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Original Article

Dexmedetomidine reduces hypoxia/reoxygenation injury by regulating mitochondrial fission in rat hippocampal neurons

Jia Liu[1], Qing Du[1], He Zhu[1], Yu Li[1], Maodong Liu[1], Shoushui Yu[2], Shilei Wang[1]

[1]*Department of Anesthesiology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China;* [2]*The People's Hospital of Rizhao, Rizhao, Shandong, China*

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Abstract: Objective: To demonstrate the effect of dexmedetomidine (DEX) on the process of hippocampal neurons in hypoxia/reoxygenation (H/R) injury of mitochondria, and then explore the possible mechanism which might provide new targets for brain protection. Methods: Hippocampal neurons were removed from the entire brain of Sprague-Dawley rats (born within 24 h) and plated on 25 cm^2 culture flasks at a density of 700,000 cells. After 8 d cultivation, the primary hippocampal neurons were randomly divided into six groups: control group (C group); vehicle group (V group); H/R group; H/R+DEX treatment groups: of P1, P2 and P3 group were added 0.1, 1, 10 μmol/L of DEX during oxygen-glucose deprivation and reperfusion period. Cultured primary hippocampal cells were subjected to oxygen-glucose deprivation (OGD) for 6 h, followed by 20 h of reperfusion. Cell apoptosis (by flow cytometry), fluorescence intensity of Ca^{2+} (using a laser scanning confocal microscope), CaN enzymatic activities (by ELISA), expression of Drp1, Fis1, CytC, caspase 3 (by western blot) were measured. Results: Compared with C group and V group, cell apoptosis, fluorescence intensity of Ca^{2+}, CaN enzymatic activities were higher in H/R group ($P<0.05$) with the expression of Drp1, Fis1, Cytc, caspase 3 increasing. However 0.1, 1, 10 μmol/L of DEX could reduce the cell apoptosis, fluorescence intensity of Ca^{2+}, CaN enzymatic activities ($P<0.05$) with lower expression of Drp1, Fis1, Cytc, caspase 3 compared with H/R group, in which 1 μmol/L could show the optimal treatment effect. Conclusion: 0.1, 1, 10 μmol/L of DEX can inhibit H/R-induced mitochondrial fission by suppressing calcium overload and then alleviate the injury of oxygen-glucose deprivation and reperfusion, of which 1 μmol/L is the optimal dose.

Keywords: Dexmedetomidine, calcium overload, mitochondrial fission, H/R injury

Introduction

Cerebral ischemia is a common occurring complication after ischemic shock, cardiac arrest or cardiac surgery, and is one of the lead causes of human death and long-term disability worldwide [1]. Recanalization following ischemia is the most effective method for treatment of acute cerebral infarct and correction of hypoxia, however, paradoxically could cause severe cerebral ischemia-reperfusion (I/R) injury [2]. Due to the lack of efficient neuroprotective therapies, I/R injury is still a major medical problem which urgently needed to be further studied.

The mitochondria are dynamic organelles that cleave and fuse consecutively to form new individual units and interconnected networks within the cell [3]. A balance between mitochondrial fission and fusion is important to mitochondrial function and form [4]. Recent evidence has indicated that the mitochondrial fission machinery actively participates in the process of programmed cell death [5]; other studies have shown that inhibition of mitochondrial fission protects the pallium from nerve injury during cerebral I/R, and that the amount of mitochondrial fission can increase during apoptosis [6]. Mitochondrial Ca^{2+} signaling has been shown to regulate mitochondrial fission through phosphorylation and dephosphorylation of dynamin-related protein 1 (Drp1) [7, 8]. Inhibiting mitochondrial uptake of Ca^{2+} may decrease mitochondrial fission [9].

Dexmedetomidine (DEX), an α2-adrenergic agonist, has been developed for human clinical use as an anesthetic and sedative [10, 11]. Recent study has shown that DEX has neuroprotective effects against ischemic cerebral injury through its activation of α2-adrenergic receptors and its binding at imidazoline 1 and 2 receptors [12, 13]. Recent research has confirmed that DEX can block the voltage-gated calcium channels, thereby inhibiting the occurrence of Ca^{2+} overload in the cerebral I/R injury [14]; and DEX can also inhibit NMDA receptors to regulate receptor-dependent Ca^{2+} channels and thus inhibit the Ca^{2+} overload [15].

However, to date, there is no research exploring whether the mechanism for Dexmedetomidine to reduce cerebral I/R injury is related to mitochondrial fission. Therefore, it was assumed that DEX regulated calcium signaling to affect mitochondrial fission, and thus played its protective role in the brain, providing a new path for the treatment of cerebral ischemic diseases, and some guidance for clinical practice.

Materials and methods

Reagents

The experiments were approved by the institution of Ethics Committee of Qingdao University Medical College (No. QUMC 2011-09). Neonatal Wistar rats (born within 24 h) were purchased from the Laboratory Animal Center of Qingdao (SCXK (Lu) 20090007). Dulbecco's modified Eagle medium (DMEM/F12), trypsin and Fetal Bovine Serum were purchased from Hyclone (USA). Neurobasal solution, B27 and Earle's Balanced salt solution (1×EBSS) were purchased from Gibco (NY, USA), poly-L-lysine were purchased from Sigma-Aldrich (St Louis, MO, USA). Fluo-3, AM was purchased from AAT Bioquest (USA). Mouse monoclonal anti-Drp1 antibody, Mouse monoclonal anti-Fis1 (TTC11) antibody were purchased from Abcam (HK). Mouse monoclonal anti- CytC and anti-caspase 3 were purchased from Abcam (HK).

Primary hippocampal neuronal culture

Primary hippocampal neurons were prepared from neonatal Wistar rats according to the methods described previously with slight modifications [16]. The rats were dealt with surface disinfection with 75% alcohol before being decapitating on ice, and then their entire brain rapidly exposed and bilateral hippocampi isolated, mechanically fragmented and digested for 20 min with 0.25% trypsin at 37°C. The dispersed cells were centrifuged at 1000 r/min for 5 min and supernatant was removed. Next, cells were re-suspended in DMEM/F12 solution containing 20% fetal bovine serum. The culture bottles were coated by 0.1 mg/ml poly-L-lysine for 30min in advance and rinsed twice by PBS before cell seeding. Cell re-suspension solution was planted at a density of $1*10^6$ cells/ml and incubated at 37°C in humidified air with 5% CO_2. After 24-hour cell inoculation, the culture medium was replaced with neurobasal medium supplemented with B27 (2%), 1 mol/L glutamine (1%) and 0.1 mol/L sodium pyruvate (1%). Every three days, half volume of medium was removed and replaced by the same fresh solution. The morphological changes and growth feature of the hippocampal neurons were observed under an inverted phase contrast microscope every day. On the 8th day, cells were identified by NSE staining, observed and photographed under fluorescent microscopy (Leica, DMI 4000 B, Japan).

Oxygen-glucose deprivation (OGD) model and experimental groups

Some studies have shown that oxygen-glucose deprivation (OGD) is an *in vitro* model used to simulate the effects of stroke and explore the effect of drugs on neurophysiologic changes which occurred in stroke [17-19]. The OGD model was prepared as follows: the culture solution was replaced with glucose-free EBSS and the culture flask was shifted to an incubator with 5% CO_2 and 95% N_2 at a temperature of 37°C.

The neurons were randomly divided into six groups: (1) Control group (C group): cultured normally without any treatment; (2) Vehicle group (V group): the culture medium mixed with Dimethylsulfoxide (DMSO, 0.1% final concentration) during OGD; (3) H/R group: after eight days of cultivation, neurons were subjected to OGD for 6 h followed by reoxygenation for 20 h but given no drugs; (4) P1 group: the culture medium mixed DEX (0.1 μm) during OGD period; (5) P2 group: the culture medium mixed DEX (1 μm) during OGD period; (6) P3 group: the culture medium mixed DEX (10 μm) during OGD period.

Protein extraction

Each group of hippocampal neurons was rinsed twice with PBS and detached from the matrix with a cell scrapper into cold PBS. The resulting cell suspensions were centrifuged at 12000 r/min for 10 min at 4°C, the supernatant was aspirated away, and cells were re-suspended in cell RIPA lysis buffer which contained protease inhibitors phenylmethanesulfonyl fluoride (PMSF). The mixture were then homogenized by vortexing for 1 min and proteins were extracted by incubation in lysis buffer on ice for 1 h, followed by sonication and centrifugation at 12000 r/min for 10 min at 4°C. Supernatants were pyrolysised for 10 min at 95°C which were joined with SDS in advance, then centrifuged at 12000 r/min for 10 min at 4°C to remove the cellular debris. The resultant supernatant was taken as whole protein and stored at -20°C. Protein concentration was measured by using a BCA kit, according to the manufacturer's instruction.

Western blot

Protein samples were electrophoresed through 8% and 15% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Then the membranes were blocked with PBST containing 5% non-fat milk for 2 hours on shaker. After that, membranes were incubated with the appropriate primary antibody over night at 4°C. The membranes were washed in PBST for 3 times and then incubated with the respective secondary antibodies (1:6000 dilution) for 1 h, subsequently washed and developed by using the ECL chemilum inescence system (VILBER Fusion FX5 Spectra, France). Results were analyzed by Quantity One Software (Bio-Rad, USA). During this process, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for internal reference, with each experiment repeated 3 times.

Flow cytometry (FCM) detection

To assess the apoptosis rate, phosphatidylserine (PS) exposure was analyzed by using Annexin V-fluorescein-5-isothiocyanate (Annexin V-FITC) apoptosis detection kit (Sigma, St Louis, MOUSA) according to the manufacturer's protocol. Briefly, cells ($5*10^5$) were harvested and rinsed with cold phosphate buffer saline (PBS) or two times. After resuspension with 500 μL of 1× binding buffer, the mixture was incubated with 5 μL of Annexin and 5 μL of propidium iodide (PI, 10 mg/L) at room temperature in the dark for 5-15 min. Then the cells were read by FCM (Becton Dickinson, San Jose, CA, USA) through FL-1 filter (530 nm) and FL-2 filter (585 nm). The medical values were determined by using CELL Quest 3.0 (Becton Dickinson, San Jose, CA). Annexin V positive and PI negative cells were considered as early apoptosis cells, whereas Annexin V positive and PI positive were regarded as late apoptotic cells.

Determination of mitochondrial calcium

The extraction of mitochondria: trypsin digested cells and centrifugal collected cells. Thereafter mitochondrial separation reagent was added in for 30 minutes, which was homogenate suspension. Samples were centrifuged under the condition of 600 g, and then liquid centrifuged again under 11 kg. The isolated mitochondria were washed by Hanks solution, and the Fluo-3, AM [17] was added in. Then samples were put at 37°C for 30 minutes to incubate. And then the samples were washed by Hanks solution 3 times to remove excess probe. Incubated at 37°C for 30 minutes again. At last a fluorescence microplate reader was used to determine the fluorescence intensity at the excitation 506 nm and emission 526 nm.

The enzyme activities of calcineurin

The calcineurin (CN) activation was associated with free calcium levels [20]. To figure out the relationship between CN and calcium levels, the CN activities were determined by using the CN detection kit via ELISA. Briefly, after cells were treated with RIPA lysis buffer, cell lysis solution collected to be centrifuged at 12,000 r/min for 10 min at 4°C. The supernatant was used to detect the enzymatic activities and protein content. Additionally, protein concentration was determined by using the BCA method according to the instructions of manufacturer. And computation method of CN was given in the direction.

Cell viability assay

Cell viability was assessed by a cell counting kit. Cells were cultured on 96-well plates. Each well was added with 100 μl culture medium mixed with 10 μl of 7Sea-Cell Counting kit and

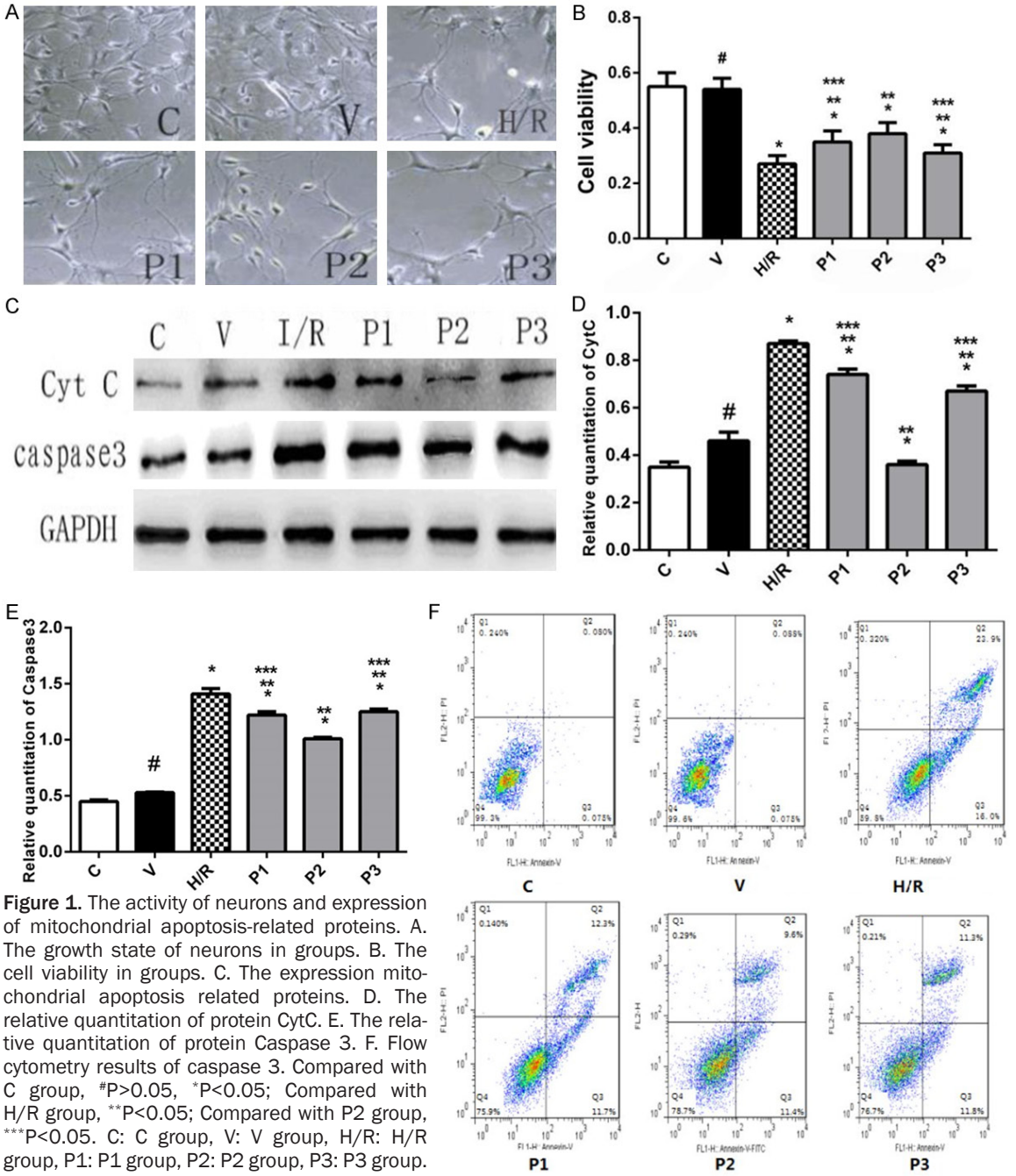

Figure 1. The activity of neurons and expression of mitochondrial apoptosis-related proteins. A. The growth state of neurons in groups. B. The cell viability in groups. C. The expression mitochondrial apoptosis related proteins. D. The relative quantitation of protein CytC. E. The relative quantitation of protein Caspase 3. F. Flow cytometry results of caspase 3. Compared with C group, #P>0.05, *P<0.05; Compared with H/R group, **P<0.05; Compared with P2 group, ***P<0.05. C: C group, V: V group, H/R: H/R group, P1: P1 group, P2: P2 group, P3: P3 group.

incubator were incubated for 2 h with 95% (vol/vol) O_2 and 5% (vol/vol) CO_2. Then absorbance was measured in a microplate reader at 450 nm. Cell viability rate =(OD treatment/OD control)*100%. Each experiment was repeated at least 3 times.

Statistical analysis

Data was expressed as mean ± standard deviation (SD) and the statistical analysis was performed with one-way ANOVA. What's more, repeated measurement was carried out by t test and ANOVA of SPSS 17.0. Differences between estimates of effects were considered significant at P<0.05.

Results

The activity of neurons and expression of mitochondrial apoptosis-related proteins

In order to choose more reasonable drug concentration of DEX to protect brain, there were

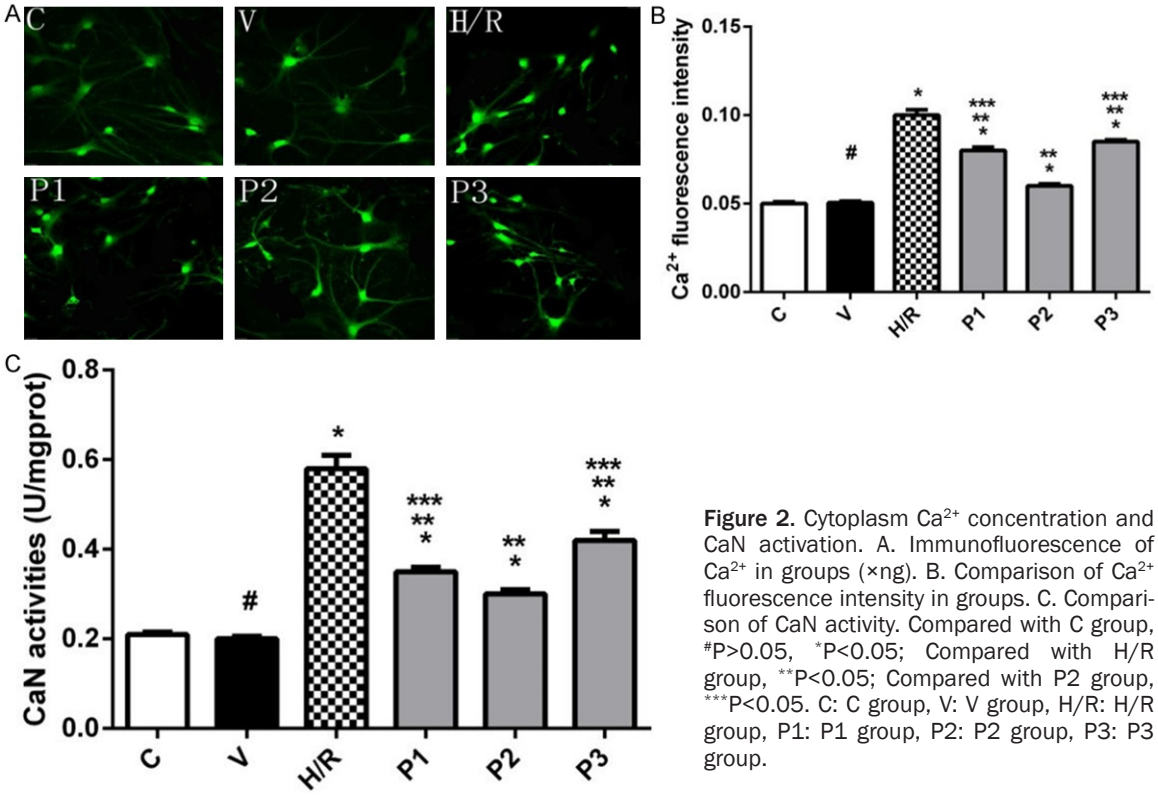

Figure 2. Cytoplasm Ca^{2+} concentration and CaN activation. A. Immunofluorescence of Ca^{2+} in groups (×ng). B. Comparison of Ca^{2+} fluorescence intensity in groups. C. Comparison of CaN activity. Compared with C group, #P>0.05, *P<0.05; Compared with H/R group, **P<0.05; Compared with P2 group, ***P<0.05. C: C group, V: V group, H/R: H/R group, P1: P1 group, P2: P2 group, P3: P3 group.

no significant difference on the growth of neurons between C group and V group, however the neurons in H/R group had worst neurons growth state with least number, and in P1, P2, P3 group, the injury degree was lighter relatively (**Figure 1A**).

The result of cell viability showed that after the treatment of 0.1 μmol/L, 1 μmol/L, 10 μmol/L of DEX, the activity of cells significantly was higher than that of H/R group and lower than that of normal group, and differences are statistically significant (P<0.05); between C group and V group, there was no significant difference on cell activity (P>0.05) (**Figure 1B**).

Compared with C group and V group, the expression of mitochondrial apoptosis-related proteins caspase 3, CytC increased (P<0.05), however, the expression of caspase 3, CytC of DEX used groups was lower than that of H/R group (P<0.05), and P2 group had the lowest expression in the three DEX used groups (P<0.05) (**Figure 1C-E**).

The result showed that after the treatment of 0.1 μmol/L, 1 μmol/L, 10 μmol/L of DEX, the expression of caspase 3 was significantly lower than that of H/R group and higher than that of C group, in which P2 group had lowest expression of caspase 3 (**Figure 1F**).

Cytoplasm Ca^{2+} concentration and CaN activation

The results of immunofluorescent assay showed that there was no significant difference about Ca^{2+} fluorescence intensity between C group and V group (P>0.05), Ca^{2+} fluorescence intensity of H/R group was higher than that of C group, and in P1, P2, P3 groups, Ca^{2+} fluorescence intensity was between group C and H/R group, and compared with other groups, 1 μmol/L of DEX could significantly reduce the intensity of Ca^{2+} fluorescence, and the differences were statistically significant (P<0.05) (**Figure 2A**, **2B**).

The CaN activity between V group and C group had no significant difference (P>0.05), however, compared with C group, CaN activity of H/R group increased significantly, and CaN activation of P1, P2, P3 groups was significantly lower activity, in which P2 had the lowest activation (P<0.05), which was consistent with the results of Ca^{2+} fluorescence intensity (**Figure 2C**).

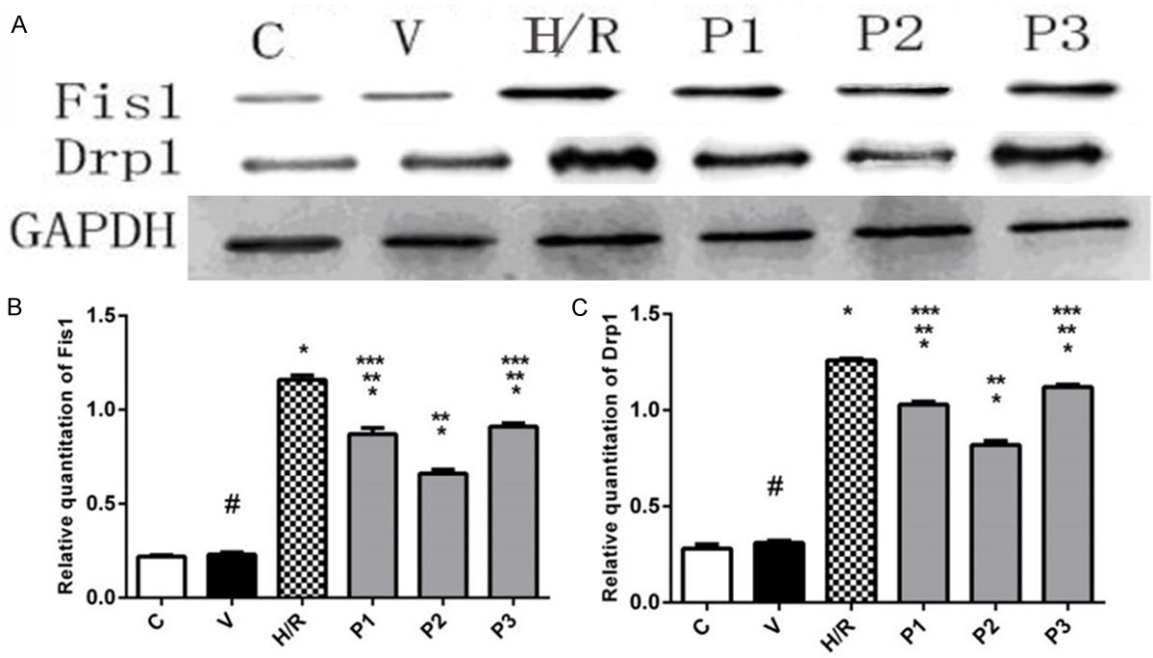

Figure 3. The expression of mitochondrial fission-relative proteins. A. The expression of Fis1 anad Drp1. B. The relative quantitation of protein Fis1. C. The relative quantitation of protein Drp1. Compared with C group, #P>0.05, *P<0.05; Compared with H/R group, **P<0.05; Compared with P2 group, ***P<0.05. C: C group, V: V group, H/R: H/R group, P1: P1 group, P2: P2 group, P3: P3 group.

The expression of mitochondrial fission-relative proteins

The change of expression of mitochondrial fission-related proteins Drp1, Fis1 was consistent with the change of apoptosis-related proteins caspase 3, CytC. The expression of Drp1, Fis1 increased (P<0.05), however, the expression of these proteins of P1, P2, P3 groups was lower than that of H/R group (P<0.05), and P2 group had the lowest expression (P<0.05) (**Figure 3A-C**).

Discussion

Hippocampus neurons are sensitive to ischemia and anoxia and thus very vulnerable to injury induced by ischemia and hypoxia [21]. The mechanism of cerebral I/R injury is complicated, and the final brain injury is presented through cell apoptosis. But no matter which mechanism operates, the calcium overload initiates cell apoptosis in the cerebral I/R injury [22]. The cells undergo apoptosis through two major pathways, namely the death receptor pathway and mitochondrial pathway [23]. It was previously considered that mitochondrial Ca^{2+} overload was an important reason for the neuron injury induced by cerebral I/R injury [24]. DEX can inhibit the stimulation of cerebral ischemia and the mitochondrial swelling caused by a high concentration of Ca^{2+} and inhibit activation of the mitochondrial apoptotic pathway by protecting mitochondrial morphology, and thus play a role in the cerebral protection [25]. Mitochondria as the core organelles regulating cell apoptosis are a hotspot of research on I/R injury. In the previous studies, it was found that the mitochondrial fission increased in the cerebral I/R injury model, so that the mitochondrial fragmentation was enhanced and the neuronal apoptosis was serious, and the inhibition of mitochondrial fission would reduce the extent of brain injury [26].

Recent study has shown that the mitochondrial calcium signaling is closely related to the mitochondrial fission [27]. The cytoplasmic calcium is considered to be an important influencer on mitochondrial activity, and its concentration is closely related to the speed of mitochondrial movement. A high concentration of Ca^{2+} can quickly stop the mitochondrial movement and simultaneously mediate mitochondrial fission. A large number of experiments showed that in the process of cell apoptosis, the cellular stim-

ulation itself may lead to a highly active state of CN. In the intrinsic pathway of mitochondria-mediated apoptosis, the mitochondria are the key initiating factor. The water-soluble protein cytochrome C (CytC) as a mitochondrial respiratory chain protein is located in the intermembrane space of mitochondria and combined with the mitochondrial inner membrane, and it will be released from the mitochondrial outer membrane when apoptosis occurs, which can cause irreversible activation of caspase cascade reaction. In the previous study [28], it was found that under the action of various apoptosis inducing factors, the reduced mitochondrial membrane potential will lead to the opening of mitochondrial permeability transition pore (MPTP) and thus result in increased permeability of the mitochondrial inner membrane. Therefore, the apoptosis inducing factors such as CytC, Apaf in the downstream cells are released from the mitochondrial inner membrane into the cytoplasm. The CytC released into the cytoplasm combines with Apaf-1 in the cytoplasm and promote its combination with caspase-9 precursor to form apoptotic body, thereby activating caspase-9. The activated caspase-9 activates the downstream effector caspase-3 to cause caspase cascade reaction and induce DNA breakage, which results in cell apoptosis [29].

So, in this experiment, the H/R injury model of rat hippocampal neurons was used to simulate cerebral I/R injury *in vitro*, and to explored whether DEX could affect the mitochondria fission in cerebral I/R injury and its possible mechanism. Meanwhile, the experimental concentration of DEX was determined as 0.1-10 μmol/L by referring to relevant fundamental experiment studies [30], and the changes in the activities of neurons treated by DEX in various groups were firstly observed before which we had demonstrated the vehicle did not injury neurons, then, the analysis of various experimental results showed that 1 μmol/L of DEX could most effectively reduce the neuron injury. The results showed that DEX could reduce the neuron injury in the H/R injury model, and inhibit mitochondrial fission, the Ca^{2+} overload in the neurons and calcineurin activity; It was also found that DEX could inhibit expressions of mitochondrial fission-related proteins such as Drp1 and Fis1 as well as expressions of cytochrome C and caspase 3, thereafter reduce neuronal apoptosis rate to protect neurons.

In conclusion, 0.1, 1, 10 μmol/L of DEX can inhibit H/R-induced mitochondrial fission by suppressing calcium overload and then alleviate the injury of oxygen-glucose deprivation and reperfusion, of which 1 μmol/L is the optimal dose.

Disclosure of conflict of interest

None.

Address correspondence to: Shilei Wang, Department of Anesthesiology, The Affiliated Hospital of Qingdao University, No. 1677 Wutaishan Road, Qingdao 266500, Shandong, China. Tel: +86-0532-82919391; Fax: +86-0532-82919391; E-mail: wangshileiahqd@126.com

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