it was significantly lower than that in I/R+2-DG

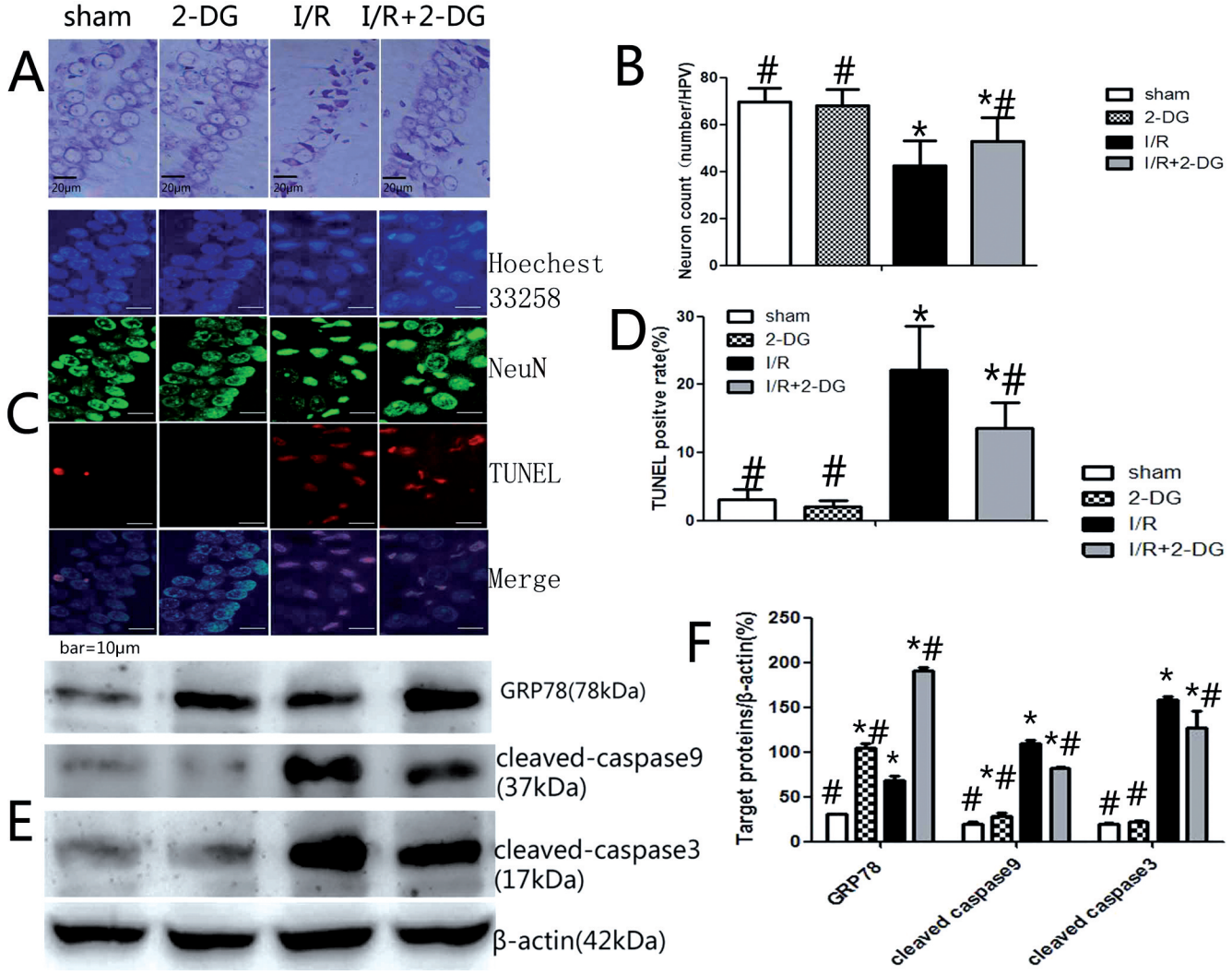

Fig. 1. Effects of 2-DG on ERS in preliminary experiment. **A.** Cresyl violet staining. **B.** Histogram of normal neurons. **C.** Rates of TUNEL-positive cells. **D.** Histogram of TUNEL-positive cells. **E.** Western blotting bands. **F.** histogram of GRP78, cleaved-caspase-9 and cleaved-caspase-3 protein expression. Notes: * indicates $P<0.01$ as compared to sham-operation group. # indicates $P<0.01$ as compared to I/R group. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose. Scale bars: A, 20 μm; C, 10 μm.

group (Fig. 1E,F). These results suggested that both 2-DG and I/R all could induce ERS, but ERS was more marked after the combination of both 2-DG and I/R (in I/R+2-DG). After I/R, the apoptosis rate and expressions of cleaved-caspase-9 and cleaved-caspase-3 significantly increased; but after treatment of 2-DG, the apoptosis rate and expression of cleaved-caspase-9 significantly decreased.

Nerve cell apoptosis detected by TUNEL/NeuN staining in each group at each time point

NeuN staining allowed nerve cell nuclei to be green but TUNEL-positive cell nuclei were red (Fig. 2). The number of TUNEL-positive cells was least in sham-operation group. Compared with sham-operation group, the number of TUNEL-positive cells significantly increased in I/R and I/R+2-DG groups (all P<0.01). In I/R group, the number of TUNEL-positive cells was least 3 h after I/R, sharply increased 6 h after I/R, reached the maximum 24 h after I/R and began decreasing 48 h after I/R. The number of TUNEL-positive cells was significantly lower in I/R+2-DG group than in I/R group at each time point (all P<0.01) (Fig. 2).

Protein expressions of GRP78, cleaved-caspase-9 and cleaved-caspase-3 in rat cerebral hippocampal CA1 area detected with immunohistochemistry

GRP78-positive cells were round or oval with brown cytoplasm (Fig. 3). There were GRP78-positive cells in sham-operation group at each time point. Compared with sham-operation group, the rate of GRP78-positive cells significantly increased in I/R and I/R+2-DG groups at

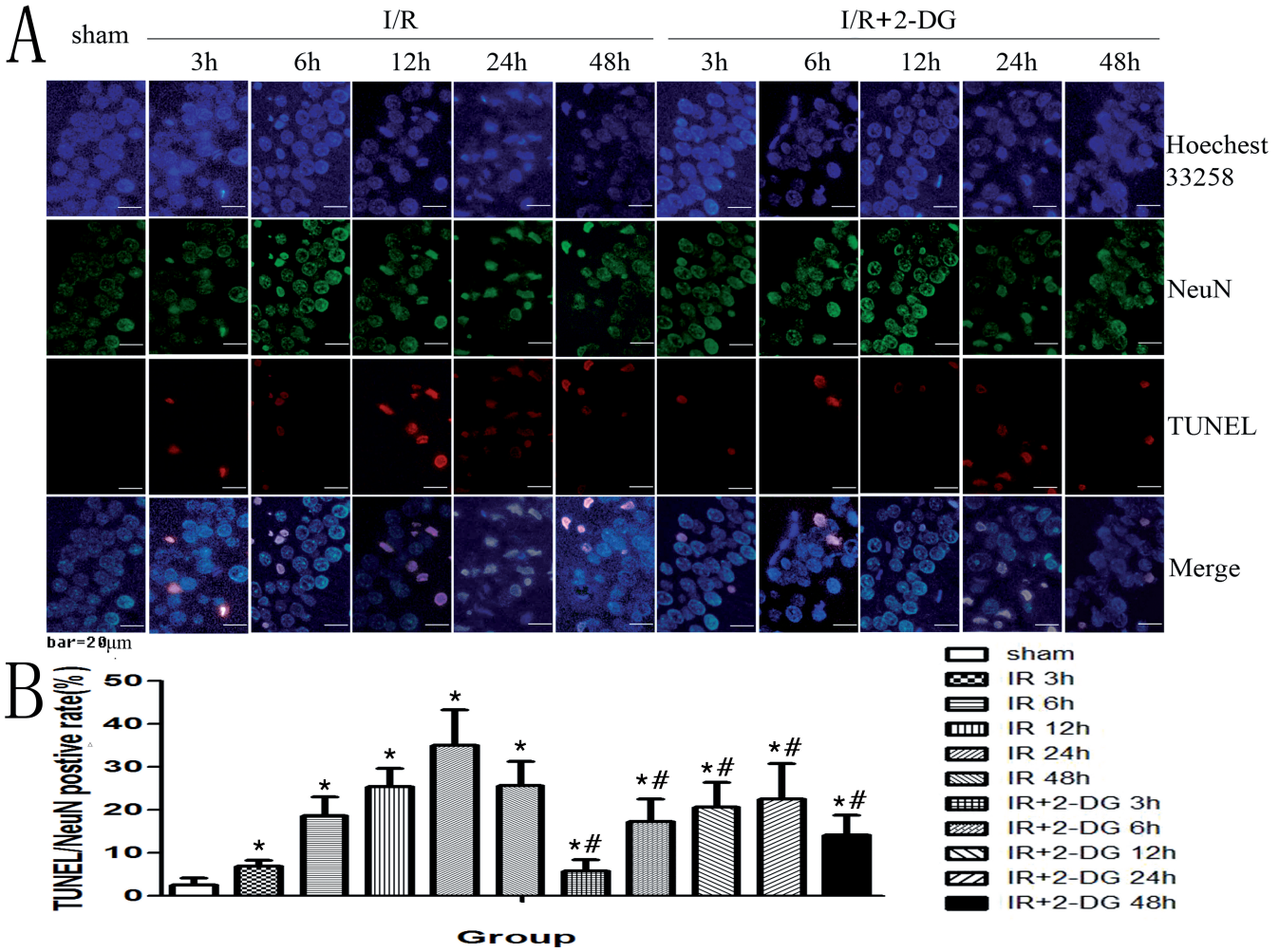

Fig. 2. Rates of TUNEL-positive cells in rat cerebral hippocampal CA1 area in each group at different time points (%). **A.** TUNEL staining imaging. **B.** Histogram of TUNEL-positive cells. Notes: * indicates P<0.01 as compared with sham-operation group. # indicates P<0.01 as compared with I/R group at the same time point. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose. Scale bars: 20 μm.

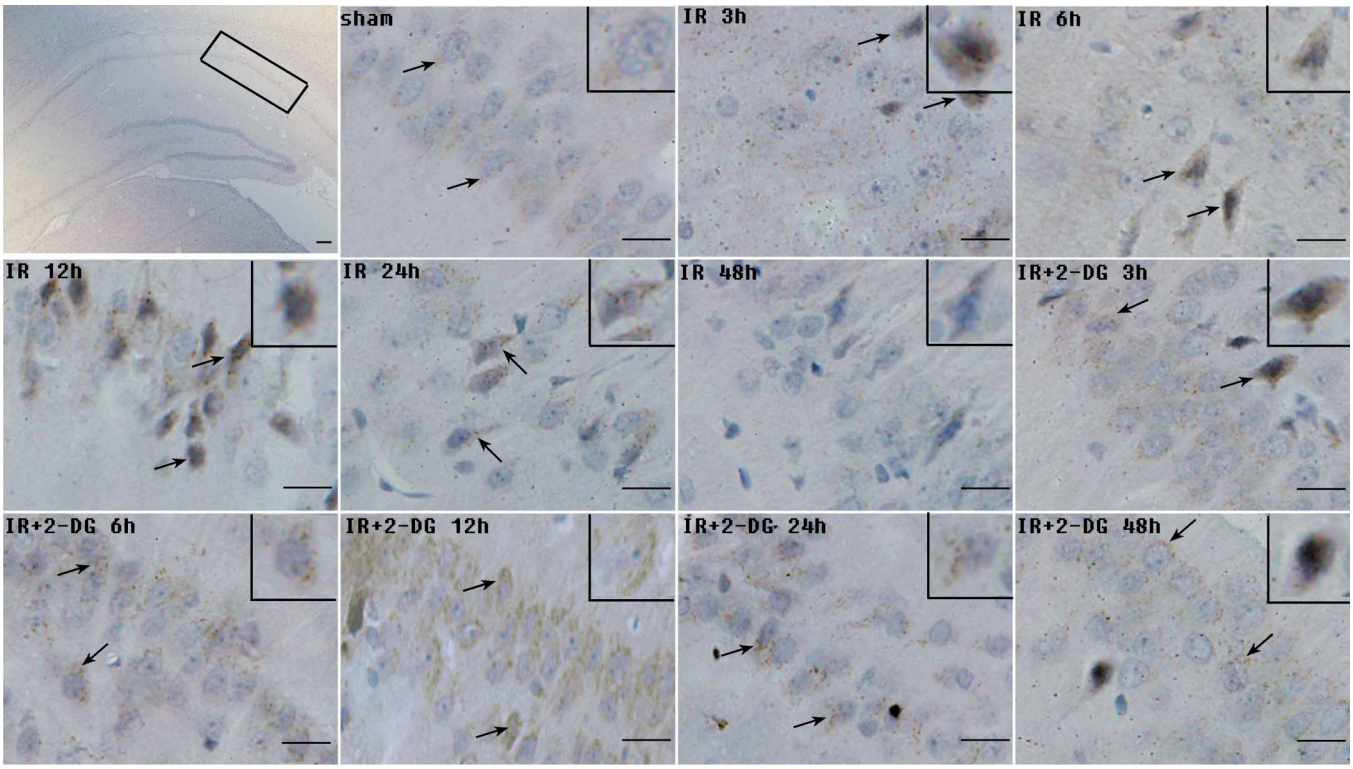

Fig. 3. Protein expression of GRP78 detected with immunohistochemistry in rat cerebral hippocampal CA1 area in each group at different time points. Notes: Arrows indicate GRP78-positive cells. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose. Scale bars: 20 μm.

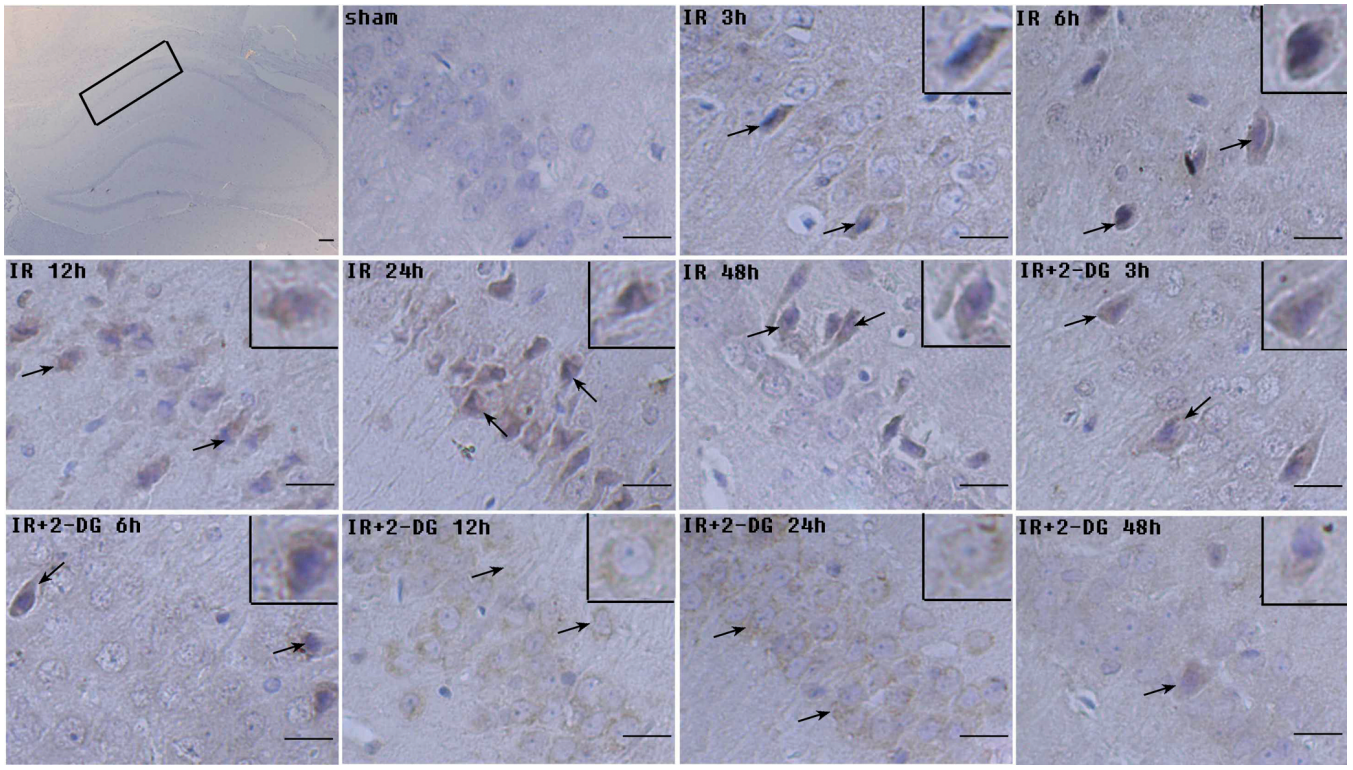

Fig. 4. Protein expression of cleaved-caspase-9 detected with immunohistochemistry in rat cerebral hippocampal CA1 area in each group at different time points. Notes: Arrows indicate cleaved-caspase-9 positive cells. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose. Scale bars: 20 μm.

each time point (all P<0.01), and reached the maximum 12 h after I/R. Compared to I/R group, the rate of GRP78-positive cells significantly increased in I/R+2-DG group at each time point (all P<0.05). Cleaved-caspase-9 and cleaved-caspase-3 positive cells became small due to shrinkage with brown nuclei (Figs. 4, 5). The rates of cleaved-caspase-9 and cleaved-caspase-3 positive cells were least in sham-operation group at each time point. Compared with sham-operation group, the rates of cleaved-caspase-9 and cleaved-caspase-3

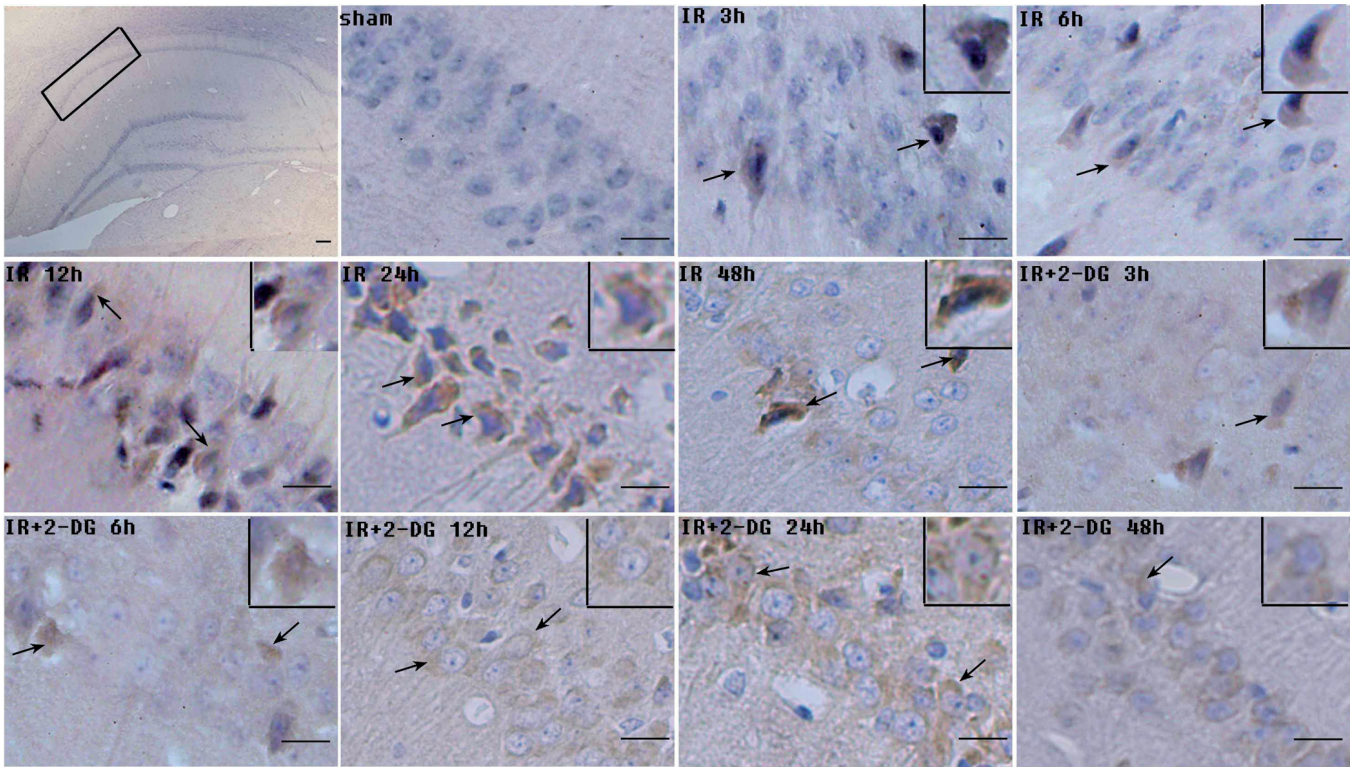

Fig. 5. Protein expression of cleaved-caspase-3 detected with immunohistochemistry in rat cerebral hippocampal CA1 area in each group at different time points. Notes: Arrows indicate cleaved-caspase-3 positive cells. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose. Scale bars: 20 μm.

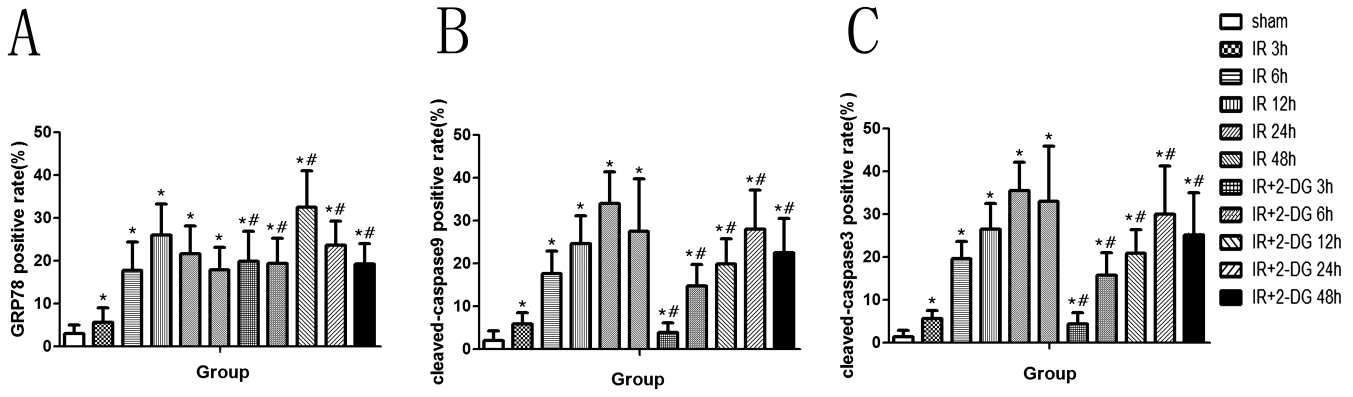

Fig. 6. Histogram of protein expression of GRP78, cleaved-caspase-9 and cleaved-caspase-3 detected with immunohistochemistry in rat cerebral hippocampal CA1 area in each group at different time points. **A.** GRP78 protein expression. **B.** Cleaved-caspase-9 protein expression. **C.** Cleaved-caspase-3 protein expression. Notes: * indicates P<0.01 as compared with sham-operation group. # indicates P<0.01 as compared with I/R group at the same time point. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose.

positive cells significantly increased in I/R and I/R+2-DG groups (all P<0.01), and reached the maximum 24 h after I/R. Compared to I/R group, the rates of cleaved-caspase-9 and cleaved-caspase-3 positive cells significantly decreased in I/R+2-DG group at each time point (all P<0.05) (Fig. 6).

Protein expressions of GRP, cleaved-caspase-3 and cleaved-caspase-9 in rat cerebral hippocampal CA1 area detected with Western blotting

The protein expressions of GRP78, cleaved-caspase-9 and cleaved-caspase-3 were least in sham-operation group at each time point. Compared with sham-operation group, the protein expressions of GRP78, cleaved-caspase-9 and cleaved-caspase-3 significantly increased in I/R and I/R+2-DG groups (all P<0.01). The protein expression of GRP78 reached the maximum 12 h after I/R and the protein expressions of cleaved-caspase-9 and cleaved-caspase-3 reached the maximum 24 h after I/R. Compared to I/R group, the protein expression of GRP78 significantly increased at each time point (all P<0.05), and the protein expressions of cleaved-caspase-9 and cleaved-caspase-3 significantly decreased in I/R+2-DG group at each time point (all P<0.05) (Fig. 7).

mRNA expressions of GRP78, caspase-9 and caspase-3 in rat cerebral hippocampal CA1 area detected with RT-PCR

The mRNA expressions of GRP78, caspase-9 and caspase-3 were least in sham-operation group at each time point. Compared with sham-operation group, the mRNA expressions of GRP78, caspase-9 and caspase-3 significantly increased in I/R and I/R+2-DG groups (all P<0.01). The mRNA expression of GRP78 reached the maximum 12 h after I/R and the mRNA expressions of caspase-9 and caspase-3 reached the maximum 24 h after I/R in I/R and I/R+2-DG groups. Compared to I/R group, the mRNA expression of GRP78 significantly increased (all P<0.05), but the mRNA expressions of caspase-9 and caspase-3 significantly decreased in

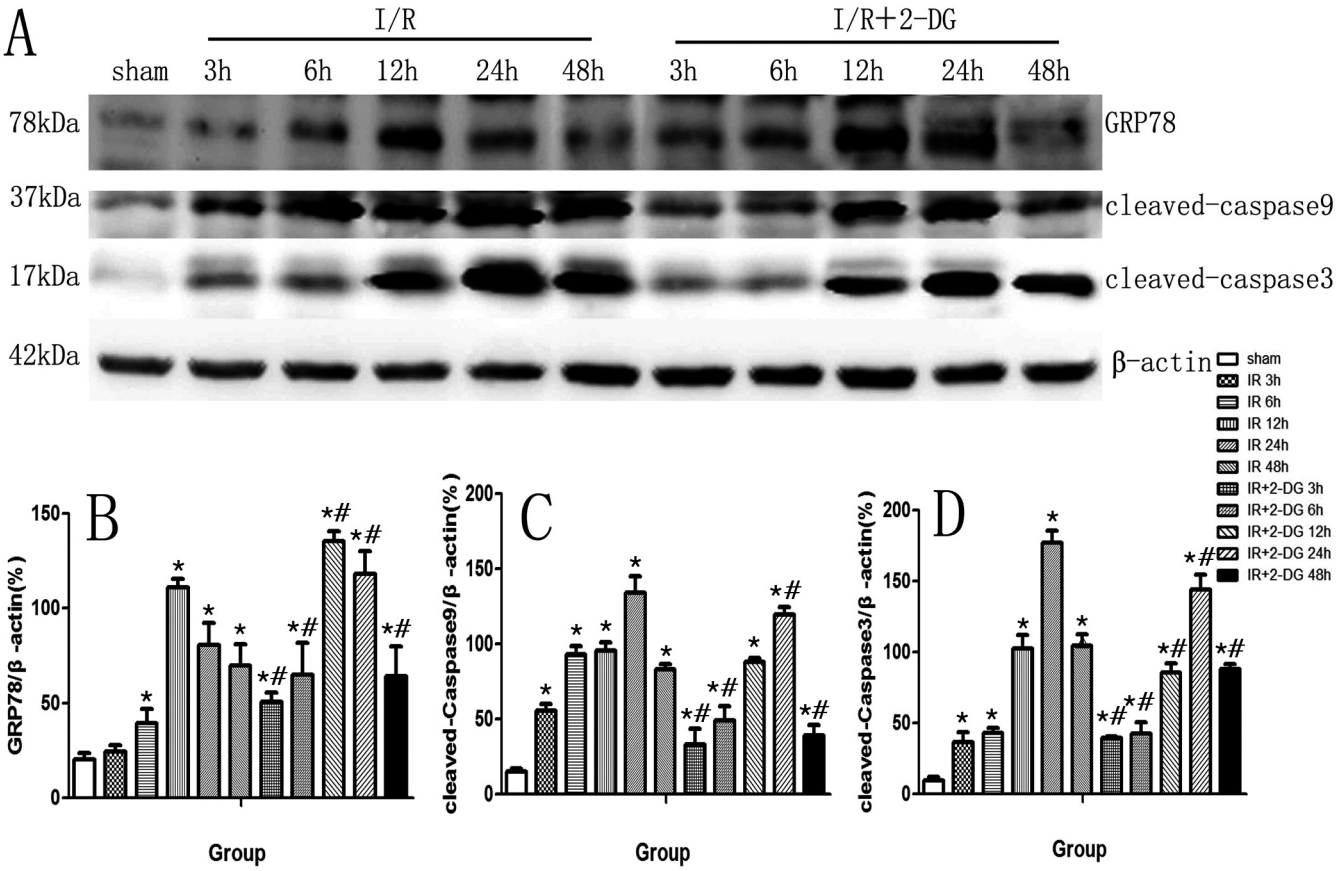

Fig. 7. Protein expression of GRP78, cleaved-caspase-9 and cleaved-caspase-3 detected with Western blotting in rat cerebral hippocampus in each group at different time points. **A.** Western blotting bands. **B.** Histogram of GRP78 protein expression. **C.** Histogram of cleved-caspase-9 protein expression. **D.** Histogram of cleved-caspase-3 protein expression. Notes: * indicates P<0.01 as compared with sham-operation group. # indicates P<0.01 as compared with I/R group at the same time point. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose.

I/R+2-DG group at each time point (all P<0.05) (Fig. 8).

Discussion

The hippocampus, a main part of the limbic system, plays an important role in higher nervous activities such as learning and memory, and has high sensitivity to ischemia (Pulsinelli et al., 1982). Pyramidal neurons in the hippocampal CA1 are one of the most sensitive areas to ischemia in the brain (Mitani et al., 1992). Based on these reasons above, we used hippocampal tissue in this study. In the preliminary experiment, the cresyl violet staining indicated marked nerve cell damage and TUNEL staining displayed severe apoptosis; and although GRP78 level increased, levels of cleaved-caspase-9 and cleaved-caspase-3 were also high in I/R group. Based on these results, we infer that I/R may trigger the activation of cleaved-caspase-9 and cleaved-caspase-3 through other pathways. However, for I/R rats, 2-DG could relieve brain injury, inhibit cell apoptosis, increase GRP78 level and decrease the levels of cleaved-caspase-9 and cleaved-caspase-3. Based on these results, we infer that 2-DG may induce ERS through activating GRP78 and inhibiting cleaved-caspase-9 and cleaved-caspase-3.

Based on the results of preliminary experiment, we set different time points to observe dynamic changes in protein level. The study indicated that sham-operation had less expression of GRP78 which may be required by normal physiological function of the endoplasmic reticulum. In I/R group, with the time extension after I/R, the expression level of GRP78 gradually increased, reached the maximum at 12 h after I/R, and then gradually decreased. This demonstrated that the ischemia, hypoxia and energy depletion triggered ERS after I/R. Early ERS shows protective effects on cells by up-regulation of GRP78 which restores the internal environment homeostasis of endoplasmic reticulum (Hu et al., 2001; Shen et al., 2004; Nakka et al., 2010; Raghubir et al., 2011). However, with the time extension after I/R, the endoplasmic reticulum injury was too severe to up-regulate GRP78. Therefore, the

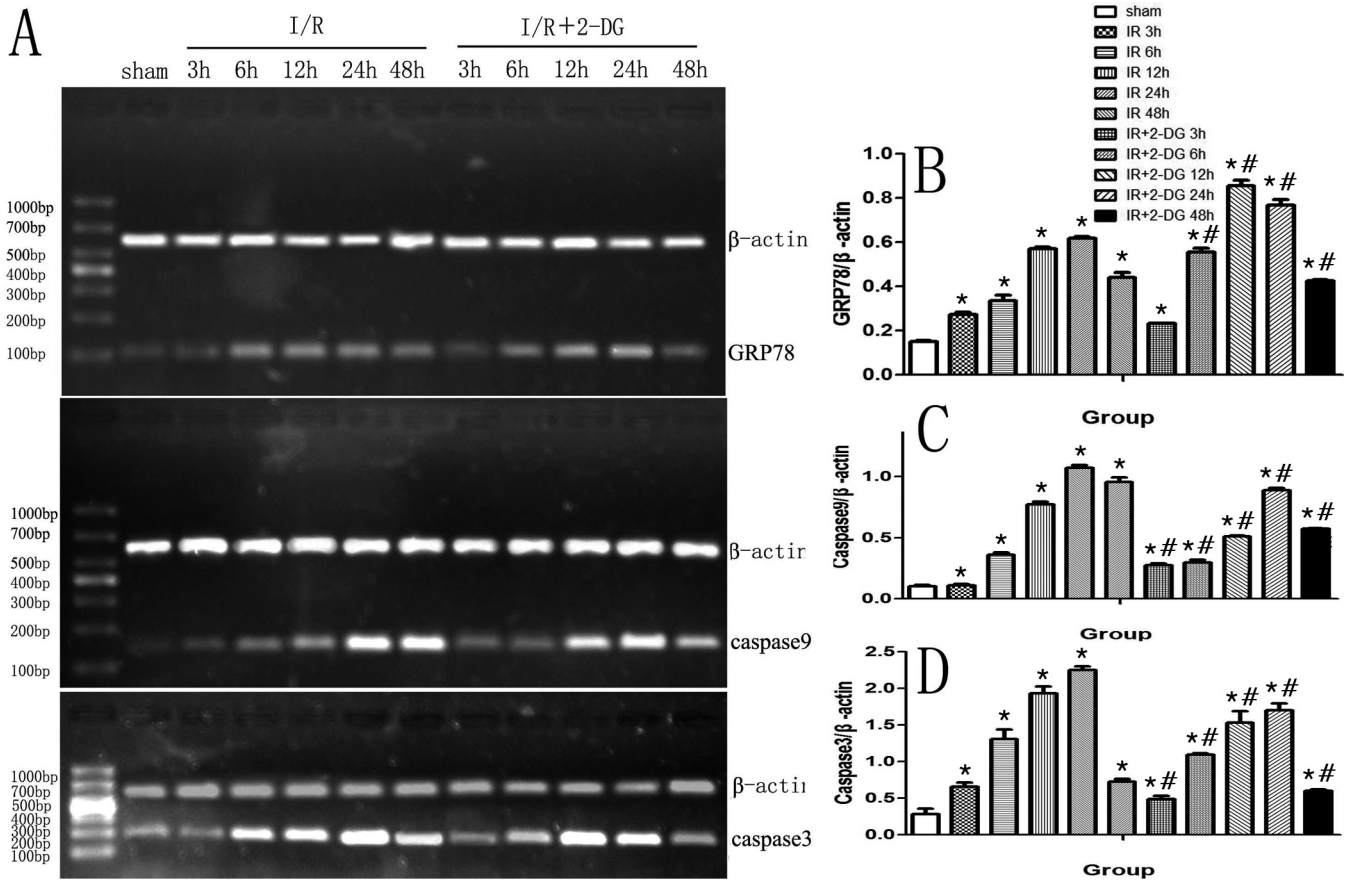

Fig. 8. mRNA expression of GRP78, cleaved-caspase-9 and cleaved-caspase-3 detected with RT-PCR in rat cerebral hippocampus in each group at different time points. **A.** RT-PCR bands. **B.** Histogram of GRP78 mRNA expression. **C.** Histogram of cleved-caspase-9 mRNA expression. **D.** Histogram of cleaved-caspase-3 mRNA expression. Notes: * indicates P<0.01 as compared with sham-operation group. # indicates P<0.01 as compared with I/R group at the same time point. I/R: ischemia-reperfusion; 2-DG: 2-deoxy-D-glucose.

endoplasmic reticulum cannot be improved by up-regulation of GRP78, and apoptosis-signal pathways are triggered (Hetz, 2002; Rasheva and Domingos et al., 2009). The expressions of protein and mRNA of GRP78 were significantly higher in I/R+2-DG group than in sham-operation and I/R groups, suggesting that 2-DG pretreatment induced ERS and up-regulated GRP78 which inhibited apoptosis.

GRP78, a glucose regulated protein, is highly conserved in evolution and has a molecular weight of 78KDa. GRP78 plays a role in protein folding and modification, degradation and transportation of protein unfolding, signal transduction, Ca^{2+} homeostasis, and the activation of transmembrane protein in ERS (Lee, 2005; Huang et al., 2006). The GRP78-activation pathway has not been completely demonstrated in mammals. However, it has been confirmed that mammalian GRP78 promoter contains some endoplasmic reticulum-stress fragments and unfolded protein response element (UPRE). UPRE is strongly associated with GRP78 formation. Transmembrane protein ATF6 also participates in the ERS-apoptosis pathway. After hydrolyses, ATF6 enters cell nucleus and activates GRP gene. In addition, other transcription factors also can activate ERS-stress fragments, leading to GRP78 gene transcription (Urano et al., 2000). GRP78 is a major cell-protective factor because it can combine with caspase-7 and caspase-12 on endoplasmic reticulum membrane to prevent release of the caspase-12 into cytoplasm. If the caspase-12 enters the cytoplasm, it will activate caspase-9 and caspase-3, leading to apoptosis (Kohrt et al., 2007). 2-DG inhibits the release of caspase-12 from endoplasmic reticulum into cytoplasm by up-regulation of GRP78, and further suppresses apoptosis, playing a protective role in brain I/R injury.

The study indicated that TUNEL-positive cells were found at 3 h after I/R in rat cerebral hippocampal CA1 area and reached the maximum at 24 h after I/R. TUNEL-positive cells significantly increased in I/R group as compared to sham-operation group, and pretreatment of 2-DG significantly relieved apoptosis. These results suggest that 2-DG has an inhibitory effect on I/R induced nerve cell apoptosis, but its mechanism is not clear yet. Therefore, we observed caspase cell apoptosis pathway to explore the mechanism that the pretreatment of 2-DG inhibits cell apoptosis.

Caspases, interleukin 1β converting enzyme/Ced homologous cysteine proteases, were named by Alnemri et al (Deasy et al., 2005). They can activate their precursor to trigger the cascade reactions, leading to programmed cell death (Wang, 2001). The ERS-induced apoptotic pathway is different from both classical mitochondrial dependent pathway (endogenous) and death-receptor pathway (exogenous), but is related to both pathways, because the three pathways trigger apoptosis eventually through the cascade reactions of caspase (Bonova et al., 2013; Manzanero et al., 2013). In general, the apoptotic signal first activates the apoptotic promoter caspase-9, and then activates apoptotic effector caspase-3, leading to protein degradation and apoptosis (Faubel and Delstein et al., 2005; Bonova et al., 2013; Manzanero et al., 2013). Caspase-3, a common action point of several apoptotic pathways, is necessary for cascade reactions. Therefore, caspase-3 is regarded as a final apoptotic executor (Ferrer and Planas, 2003).

The study displayed that the expressions of protein and mRNA of caspase-9 and caspase-3 began occurring 3 h after I/R, and reached the maximum 24 h after I/R. The expressions of protein and mRNA of caspase-9 and caspase-3 significantly decreased in 2-DG group as compared to I/R group at each time point, suggesting that 2-DG induced ERS can effectively inhibit expressions of endoplasmic reticulum-apoptotic proteins caspase-9 and caspase-3, and relieve nerve cell apoptosis after I/R. The mechanism may be that ERS up-regulates GRP78 expression, and then GRP78 and caspase-12 form complexes to prevent release of caspase-12 from the endoplasmic reticulum, interfering with caspase-9 activation (Cao et al., 2002; Mouw et al., 2002). Therefore, we think that 2-DG has protective effects on nerve cell after I/R through down-regulating protein expressions of caspase-9 and caspase-3, reducing apoptosis. It remains to be further studied how 2-DG interferes with upstream signals of caspase-9 and caspase-3.

Based on our results, we infer that 2-DG may become an effective component for prevention and treatment of brain I/R injury.

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