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Protective effect of dexmedetomidine against diabetic hyperglycemia-exacerbated cerebral ischemia/reperfusion injury: An *in vivo* and *in vitro* study

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ABSTRACT

Aims: Dexmedetomidine (Dex) has been noted to have neuroprotective effect against cerebral ischemia-reperfusion (I/R) injury. However, the effect of Dex in diabetic hyperglycemia-exacerbated cerebral I/R injury and its underlying mechanism remain unclear.
Main methods: The infarct volume and brain edema were evaluated by 2,3,5-triphenyltetrazolium chloride staining and standard wet-dry method. Modified neurological severity score was utilized to assess the neurological deficits. The oxidative stress and inflammation were evaluated by detecting reactive oxygen species (ROS), malondialdehyde (MDA), tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay and cell count kit-8 were applied to measure cell apoptosis and viability.
Key findings: Dex treatment reduced infarct volume, decreased brain water content and improved neurological deficit in middle cerebral artery occlusion/reperfusion (MCAO/R) mice. Dex treatment reduced the levels of ROS, MDA, TNF-α and IL-1β in the entire middle cerebral artery territory of diabetic mice subjected to MCAO/R, as well as in primary culture of mouse hippocampal neurons stimulated with 50 mM glucose and oxygen glucose deprivation/reperfusion. Dex treatment inhibited neuronal apoptosis induced by diabetic hyperglycemia-exacerbated cerebral I/R injury. Dex upregulated nuclear factor of activated T-cells 5 (NFAT5) and Sirtuin 1 (SIRT1) expression, induced NF-E2-related factor 2 (Nrf2) translocation from cytoplasm to nucleus and inhibited the acetylation of Nrf2. However, these changes triggered by Dex treatment were abrogated by NFAT5 knockdown.
Significance: Dex protects against diabetic hyperglycemia-exacerbated cerebral I/R injury through attenuation of oxidative stress, inflammation and apoptosis. The underlying mechanism is at least the NFAT5/SIRT1/Nrf2 signaling pathway dependent.

1. Introduction

Stroke is a common neurological disease caused by acute cerebral vascular circulation disorders, and is a major cause of death in the world [1]. Stroke can be divided into ischemic stroke and hemorrhagic stroke in terms of the pathogenesis. Ischemic stroke results from a blockage of blood vessel, which ultimately leads to ischemia and hypoxia of the brain. While hemorrhagic stroke is caused by a rupture of blood vessel [2,3]. According to the statistics, about 80% of stroke patients are ischemic stroke [4]. Currently, thrombolytic therapy is the best treatment for ischemic stroke [5]. However, thrombolytic therapy might cause the occurrence and development of cerebral ischemia/reperfusion (I/R) injury [6]. Diabetes mellitus is one of the most common risk factors for ischemic stroke, which may aggravate the progression of

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ischemic stroke and result in poor prognosis [7]. Surprisingly, little attention has been devoted to the pathogenesis of hyperglycemia-exacerbated cerebral I/R injury.

Dexmedetomidine (Dex), a highly selective α2-adrenergic agonist, can decrease the activity of sympathetic nerve and has effects of sedation, analgesia. It is used clinically as an anesthetic adjuvant drug [8]. Several lines of evidence have shown that Dex plays a protective role in I/R injury, including cerebral I/R injury [9]. For instance, Wang et al. showed that Dex treatment attenuated cerebral I/R injury in middle cerebral artery occlusion/reperfusion (MCAO/R) rats, as indicated by a lower degree of cerebral I/R injury and a marked decrease in the number of TUNEL-positive cells. Moreover, Dex preconditioning strikingly suppressed the expression of glial fibrillary acidic protein, toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-κB), and elevated the expression of TIR-domain-containing adapter-inducing interferon-β in MCAO/R rats [10]. Kim et al. suggested that Dex treatment mitigated MCAO/R-induced apoptosis and inflammation, as evidenced by reduced number of necrotic and apoptotic cells and decreased levels of TLR4, NF-κB, caspase-3, tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) [11]. Luo et al. documented that Dex inhibited cell apoptosis and autophagy in *in vivo* and *in vitro* models of cerebral I/R injury, as shown by an increase in cell viability, an elevation in B-cell lymphoma-2 (Bcl-2) and p62 levels, and a reduction in microtubule-associated protein 1 light chain 3 and Beclin 1 levels. Furthermore, Dex suppressed neuronal autophagy by upregulating hypoxia-inducible factor-1α [12]. However, the protective effect of Dex on hyperglycemia-exacerbated cerebral I/R injury and its underlying mechanism are poorly understood.

Oxidative stress results from an imbalance of pro-oxidants and antioxidants and is acknowledged as a pivotal causative factor of cerebral I/R injury [13,14]. During cerebral I/R injury, the anti-oxidation system is damaged and the reactive oxygen species (ROS) is overproduced. The excessive production of ROS induces the mitochondrial lipid peroxidation, leading to mitochondrial dysfunction of mitochondrial respiratory chain complexes, which further promotes ROS production [15]. Also, the superfluous accumulation of ROS causes the mitochondrial permeability transition pore opening and contributes to cerebral microcirculation disorder [16]. Furthermore, the overproduction of ROS evokes the increase of the release of excitatory amino acid and inhibits the reuptake of excitatory amino acid, ultimately leading to neurotoxic injury [17]. Besides, excessive ROS can mediate inflammatory reaction and immune responses by stimulating the expression of cytokines and adhesion molecules, further aggravating cerebral I/R injury [18]. However, little is known regarding the molecular mechanism by which Dex protects against oxidative stress during cerebral I/R injury.

In this study, we found that Dex protected against cerebral I/R injury in diabetic mice *via* attenuation of oxidative stress, inflammation and apoptosis. These findings suggest that Dex promises to become a potential agent for diabetic hyperglycemia-exacerbated cerebral I/R injury.

2. Material and methods

2.1. Animals and treatment

All animal procedures were in line with the Guide for the Care and Use of Laboratory Animals of the Affiliated Taizhou Hospital of Wenzhou Medical University. Male C57BL/6 J mice (Eight- to ten-week-old; Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China) were kept under specific pathogen-free conditions. After a fast of 12 h, mice received intraperitoneal injection of 55 mg/kg/d streptozotocin (Solarbio, Beijing, China) or equal volume of citrate buffer (0.1 M, pH = 4.5) respectively for 3 d. Mice with a fasting plasma glucose level > 16 mmol/L were chosen to use in this study. Mice were randomly divided into 3 groups: sham group, MCAO/R group and MCAO/R + Dex group. Mice in the MCAO/R + Dex group were injected intraperitoneally with Dex (25 μg/kg) after MCAO/R surgery.

2.2. Middle cerebral artery occlusion model

To prepare an *in vivo* model of cerebral ischemia/reperfusion injury, diabetic mice were initially anesthetized with 3% isoflurane. The MCAO/R procedure was performed as previously reported [19] with minor modifications. During the operation, anesthesia was maintained with 1–2% isoflurane and the rectal and temporalis muscle temperatures were kept at 37.0 ± 0.5 °C using a heating pad. The surgical area was exposed with a midline neck incision, and a 6–0 nylon monofilament suture with a silicon-coated tip was inserted through external carotid artery into the terminal internal carotid artery to clog the origin of the right middle cerebral artery and maintained for 2 h. Afterwards, the monofilament suture was pulled out to allow reperfusion. Then, the incision was closed and mice were returned into the cage. In the sham group, mice underwent the same surgical procedures, except for the insertion of a monofilament suture.

2.3. Measurement of infarct volume

At 48 h after Dex treatment, mice were sacrificed, and the brains were cut into six sections. The procedure was performed as a previously described method [20] with slight modifications. The sections were immersed in 2% 2,3,5-Tripheny-ltetrazolium chloride (TTC) solution for 30 min at 37 °C, and then fixed in 4% paraformaldehyde overnight at room temperature. Normal tissues were stained to red, and the white area was identified as infarct tissues. The total infarct volume was measured using Image J software (NIH, Rockville, MD, USA).

2.4. Brain edema measurement

The brain edema was determined using the standard wet-dry method [20]. At 48 h after Dex treatment, mice were sacrificed. The brains of mice were excised and weighed to obtain its wet weight (WW). Afterwards, the brains were dried at 110 °C for 24 h and weighed to get dry weight (DW). The brain water content was calculated as follows: $[(WW - DW)/WW] \times 100$.

2.5. Modified neurological severity score (mNSS)

At 48 h after Dex treatment, the severity of neurological deficit was assessed with mNSS [21]. mNSS was utilized to give an overall evaluation of motor, sensory, and balance functions. The severity of neurological deficits was graded on a 0–18 scale (normal score, 0; maximal deficit score, 18). The higher the mNSS score is, the more severe the neurological deficit is.

2.6. Cell culture and oxygen-glucose deprivation/reoxygenation (OGD/R) model

One-week-old C57BL/6 J mice were sacrificed and the brains were removed. The hippocampus tissues were dissected, trypsinized, triturated and dissociated in neurobasal medium contained 1× B27, 1× N2, 2.4 mM L-glutamine and 100 units/ml penicillin/streptomycin as described previously [22]. Primary culture of mouse hippocampal neurons were grown in neurobasal medium in a 5% CO_2 incubator at 37 °C, and the medium was exchanged every 3 days.

To mimic diabetic hyperglycemia-I/R injury *in vitro*, primary culture of mouse hippocampal neurons were pre-treated with 50 mM glucose for 72 h [23]. Subsequently, primary culture of mouse hippocampal neurons were cultured in glucose-free neurobasal medium and kept at 37 °C in a multigas incubator with 94% N_2, 5% CO_2 and 1% O_2. Following OGD, primary culture of mouse hippocampal neurons were incubated in neurobasal medium plus HG and 1 μM Dex in a humidified incubator at 37 °C under 5% CO_2/95% air.

2.7. Cell transfection

For knockdown of nuclear factor of activated T-cells 5 (NFAT5), small interfering RNA against NFAT5 (si-NFAT5), si-Sirtuin 1 (si-SIRT1) and negative control (si-NC) were designed and synthesized by GenePharma (Shanghai, China), and transfected into primary culture of mouse hippocampal neurons using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications.

2.8. ROS and MDA measurement

The content of ROS was determined by use of ROS Assay Kit (Nanjing Jiancheng Bioengineering institute, Nanjing, China) according to the manufacturer's instructions. At 48 h after Dex treatment, the entire middle cerebral artery (MCA) territory (ischemic side) of mice was homogenized and incubated with 2′,7′-dichlorofluorescin diacetate for 30 min at 37 °C. After washing, the content of ROS was determined using flow cytometry. The level of malondialdehyde (MDA) was detected using MDA Assay Kit (Solarbio) following the manufacturer's specifications.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α and IL-1β were measured by mouse TNF-αELISA Kit (Beyotime, Haimen, China) and mouse IL-1β ELISA Kit (Beyotime), respectively, according to the manufacturer's instructions. At 48 h after Dex treatment, the entire MCA territory (ischemic side) of mice was homogenized. The homogenate was utilized to analyze the levels of TNF-α and IL-1β, according to the instruction of manufacturer.

2.10. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

Cell apoptosis was evaluated by TUNEL assay using an *In Situ* Cell Death Detection Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Briefly, cells smears or tissue sections were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and permeated with 0.3% Triton X-100 for 5 min at room temperature. Subsequently, samples were washed with PBS, followed by incubation with TUNEL reaction mixture for 1 h at 37 °C in the dark. Then, samples were washed with PBS and stained with 6-diamidino-2-phenylindole. TUNEL-positive cells were photographed under a fluorescent microscope.

2.11. Western blot analysis

Total protein was extracted from cells or tissues using RIPA buffer. The cytosolic protein was extracted using hypotonic lysis buffer (KeyGEN, Nanjing, China) and the nuclear protein was extracted using hypertonic lysis buffer (KeyGEN). The concentration of protein was qualified with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). A total of 30 μg protein lysate was resolved by 14% sodium dodecyl sulfate-polyacrylamide gel, followed by transfer onto a polyvinylidene difluoride membrane. Membranes were blocked with 5% free-fat milk, followed by incubation with primary antibodies overnight at 4 °C. The primary antibodies used in this study were as follows: anti-Bcl2-associated X (Bax; 1: 1000; 21 kDa; Abcam, Cambridge, MA, USA), anti-Bcl-2 (1: 1000; 26 kDa; Abcam), anti-NFAT5 (1: 1000; 170 kDa; Novus, Littleton, CO, USA), anti-SIRT1 (1: 500; 81 kDa; Novus), anti-NF-E2-related factor 2 (Nrf2; 1: 1000; 60 kDa; Santa Cruz, CA, USA), and anti-β-actin (1: 1000; 42 kDa; Boster, Wuhan, China) antibodies. After washing, membranes were probed with secondary antibody conjugated with horseradish peroxidase (1: 2000; Boster) for 1 h at room temperature, and the bands were visualized by chemiluminescence (Boster) and analyzed by Image J software.

2.12. Detection of acetylated Nrf2

Primary culture of mouse hippocampal neurons were transfected with si-NFAT5 or si-NC, and then exposed to OGD/R. After OGD/R, primary culture of mouse hippocampal neurons were incubated with 1 μM of Dex for 2 h. The procedure was performed as a previously described method [24] with slight modifications. Primary culture of mouse hippocampal neurons were treated with deacetylases inhibitors (66 nM trichostatin A and 10 mM NAM) for 6 h and then harvested. Primary culture of mouse hippocampal neurons were mixed with nondenaturing lysis buffer composed of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μl/ml protease inhibitor. The solution was probed with antibody against Nrf2, followed by incubation with anti-acetyl-lysine antibody (Santa Cruz). The supernatant solution was subjected to 14% SDS-PAGE and Immunoblotting.

2.13. SIRT1 activity assay

The activity of SIRT1 was detected using SIRT1 Activity Assay Kit (Abcam, Cambridge, MA, USA) following the manufacturer's specification. Tissue homogenate or cell lysates were incubated with antibody against SIRT1 for 2 h at 4 °C, followed by incubation with protein A-conjugated Sepharose for 1.5 h at 4 °C. Samples were microcentrifuged, washed, and treated with Fluoro-Substrate Peptide Solution, NAD+ and SIRT1 Assay Buffer. SIRT1 activity was determined using a microtitre plate fluorometer.

2.14. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA from tissues or cells was isolated using TRIzol reagent (Invitrogen), and then reverse-transcribed to synthesize cDNA using One Step PrimeScript cDNA kit (Qiagen, Hilden, Germany). RT-qPCR analysis was carried out on a CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad). The experiment was carried out according to the manufacturer's instructions. β-actin was selected as the internal standard for normalization. The $2^{-\Delta\Delta Ct}$ method was utilized to determine the relative expression level of NFAT5. Gene-special primers were listed as follows: NFAT5 forward, 5′-CAG AGC TGC AGT ATG TG-3′ and reverse, 5′-CCT CTG CTT TGG ATT TCG-3′; β-actin forward, 5′-CTA AGG CCA ACC GTG AAA AG-3′ and reverse, 5′-ACC AGA GGC ATA CAG GGA CA-3′.

2.15. Cell counting kit-8 (CCK-8)

The viability of primary culture of mouse hippocampal neurons was evaluated using CCK-8 reagent (Solarbio) according to the manufacturer's instructions. Primary culture of mouse hippocampal neurons were transfected with si-NFAT4 or si-NC, and then subjected to OGD/R. After OGD/R, primary culture of mouse hippocampal neurons were treated with 1 μM of Dex for 2 h. After incubation for 48 h, primary culture of mouse hippocampal neurons were incubated with CCK-8 reagent, and the absorbance of each well was detected at 450 nm employing a microplate reader.

2.16. Statistical analysis

All data were shown as the mean ± standard error of the mean (SEM) of three replicates, and analyzed by one-way ANOVA followed by Tukey *post hoc* test using SPSS 20.0 statistical analysis software (Chicago, IL, USA). A *P*-value of 0.05 or less was deemed significant.

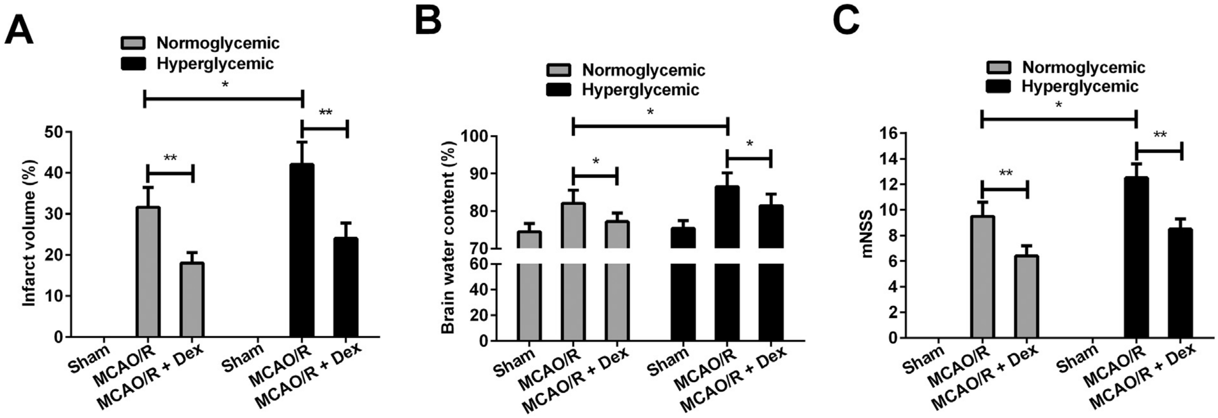

Fig. 1. The protective effect of Dex against diabetic hyperglycemia-exacerbated cerebral I/R injury in mice. Normoglycemic and hyperglycemic mice were injected intraperitoneally with Dex (25 μg/kg) after MCAO/R surgery.The infarct volume (A) and brain water content (B) were reduced following Dex treatment. (C) MCAO/R mice showed reduced neurological deficits. (n = 6). *P < 0.05 and **P < 0.01.

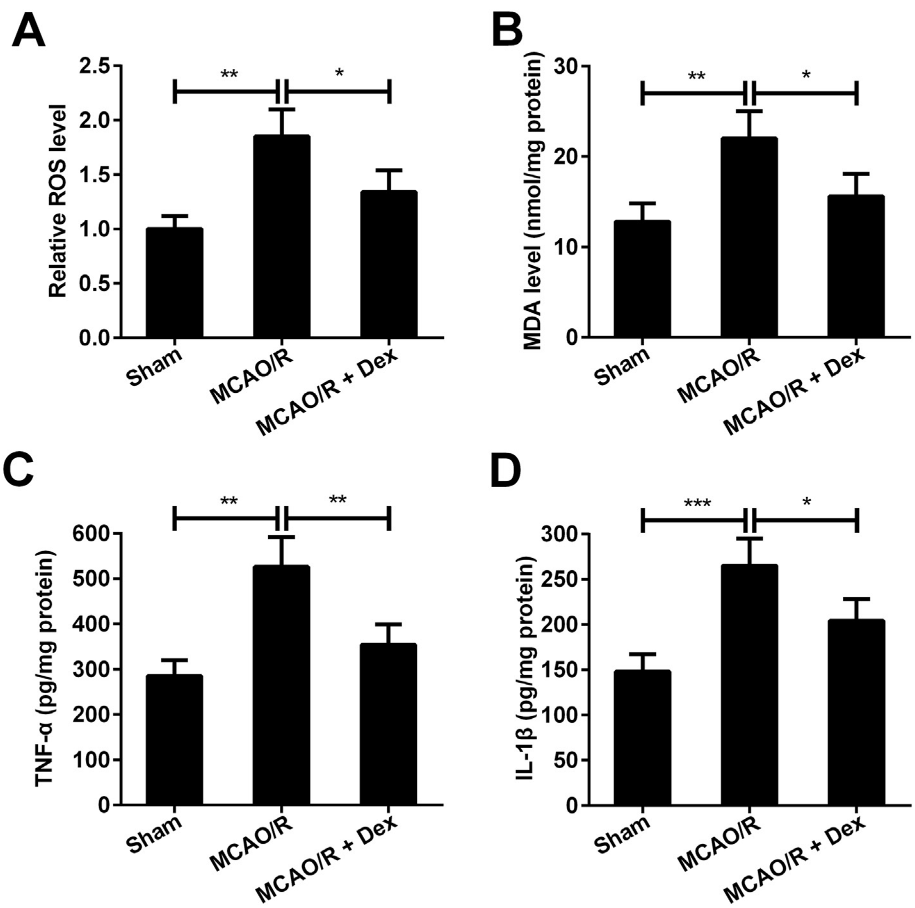

Fig. 2. Dex mitigates oxidative stress and inflammation in diabetic mice with cerebral I/R injury. Diabetic mice were injected intraperitoneally with Dex (25 μg/kg) after MCAO/R surgery. The levels of ROS (A), MDA (B), TNF-α (C) and IL-1β (D) in the entire MCA territory (ischemic side) of mice were obviously increased following MCAO/R surgery and were partially restored after Dex treatment.(n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001.

3. Results

3.1. The protective effect of Dex against diabetic hyperglycemia-exacerbated cerebral I/R injury in mice

To investigate the protective effect of Dex against diabetic hyperglycemia-exacerbated cerebral I/R injury, diabetic mice were injected intraperitoneally with Dex (25 μg/kg) for 3 d after MCAO/R surgery. At 48 h after Dex treatment, the infarct volume and brain water content were measured using TTC staining and standard wet-dry method. As a result, no infarct was discovered in the brains of diabetic mice in the sham group. After MCAO/R surgery, hyperglycemic mice displayed increased infarct volume and brain water content relative to normoglycemic mice. Additionally, the infarct volume and brain water content were reduced in the MCAO/R + Dex group in comparison with that in the MCAO/R group (Fig. 1A and B). At 48 h after Dex treatment, neurological deficits were evaluated by use of mNSS. As illustrated in Fig. 1C, hyperglycemic mice displayed higher mNSS score after MCAO/R surgery compared with normoglycemic mice. Compared to the mice in the sham group, MCAO/R mice exhibited severe neurological deficits. Administration of Dex caused a marked reduction in mNSS score compared with that in the MCAO/R group. Together, these findings indicated that Dex protected mice against diabetic hyperglycemia-exacerbated cerebral I/R injury.

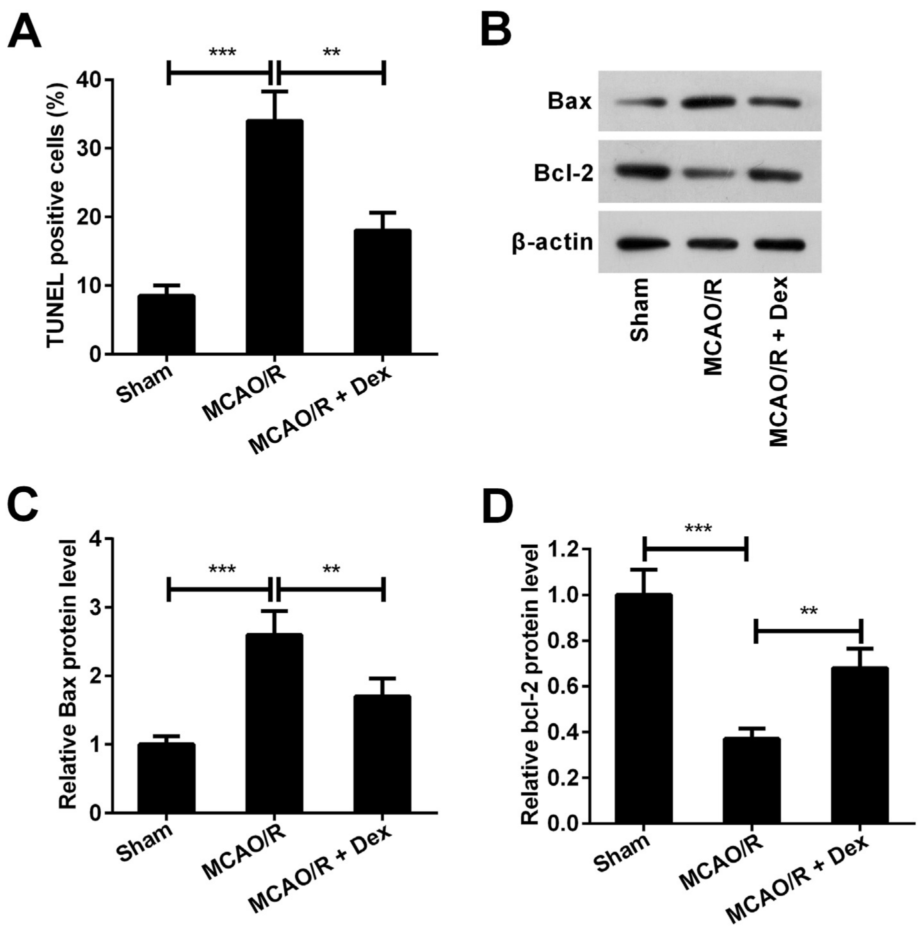

Fig. 3. Dex inhibits neuronal apoptosis in diabetic mice with cerebral I/R injury. (A) Quantification of the number of TUNEL-positive neurons. (B) Representative images of immunoblots for Bax and Bcl-2 in the entire MCA territory (ischemic side) of mice after Dex treatment. Quantification of Bax (C) and Bcl-2 (D) protein levels in panel B.($n = 6$). $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.2. Dex mitigates oxidative stress and inflammation in diabetic mice with cerebral I/R injury

To determine the effect of Dex on the oxidative stress and inflammation *in vivo*, the levels of ROS, MDA, TNF-α and IL-1β were examined using ROS assay kit, MDA assay kit and ELISA assay at 48 h after Dex treatment. As shown in Fig. 2, the levels of ROS, MDA, TNF-α and IL-1β were strikingly elevated in MCAO/R mice relative to sham mice. Furthermore, Dex treatment led to an obvious decrease in ROS, MDA, TNF-α and IL-1β levels compared with those in MCAO/R mice. Collectively, these results suggested that Dex may protect mice against diabetic hyperglycemia-exacerbated cerebral I/R injury by mitigating the oxidative stress and inflammation.

3.3. Dex inhibits neuronal apoptosis in diabetic mice with cerebral I/R injury

To study the effect of Dex on neuronal apoptosis *in vivo*, TUNEL assay was performed at 48 h after Dex treatment. We found that MCAO/R treatment induced an increase in the number of TUNEL positive cells in the entire MCA territory (ischemic side) of mice, which was partially conversed by Dex administration (Fig. 3A). To further address the mechanism by which Dex protected mice against MCAO/R-induced apoptosis, the expression of Bax and Bcl-2 in the entire MCA territory (ischemic side) of mice was measured by Western blot. Compared to the sham group, MCAO/R mice exhibited an increased level of Bax and a decreased level of Bcl-2 in the entire MCA territory (ischemic side) of mice. However, these changes induced by MCAO/R were blocked by Dex treatment (Fig. 3B-3D). Overall, these results indicated that Dex may protect mice against diabetic hyperglycemia-exacerbated cerebral I/R injury by inhibiting neuronal apoptosis.

3.4. Effect of Dex on the NFAT5/SIRT1/Nrf2 signaling pathway during cerebral I/R injury in diabetic mice

To detect the impact of Dex on the NFAT5/SIRT1/Nrf2 signaling pathway, we determined the levels of NFAT5, SIRT1, Nrf2 and ac-Nrf2 using RT-qPCR and Western blot. The mRNA and protein expression levels of NFAT5 were prominently decreased in the MCAO/R group compared with those in the sham group, but increased in the MCAO/R + Dex group compared with those in the MCAO/R group (Fig. 4A-C). Furthermore, MCAO/R reduced the activity and protein expression of SIRT1, whereas, the reduction was partially overturned in the presence of Dex (Fig. 4C and D). In addition, MCAO/R elevated the protein level of Nrf2 in nuclear fraction and reduced the protein level of Nrf2 in cytosolic fraction, which was conspicuously reinforced by Dex treatment (Fig. 4E and F). MCAO/R promoted the acetylation of Nrf2, which was prevented by Dex treatment (Fig. 4G and H). Taken together, these findings revealed that Dex protected mice against diabetic hyperglycemia-exacerbated cerebral I/R injury potentially by regulating the NFAT5/SIRT1/Nrf2 signaling pathway.

3.5. Dex protects neurons against HG and OGD/R-induced injury by activating the NFAT5/SIRT1/Nrf2 pathway in vitro

To further clarify the underlying mechanism, we investigated the role of NFAT5 in the neuroprotective effect of Dex *in vitro*. Primary culture of mouse hippocampal neurons were treated with HG, followed by transfection with si-NFAT5, si-SIRT1, or si-NC. After transfection, primary culture of mouse hippocampal neurons were exposed to OGD/R, and then incubated with 1 μM of Dex for 2 h. As detected by RT-qPCR and Western blot, the expression of NFAT5 was decreased in primary culture of mouse hippocampal neurons after HG and OGD/R insults,

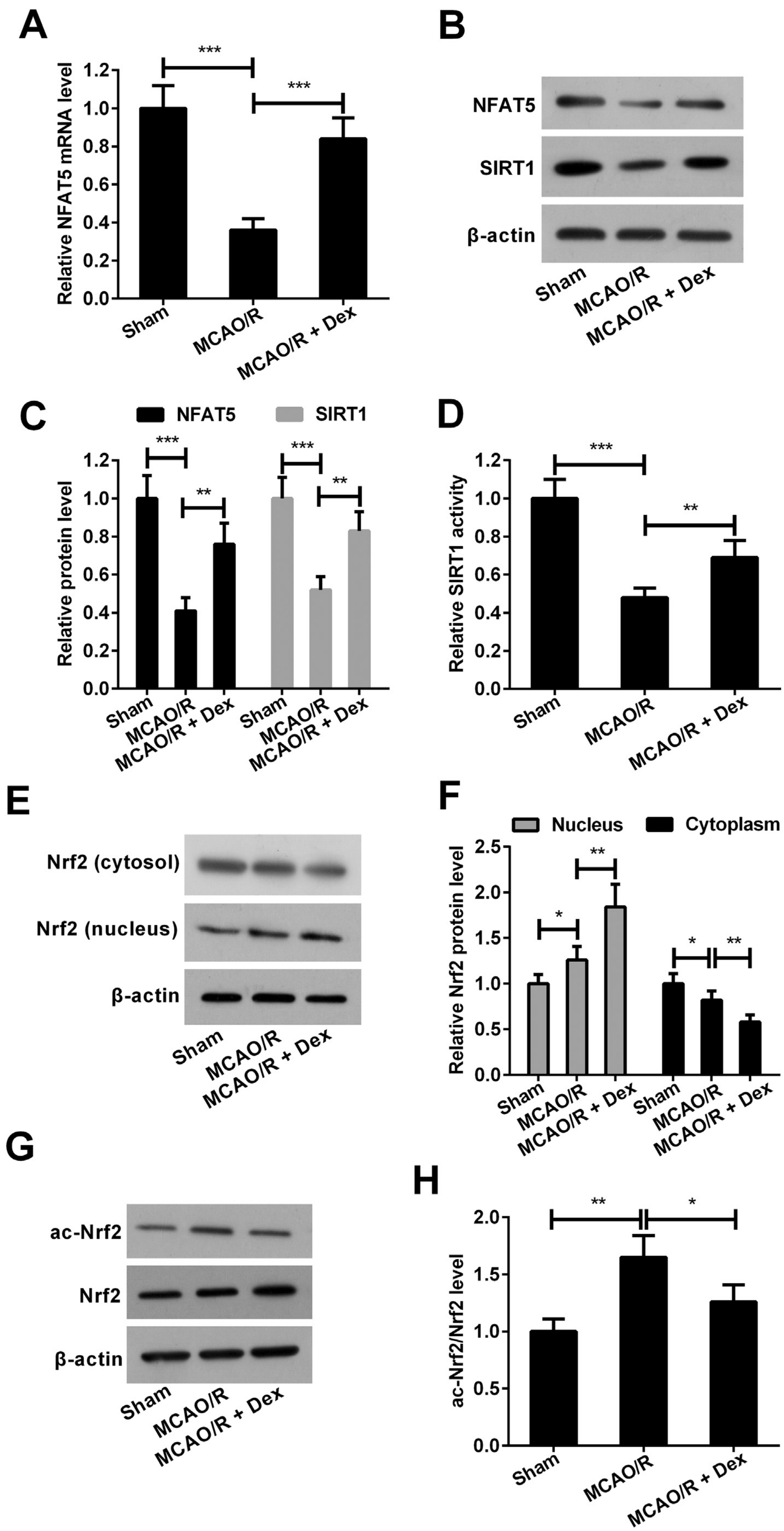

Fig. 4. Effect of Dex on the NFAT5/SIRT1/Nrf2 signaling pathway during cerebral I/R injury in diabetic mice. (A) Comparison of NFAT5 mRNA expression in Sham, MCAO/R and MCAO/R mice treated with Dex. (B) Representative Western blots of NFAT5 in the entire MCA territory (ischemic side) of mice. (C) Quantification of NFAT5 and SIRT1 protein levels in panel B. (D) Evaluation of SIRT1 activity and protein expression in Sham, MCAO/R and MCAO/R mice treated with Dex. (E) Representative Western blots of Nrf2 in nuclear fraction and cytosolic fraction. (F) Quantification of Nrf2 protein level in panel E. (G) Representative Western blots of Nrf2 and acetylated Nrf2 (ac-Nrf2) in the entire MCA territory (ischemic side) of mice. (H) Quantification of Nrf2 and ac-Nrf2 protein levels in panel G.($n = 6$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

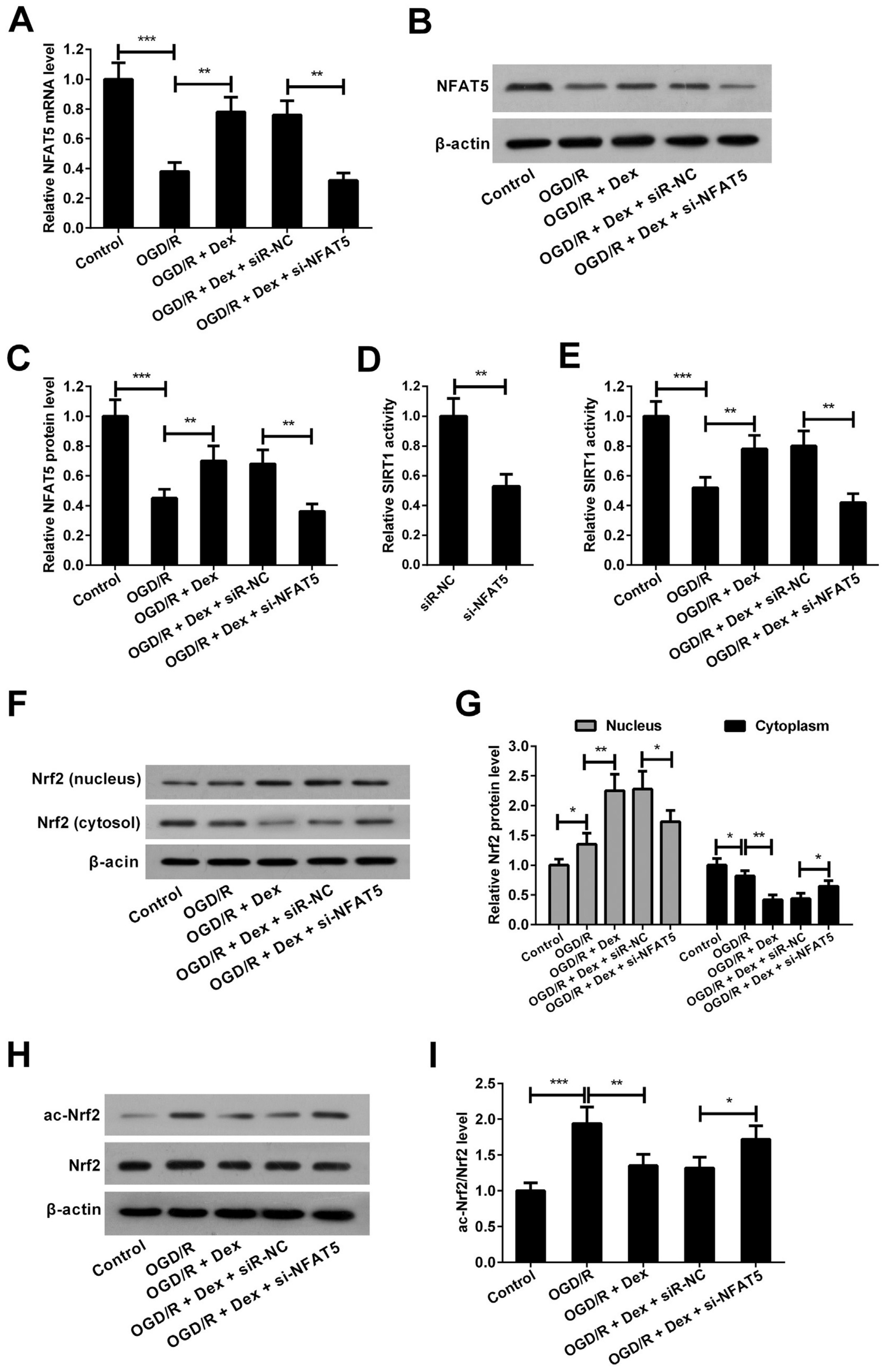

Fig. 5. Dex protects neurons against HG and OGD/R-induced injury by activating the NFAT5/SIRT1/Nrf2 pathway *in vitro*. (A) Determination of NFAT5 mRNA expression after si-NFAT5 transfection. (B) Representative Western blots of NFAT5 in primary culture of mouse hippocampal neurons. (C) Quantification of NFAT5 protein level in panel B. (D and E) Evaluation of SIRT1 activity in primary culture of mouse hippocampal neurons. (F) Representative Western blots of Nrf2 in nuclear fraction and cytosolic fraction. (G) Quantification of Nrf2 protein level in panel F. (H) Representative Western blots of Nrf2 and ac-Nrf2 in primary culture of mouse hippocampal neurons. (I) Quantification of Nrf2 and ac-Nrf2 protein levels in panel H. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

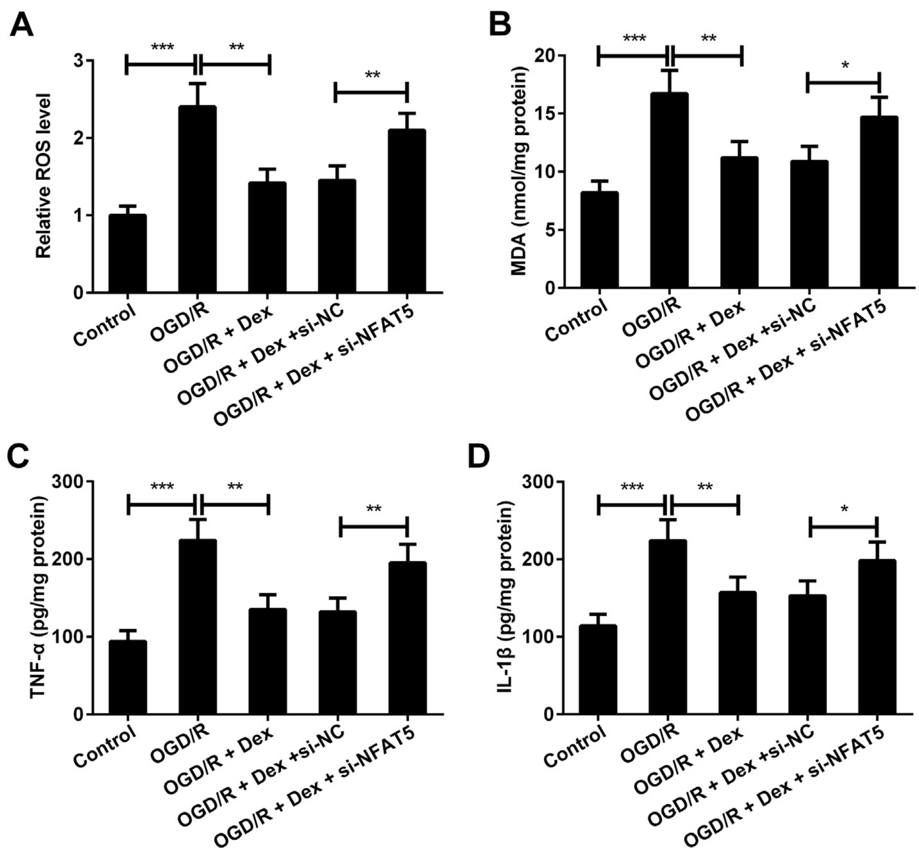

Fig. 6. NFAT5 knockdown reverses the anti-inflammatory and anti-oxidative effect of Dex *in vitro*. The reductions of ROS (A), MDA (B), TNF-α (C) and IL-1β (D) caused by Dex treatment were reversed by silencing of NFAT5 in primary culture of mouse hippocampal neurons exposed to OGD/R. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

which was partially reversed by Dex treatment. The mRNA and protein expression levels of NFAT5 were strikingly decreased in the si-NFAT5 group relative to the si-NC group (Fig. 5A-C). NFAT5 knockdown reduced SIRT1 activity in primary culture of mouse hippocampal neurons (Fig. 5D). The SIRT1 activity was reduced in primary culture of mouse hippocampal neurons stimulated with HG and OGD/R, but increasedin the presence of Dex treatment. However, the effects of Dex were overturned by downregulation of NFAT5 (Fig. 5E). OGD/R elevated the protein level of Nrf2 in nuclear fraction and reduced the protein level of Nrf2 in cytosolic fraction, which was enhanced by Dex treatment. OGD/R promoted the acetylation of Nrf2, which was attenuated by Dex treatment. However, NFAT5 knockdown could block the changes triggered by Dex treatment (Fig. 5F-I). Hence, we concluded that Dex protected against diabetic hyperglycemia-exacerbated cerebral Iv/R injury by upregulating SIRT1 expression, promoting Nrf2 nuclear translocation and inhibiting acetylation of Nrf2 through upregulation of NFAT5.

3.6. NFAT5 knockdown reverses the anti-inflammatory and anti-oxidative effect of Dex in vitro

To explore the role of NFAT5 in the anti-inflammatory and anti-oxidative effect of Dex *in vitro*, the levels of ROS, MDA, TNF-α and IL-1β were examined. As displayed in Fig. 6, the levels of ROS, MDA, TNF-α and IL-1β were dramatically increased in primary culture of mouse hippocampal neurons exposed to HG and OGD/R, while the levels of ROS, MDA, TNF-α and IL-1β were subsequently dropped with Dex administration. However, the inhibitory effect of Dex was abrogated by knockdown of NFAT5. Therefore, our results clarified that NFAT5 knockdown reversed the anti-inflammatory and anti-oxidative effect of Dex *in vitro*.

3.7. NFAT5 knockdown reverses the anti-apoptosis effect of Dex in vitro

To investigate the role of NFAT5 in the anti-apoptosis effect of Dex *in vitro*, the apoptosis and viability of primary culture of mouse hippocampal neurons were determined by TUNEL assay and CCK-8 assay. OGD/R induced a dramatic increase in the number of TUNEL positive cells, while Dex treatment led to a marked decrease in the number of TUNEL positive cells. However, NFAT5 knockdown could abrogate the effect of Dex on OGD/R-induced apoptosis (Fig. 7A and B). In parallel, the viability of primary culture of mouse hippocampal neurons was reduced after OGD/R, but increased following Dex treatment. Whereas, the effect of Dex on the viability of primary culture of mouse hippocampal neurons was abolished by transfection of si-NFAT5 (Fig. 7C). As detected by Western blot, OGD/R increased the Bax level and decreased the Bcl-2 level, which was conversed by Dex treatment. However, the changes induced by Dex treatment were blocked by silencing of NFAT5 (Fig. 7D-F). Accordingly, these results showed that NFAT5 knockdown reversed the anti-apoptosis effect of Dex *in vitro*.

4. Discussion

A number of studies have demonstrated that Dex can protect against cerebral I/R injury [25]. However, the role of Dex on the diabetic hyperglycemia-exacerbated cerebral I/R injury remains elusive. In this study, we discovered that administration of Dex reduced infarct volume, decreased brain water content and improved neurological deficit in MCAO/R mice. Dex treatment reduced the levels of ROS and MDA in the entire MCA territory (ischemic side) of hyperglycemic mice subjected to MCAO/R and primary culture of mouse hippocampal neurons stimulated with 50 mM glucose and OGD/R. These results showed that Dex has the anti-apoptotic effect in *in vivo* and *in vitro* models of diabetic hyperglycemia-exacerbated cerebral I/R injury.

Accumulating recent evidence has shown that inflammation serves as a vital contributor for cerebral I/R injury [26]. Cerebral I/R triggers

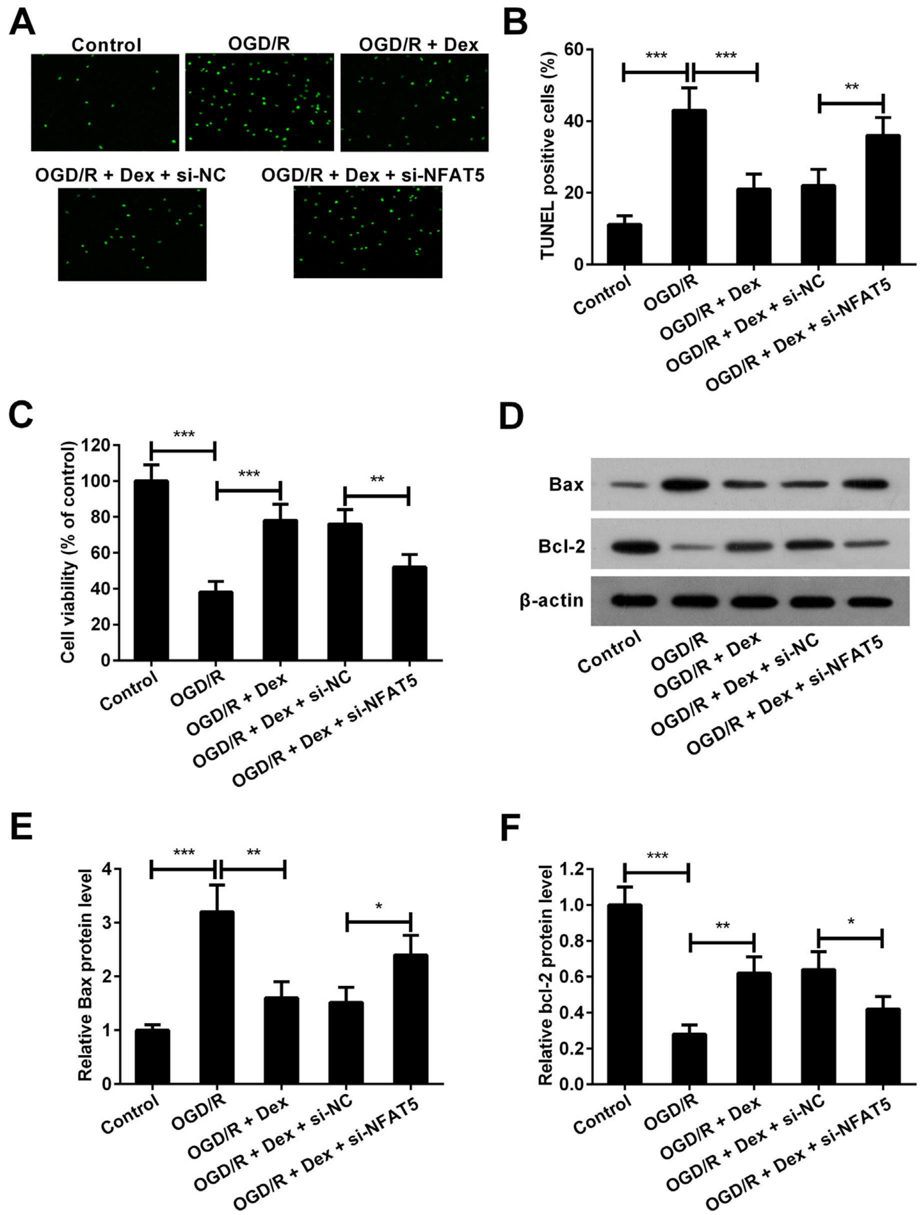

Fig. 7. NFAT5 knockdown reverses the anti-apoptosis effect of Dex *in vitro*.(A and B) NFAT5 knockdown reverses the anti-apoptosis effect of Dex in primary culture of mouse hippocampal neuronsin treated with HG and OGD/R.. (C) NFAT5 knockdown reverses the promotory effect of Dex on cell viability in primary culture of mouse hippocampal neuronsin treated with HG and OGD/R.. (D) Representative images of immunoblots for Bax and Bcl-2 in primary culture of mouse hippocampal neurons. (E and F) Quantification of Bax and Bcl-2 protein levels in panel D. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

the activation of microglia cells and induces the infiltration of peripheral leukocytes, thus exacerbating the cerebral I/R injury [27]. Moreover, inflammatory response causes oxidative stress and releases the inflammatory factors, further aggravating brain damage. Inflammatory cytokines, including TNF-α and IL-1β, are tightly associated with the inflammatory response in cerebral I/R injury [28]. Hence, anti-inflammation is one of the therapeutic strategies of cerebral I/R injury. In this study, we found that inflammation was activated in MCAO/R mice, suggesting that inflammation is one of the pathological mechanisms of cerebral stroke. Similarly, the levels of TNF-α and IL-1β were increased in primary culture of mouse hippocampal neurons stimulated with HG and OGD/R relative to control group. Furthermore, Dex treatment inhibited inflammation induced by diabetic hyperglycemia-exacerbated cerebral I/R injury in *in vivo* and *in vitro*. However, the *in vitro* effect of Dex could be blocked by knockdown of NFAT5, indicating that NFAT5 was involved in the protective mechanism of Dex on diabetic hyperglycemia-exacerbated cerebral I/R injury.

NFAT5, also known as tonicity-responsive enhancer-binding protein, belongs to the Rel family and is composed of NF-κB and NFATc proteins. Several reports have shown that NFAT5 serves as a central role in inducible gene transcription in response to immune and hyperosmolar stimuli [29]. NFAT5 has been shown to be closely correlated with neuronal function, including neuronal development, excitability, axonal growth, and neuronal survival [30]. NFAT5 has been documented to be downregulated in spinal cord of I/R rats. Moreover, upregulation of NFAT5 attenuated oxidative stress and cell apoptosis conferred by OGD/R damage in astrocytes by activating the SIRT1/Nrf2 pathway [31]. In addition, NFAT5 knockdown caused an obvious increase in neuronal apoptosis and ROS generation in neurons under hypoxia/ischemia condition [32]. However, the role of NFAT5 in diabetic hyperglycemia-exacerbated cerebral I/R injury is still unclear. In our study, Dex treatment increased the expression of NFAT5 in MCAO/R mice. Knockdown of NFAT5 abrogated the inhibitory effects of Dex on oxidative stress, cell apoptosis and inflammation in primary culture of mouse hippocampal neurons stimulated with HG and OGD/R. In parallel, Dex administration upregulated SIRT1 expression, induced Nrf2 translocation from cytoplasm to nucleus and inhibited the acetylation of Nrf2 in *in vivo* and *in vitro* diabetic hyperglycemia-exacerbated cerebral I/R injury models. But, these regulatory effects of Dex on SIRT1 and Nrf2 could be abolished by NFAT5 silencing. Thus, we concluded that Dex exerted its roles potentially by regulating the NFAT5/SIRT1/Nrf2 signaling pathway.

SIRT1, also known as NAD-dependent deacetylase sirtuin-1, is a member of the sirtuin family of proteins and acts as a crucial role in lipid metabolism, signal transduction, cell senescence, stress response and cell apoptosis [33,34]. SIRT1 has been shown to interact with NFAT5 [35,36]. It has been documented that SIRT1 promotes Nrf2 transcription and then regulates the expression of downstream genes of Nrf2 [37]. Compelling evidence has established that SIRT1 protects neurons against cerebral I/R injury. As an example, SIRT1 was downregulated in striatal region of MCAO/R rats [38]. SIRT1 has been postulated to be involved in the impaired learning and memory abilities of MCAO/R rats [39]. Overexpression of SIRT1 attenuated MCAO/R-induced apoptosis, inflammation and oxidative by inhibiting the p53/microRNA-22 axis [40]. Besides, downregulation of SIRT1 could reverse the neuroprotective effect of Dex in MCAO/R rats [41]. Here, we found that MCAO/R decreased the activity of SIRT1, while Dex treatment increased the activity of SIRT1. Nevertheless, the effect of Dex treatment was abrogated by deletion of NFAT5. These findings indicated that NFAT5-mediated SIRT1 expression was critical for the neuroprotective effects of Dex.

Nrf2, a basic leucine zipper protein, is ubiquitously expressed in the body tissues and participates in modulation of physiological processes [42]. Nrf2 is capable of regulating the expression of antioxidant proteins that protect against oxidative stress-induced damage [43]. Normally, Nrf2 is kept in the cytoplasm by Kelch like-ECH-associated protein 1 (Keap 1), an adapter substrate of Cullin3 (Cul3) ubiquitin E3 ligase complex. Under oxidative stress, the Keap 1-Cul3 ubiquitination system is disrupted, and Nrf2 is dissociated from Keap 1. But, in the oxidative stress state, Nrf2 is activated, translocates into nucleus and initiates transcription of antioxidative genes [44,45]. Notably, Nrf2 has been proposed to be involved in the progression of cerebral I/R injury [46]. Deletion of Nrf2 led to a marked aggravation of neurological deficit, inflammation and infarct volume in MCAO/R mice [47]. In this study, we found that MCAO/R elevated the protein level of Nrf2 in nuclear fraction and reduced the protein level of Nrf2 in cytosolic fraction, which were conspicuously reinforced by Dex treatment. Furthermore, MCAO/R promoted the acetylation of Nrf2, which was prevented by Dex treatment. However, the changes induced by Dex treatment were blocked by transfection with si-NFAT5, suggesting that the protective role of Dex was at least mediated by NFAT5.

5. Conclusion

In general, this study found that Dex exerted a neuroprotective effect against diabetic hyperglycemia-exacerbated cerebral I/R injury *in vivo* and *in vitro*, which was mediated by the NFAT5/SIRT1/Nrf2 signaling pathway. These results imply that Dex promises to become an effective agent for the treatment of diabetic hyperglycemia-exacerbated cerebral I/R injury and offer strong support to its clinical application.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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