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# Dexmedetomidine protects hippocampal neurons against hypoxia/reoxygenation-induced apoptosis through activation HIF-1α/p53 signaling

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ABSTRACT

*Purpose:* To observe the effect of dexmedetomidine (DEX) on mitochondrial apoptosis of hippocampal neurons in hypoxia/reoxygenation (H/R) brain injury in developing rats, and to investigate its regulatory mechanism on HIF-1α/p53 signaling pathway.

*Methods:* Hypoxia/reoxygenation model was used in this study. TUNEL assay was performed to detect cell apoptosis. Immunohistochemical analysis and Western-blotting analysis were conducted to detect Cytochrome-C (Cyt-c), APAF-1, Caspase-3, Neuroglobin (Ngb), HIF-1α and p53 expression. After 28 days, Morris water maze (MWM) was performed.

*Results:* 50 μg/kg DEX improved H/R-induced brain injury and inhibited mitochondrial apoptosis in rats. Western-blotting and Immunohistochemical results demonstrated that DEX could up-regulate Ngb through $\alpha_2$ receptor to inhibit H/R-induced mitochondrial apoptosis. In addition, by adding inhibitors yohimbine and 2-methoxyestradiol (2ME2), we found that DEX could activate HIF-1α/p53 signaling pathway. MWM test showed that DEX could enhance long-term learning and memory of H/R brain injury rats.

*Conclusion:* DEX alleviates H/R-induced brain injury and mitochondrial apoptosis in developing rats through $\alpha_2$ receptor, which may be related to activation of HIF-1α/p53 signaling pathway to up-regulate the expression of Ngb.

## 1. Introduction

Ischemia/reperfusion (I/R) injury is a complex pathophysiological process involving a variety of factors and signaling pathways, including oxygen free radicals, calcium overload, inflammation, and apoptosis [1]. Of these, the hypoxia/reoxygenation (H/R) injury is a major component of I/R-induced tissue injury. Transient ischemic hypoxia can cause severe acute brain injury, especially in hippocampus, which is highly susceptible to ischemic hypoxia. During the process of reoxygenation, induction of a series of complex mechanisms were also reported to initiate neuronal apoptosis and cause delayed neurological damage [2]. Currently, calcium antagonists, excitatory amino acid antagonists and free radical scavengers are commonly used in clinical prevention and treatment of H/R brain injury, but the efficacy is difficult to be affirmed.

Neuroglobin (Ngb) is a recently discovered tissue globin with a high affinity for oxygen that is widely expressed in vertebral central and peripheral nerve systems [3]. As a newly discovered member of globin family, Ngb has been considered as the brain or nerve equivalent of tissue haemoglobin [4]. Previous studies have demonstrated that overexpression of Ngb is neuroprotective against hypoxic/ischemic brain injuries [5,6]. As an endogenous protective protein, Ngb has become a focus of research in cerebral ischemia/hypoxia injury. However, the mechanism of Ngb on hypoxia/reoxygenation brain injury has not been clarified.

Dexmedetomidine (DEX) is a highly selective $\alpha_2$-adrenergic receptor agonist that has sedative, analgesic, and anxiolytic properties and it is commonly used as a sedative and anesthetic. Clinical evidence has suggested that DEX preconditioning could improve outcomes in patients after cardiac and noncardiac surgeries [7,8]. In addition, there are many studies have shown that DEX improves neuronal survival after transient global or focal cerebral ischemia in rats [9,10], which might be regulated by central catecholamine and glutamate release, hypothermia, and inhibition of apoptosis and neural cell death [11].

Hypoxia induction factor 1 alpha (HIF-1α), a master regulator of the response to hypoxia regulates the expression of a broad range of genes

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that facilitate the adaptation to, and survival of cells to low oxygen environments [12,13]. It is a basic helix-loop-helix (bHLH) transcription factor that is degraded during normoxia. In hypoxic conditions, the hydroxylation modification declines and HIF-1α is stabilized for its transcriptional activities [14]. HIF-1α signaling pathway has been found to be closely related to the pathogenesis of hypoxic encephalopathy in recent years, and its activation is believed to be related to the inhibition of neuronal apoptosis. Herein, we investigated whether the protective effect of DEX on H/R injury was related to the expression level of Ngb and the regulation of HIF-1α/p53 signaling pathway. This would further amplify our understanding of the underlying mechanism and molecular roles of DEX in clinical management of H/R injury.

## 2. Materials and methods

### 2.1. Animals and experimental groups

Sprague-Dawley (SD) rats (7 days old) weighing 16 to 19 g were randomly divided into control group (C group, n = 22), Hypoxia/Reoxygenation group (H/R group, n = 22), Hypoxia/Reoxygenation + 50 μg/kg Dexmedetomidine group (D group, n = 22), Hypoxia/Reoxygenation group + 50 μg/kg Dexmedetomidine + 0.5 mg/kg Yohimbine group (DY group, n = 22) and Hypoxia/Reoxygenation group + 50 μg/kg Dexmedetomidine + 16 mg/kg 2-methoxyestradiol group (DM group, n = 22). SD rats were provided by the Experimental Animal Centre of China Medical University. All the animal experimental procedures were approved by the Experimental Animal Ethics Committee of Shengjing Hospital affiliated to China Medical University (No. 2018PS406K). All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### 2.2. Hypoxia/reoxygenation (H/R) model

A hypoxic environment was induced in a humidified and closed glass vessel. The rats were placed into the hypoxic vessel filled with a mixture of 8% $O_2$ and 92% $N_2$ for 2 h and then turn to supply 50% $O_2$ for 30 min. In D group, rats were intraperitoneally injected with 50 μg/kg DEX (HENGRUI MEDICINE, Jiangsu, China). In DY group, rats were intraperitoneally injected with 50 μg/kg DEX and 0.5 mg/kg yohimbine (J&k Scientific, Beijing, China). In DM group, rats were intraperitoneally injected with 50 μg/kg DEX and 16 mg/kg 2-methoxyestradiol (Meilun Biotechnology Co., LTD, Dalian, China).

### 2.3. TUNEL assay for apoptosis

Cell apoptosis was determined using TUNEL assay with an detection kit (One Step TUNEL Apoptosis Assay Kit, Roche Applied Science) in accordance with the manufacturer's protocol. Paraffin sections were deparaffinized and soaked in PBS for 5 min. The samples were incubated with proteinase K (20 μg/ml) for 12 min at 37 °C, then rinsed three times with PBS for 5 min each time. The samples were treated with 0.1% tritol for 10–30 min at room temperature and then rinsed as above using PBS, following incubation with TUNEL reagents 1 and 2 (1:10) in a cassette at 4 °C overnight. The cells were counterstained with DAPI, and apoptosis was observed under a fluorescence microscope (Olympus, Tokyo, Japan) and the hippocampal cell apoptosis index was calculated.

### 2.4. Immunohistochemical analysis

Briefly, endogenous peroxidase activity within the sections was quenched by incubating the sections with 3% $H_2O_2$ for 10 min after dewaxing and hydration. Hippocampal tissues were incubated in a humidified chamber with primary antibodies directed against Ngb (1:200, N7162, Sigma-Aldrich Co., USA), HIF-1α (1:100, ab2185, Abcam, England). On the following day, the hippocampal tissue were washed with PBS and incubated with secondary antibody. In the negative controls, the primary antibody was replaced with PBS. They were counterstained with DAB.

### 2.5. Western-blotting analysis

Total proteins were extracted using a total Protein Extraction kit (BestBio Science, China), according to the manufacture's instruction. Concentrations of the total proteins were determined by using Bioepitope Bicinchoninic Acid Protein Assay kit (Bioworld Technology CO., Ltd., USA). Protein samples were separated on polyacrylamide-SDS gels and electroblotted onto nitrocellulose membrane (Millipore, USA). After blocking with TBS, 5% nonfat dry milk for 2 h, the membrane incubated overnight with primary antibodies: Ngb (1:1000, GTX54552, GeneTex), Cytochrome-C (1:500, 10993-1-AP, Proteintech Group, Inc., Wuhan, China), APAF-1 (1:500, 21710-1-AP, Proteintech Group, Inc., Wuhan, China), Caspase-3 (1:1000, 9662S, Cell Signaling Technology, Inc., China), HIF-1α (1:1000, ab2185, Abcam, England) and p53(1:500, 10442-1-AP, Proteintech Group, Inc., Wuhan, China). After washing in TBST three times, they were incubated with HRP-conjugated secondary antibody for 45 min at room temperature. The immunoblots were developed using the enhanced chemiluminescence detection system. The band intensity was quantified by Image J 1.39u software (National Institutes of Health, NIH, USA).

### 2.6. Morris water maze (MWM)

In order to minimize the effect of the stress response on the experimental results, the day before the training, all the experimental rats were placed one by one in the water maze of the removal platform for about 2 min to familiarize themselves with the water environment to reduce the stress response. The MWM test was carried out 28 days after successful modeling for 6 consecutive days. During the 6 consecutive days, each rat was run for total of 4 trials per day, with a 3–5 min intertrial interval. The rats were given a maximum of 90 s to find the platform and remain seated for at least 15 s. If the rat was unable to find the platform within the 90 s, it was placed directly on the platform for 15 s and then returned to its cage. On the last day of the cycle, the platform was removed, and the same water inlet point was selected. The 90 s swimming trajectory of the rats was photographed by a camera, and the number of crossings of the rats was calculated.

### 2.7. Statistical analysis

SPSS 17.0 (IBM, New York, USA) was applied to analyze all data. Results are expressed as means ± SEM. Differences among multiple groups were statistically analyzed using one-way ANOVA and post hoc comparisons (Tukey's test). The escape latency of rats was analyzed using two-way ANOVA. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. DEX improved H/R-induced brain injury and inhibited mitochondrial apoptosis in rats

Western-blotting results showed that compared with C group, the protein expression of Cyt-c, APAF-1 and Caspase-3 were significantly increased in H/R group ($P < 0.05$). However, when DEX was added, the protein expressions of these three proteins were significantly downregulated ($P < 0.05$; Fig. 1A). Fig. 1B is the results of TUNEL assay. As shown in Fig. 1B, the degree of staining in group H/R was deepened in comparison with C group, while that of D group was significantly decreased when DEX was added. More specifically, compared with C group, the apoptosis of hippocampal neurons in H/R group was

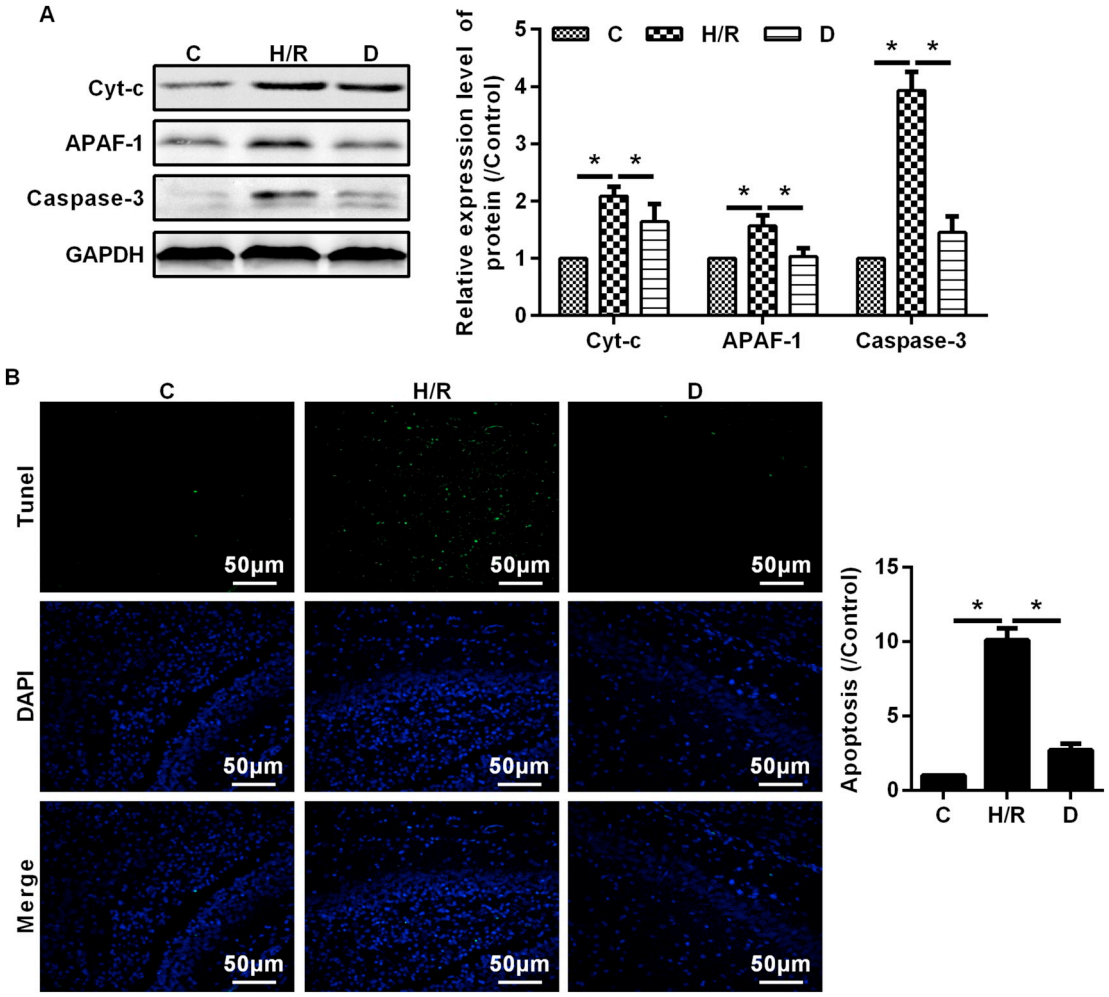


**Fig. 1.** The protein expressions of Cyt-c, APAF-1, Caspase-3 and apoptosis of hippocampal neurons in each group. (A) Protein expressions of Cyt-c, APAF-1, Caspase-3 were detected by Western-Blotting analysis. *Indicated H/R group compared with control group and D group compared with H/R group. (B) TUNEL assay detects mitochondrial apoptosis in hippocampal neurons. *Indicated H/R group compared with control group and D group compared with H/R group. Scale bar = 50 μm. Cytochrome-C, Cyt-c; apoptotic protease activating factor-1, APAF-1; all data were obtained from three independent experiments.

significantly increased ($P < 0.05$). After the addition of DEX in D group, the apoptosis was significantly reduced in comparison with group H/R ($P < 0.05$; Fig. 1B).

### 3.2. DEX up-regulated the expression of Ngb in H/R-induced brain

On the basis of the H/R-induced brain injury model, DEX was administered and samples were taken 24 h later. The expression of Ngb was detected by western-blotting and immunohistochemical. Western-blotting results showed that compared with C group, the Ngb protein expression was significantly increased in H/R group ($P < 0.05$). More interestingly, when DEX was added, the expression of Ngb protein in D group was more significant ($P < 0.05$; Fig. 2A).

A similar pattern change was observed in immunohistochemical, which was markedly increased in H/R group and D group at 24 h when compared with C group (Fig. 2B). Accordingly, the mean density of Ngb in the hippocampal neurons brain tissue increased significantly in the H/R group and D group when compared with C group, respectively ($P < 0.05$; Fig. 2B).

### 3.3. DEX alleviated H/R-induced mitochondrial apoptosis through $\alpha_2$ receptor

To explore the mechanism of neuroprotection induced by DEX itself, and whether it is $\alpha_2$ receptor-dependent, yohimbine, an inhibitor of $\alpha_2$ receptor, was given to detect its effect on Cyt-c, APAF-1 and Caspase-3 level by TUNEL staining and Western-blotting, to assess the extent of damage in hippocampal neurons of brain tissue. As shown in Fig. 3A, compared with D group, the protein expressions of Cyt-c, APAF-1 and Caspase-3 were all increased significantly after yohimbine treatment ($P < 0.05$). The quantized histogram can more directly show the protein changes (Fig. 3A).

In order to further verify this conclusion, we conducted the TUNEL staining. It was found that the degree of staining was significantly deepened in group DY as compared with group D when treated by yohimbine. By observing the apoptosis of different groups of cells, we found that the degree of apoptosis is directly proportional to the degree of staining ($P < 0.05$; Fig. 3B).

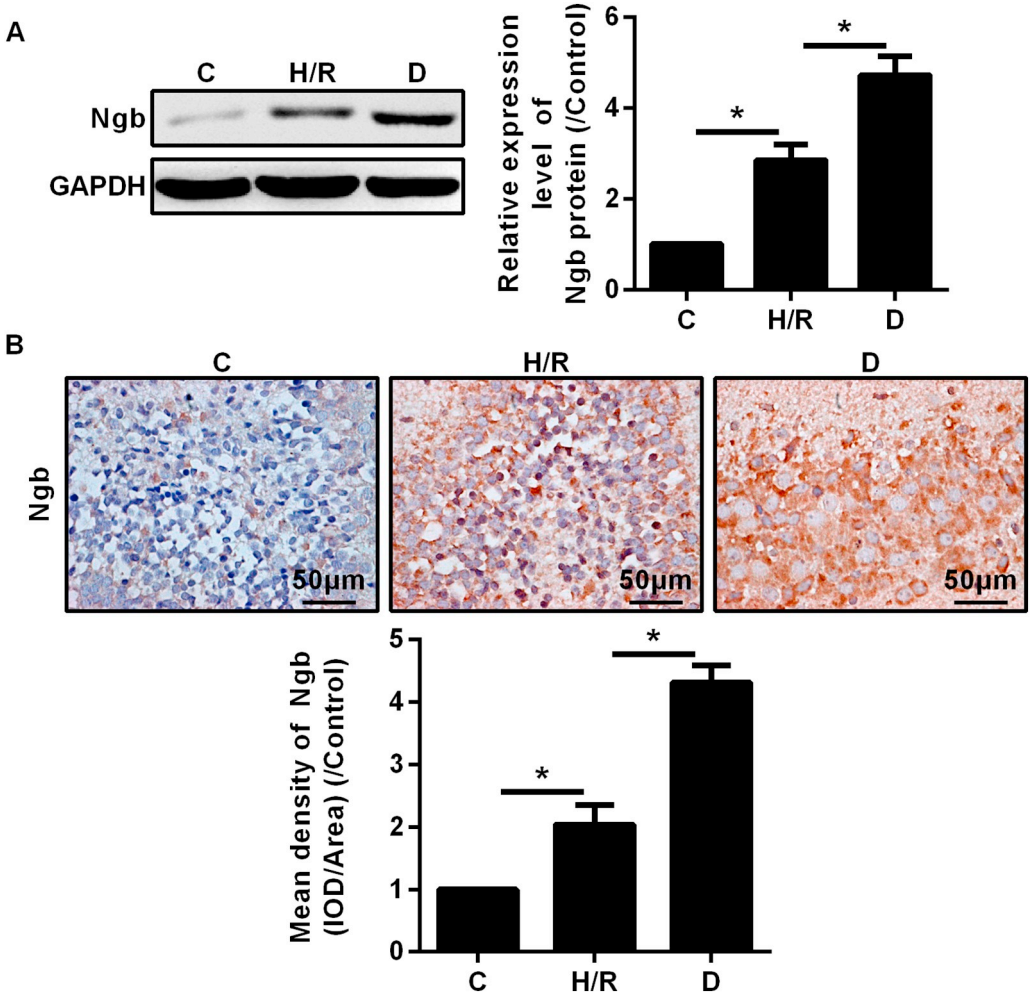


**Fig. 2.** Ngb protein expression of hippocampal neurons in each group. (A) Protein expressions of Ngb was detected by Western-Blotting analysis. *Indicated H/R group compared with control group and D group compared with H/R group. (B) Immunohistochemical analysis of Ngb. *Indicated H/R group compared with control group and D group compared with H/R group. Scale bar = 50 μm. Neuroglobin, Ngb. All data were obtained from three independent experiments.

### *3.4. DEX activated HIF-1α/p53 signaling pathway through Ngb regulated by $\alpha_2$ receptor*

In D group, up-regulated protein expressions of HIF-1*α*, p53 and Ngb were observed in the hippocampal neurons when compared with H/R group. After treatment with yohimbine, however, the protein expressions of HIF-1*α*, p53 and Ngb in DY group were markedly decreased in comparison to D group ($P < 0.05$; Fig. 4A).

Strong HIF-1*α* and Ngb immunoreactivity were detected in the D group in the hippocampal neurons when compared with the H/R group. However, after treatment with yohimbine, HIF-1 and Ngb immunoreactivity were significantly decreased when compared with the matching D group ($P < 0.05$; Fig. 4B).

### *3.5. DEX improved H/R-induced brain injury by HIF-1α/p53 signaling pathway in rats*

To further investigate the relationship between DEX and HIF-1α/p53 signaling pathway in improving H/R-induced brain injury in rats, the HIF-1α inhibitor 2ME2 was given immediately after DEX administration. The Western-blotting results showed that when 2ME2 was added, the protein expression of Ngb, HIF-1α and p53 were decreased significantly ($P < 0.05$; Fig. 5A). A similar pattern change was observed in immunohistochemical, which was markedly decreased in DM group when compared with D group ($P < 0.05$; Fig. 5B).

As shown in Fig. 5C, the protein expressions of Cyt-c, APAF-1 and Caspase-3 in DM group were significantly increased in comparison with D group. Accordingly, from quantitative data we can see that Cyt-c, APAF-1 and Caspase-3 protein expression were significantly up-regulated after treatment with 2ME2 ($P < 0.05$). TUNEL results showed that the staining intensity of D group was lower than that of DM group, and the corresponding apoptosis was also less than that of DM group. ($P < 0.05$; Fig. 5D).

### *3.6. DEX enhanced long-term learning and memory in H/R-induced brain injury rats*

To investigate the effect of DEX on long-term learning and memory in rats, the MWM test was performed on the 28th day of neonatal rats. It was found that in D group, the fifth day of the rats escape latency was

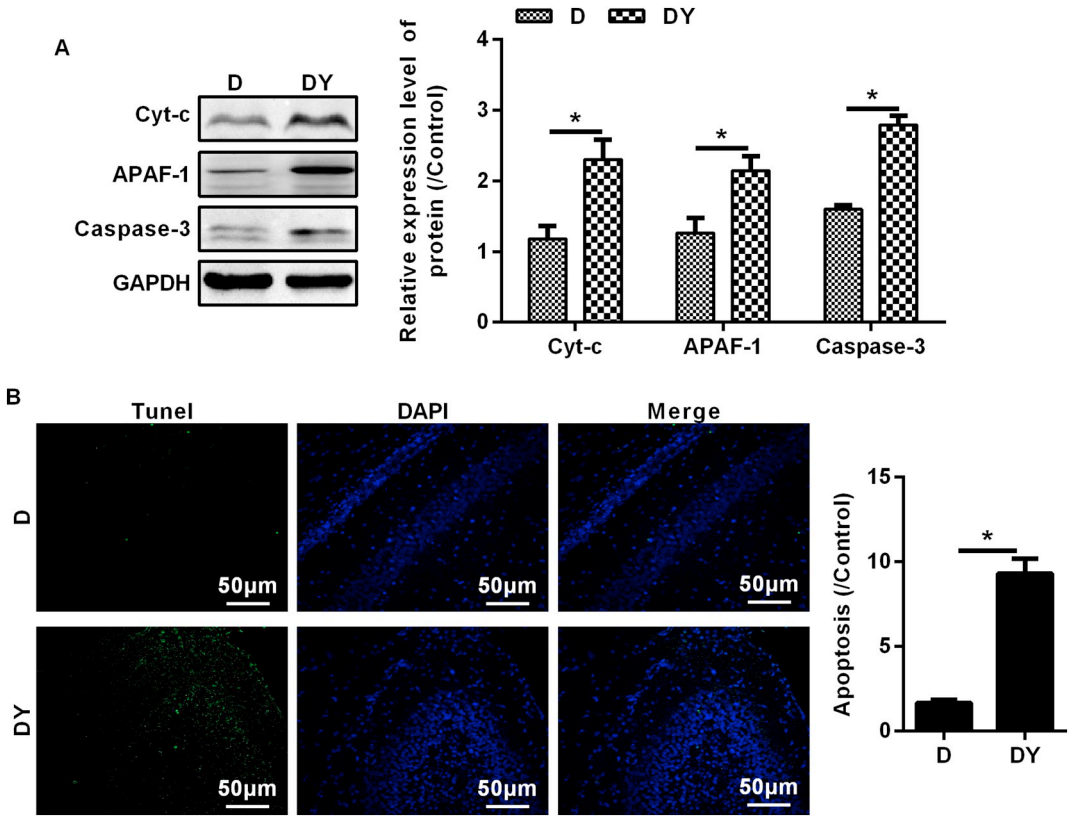


**Fig. 3.** Cyt-c, APAF-1 and Caspase-3 protein expression changes and the apoptosis of hippocampal neurons after treatment with α2 receptor inhibitor-yohimbine. (A) Protein expressions of Cyt-c, APAF-1, Caspase-3 were detected by Western-Blotting analysis after treatment with α2 receptor inhibitor-yohimbine. *Indicated DY group compared with D group; (b) TUNEL assay detects mitochondrial apoptosis in hippocampal neurons after treatment with α2 receptor inhibitor -yohimbine. *Indicated DY group compared with D group; scale bar = 50 μm. Cytochrome-C, Cyt-c; apoptotic protease activating factor-1, APAF-1; all data were obtained from three independent experiments.

significantly shortened, and the number of crossing platforms on the sixth day increased significantly in comparison with H/R group ($P < 0.05$). While compared with D group, the escape latency on the fifth day was significantly prolonged and the number of crossing the platform was significantly reduced in DY group and DM group ($P < 0.05$; Fig. 6).

## 4. Discussion

H/R-induced brain injury is a common problem in neonates, especially premature infants. It is mainly caused by perinatal asphyxia with a series of central nervous system abnormalities. Its pathogenesis is a process involving multiple factors, and apoptosis is the main cause of perinatal brain injury [15]. Most of the studies on perinatal H/R brain injury are carried out by using animal models to simulate the pathological process.

DEX is an $\alpha_2$ adrenergic receptor agonist that acts extensively on the mammalian brain. When the $\alpha_2$ adrenergic receptor located in the synaptic membrane is activated, it can produce sympathetic blockade and sedative analgesic effects, and its neuroprotective effect has attracted more and more attention [16]. In this study, a H/R -induced brain injury model was established in neonatal rats and the effect of DEX on the expression of injury and anti-injury factors in brain tissue was investigated at the molecular level. It has been reported that the anti-infective and anti-oxidative effects of dexamethasone are protective mechanisms against nerve damage. Inhibiting apoptosis and promoting neurogenesis are also important protective mechanisms of dexamethasone on the brain [17–20]. Yang et al. has proved that sevoflurane exposure in late pregnancy caused damage to the nervous system of rats, and abnormalities in autophagy and mitochondrial kinetics were associated with this neurotoxicity, which was antagonistic by DEX [21]. In our study, the protein expression of Cyt-c, APAF-1 and Caspase-3 in the D group was significantly decreased as compared with H/R group. In addition, hippocampal neuronal apoptosis rate was significantly reduced, indicating that DEX exerts neuroprotective effects by reducing mitochondrial apoptosis. Yan et al. [22] found that DEX can promote the secretion of glioblastogenic neurotrophic factor (BDNF) by astrocytes through α2 receptor, which is time-dependent and dose-dependent. This effect can be blocked by the α2 receptor antagonist-yohimbine, but not by the α1 receptor blocker piprazosin, suggesting that the neuroprotective effect of DEX is the α2 receptor dependent pathway. In our study, we added the α2 receptor antagonist-yohimbine. Yohimbine intervention results showed that the expression of Cyt-c was significantly increased in the APAF-1 and Caspase-3 DY groups compared with the D group. This result also confirms the previous conclusions.

Ngb is a novel endogenous neuroprotective factor with obvious functions of protecting neurons and belongs to anti-injury factors. Its high affinity to O2 and its high specificity in the nervous system suggest that Ngb may have protective effects against hypoxic-ischemic brain damage [23–25]. Sun et al. [26] found for the first time that ischemia and hypoxia can induce the expression of Ngb in nerve cells and inhibit the viability of nerve cells after Ngb expression. They also found that after inhibiting the expression of Ngb, the infarcted area of brain tissue increased and the neurological defects were aggravated, while increasing the expression of Ngb could reduce the infarcted area of brain tissue and improve the neurological defects. In addition, a previous has proved that Ngb can promote axonal regeneration through p38 during ischemia-reperfusion [27] to play a protective role, and also another study demonstrated that Ngb can promote neurogenesis through wnt

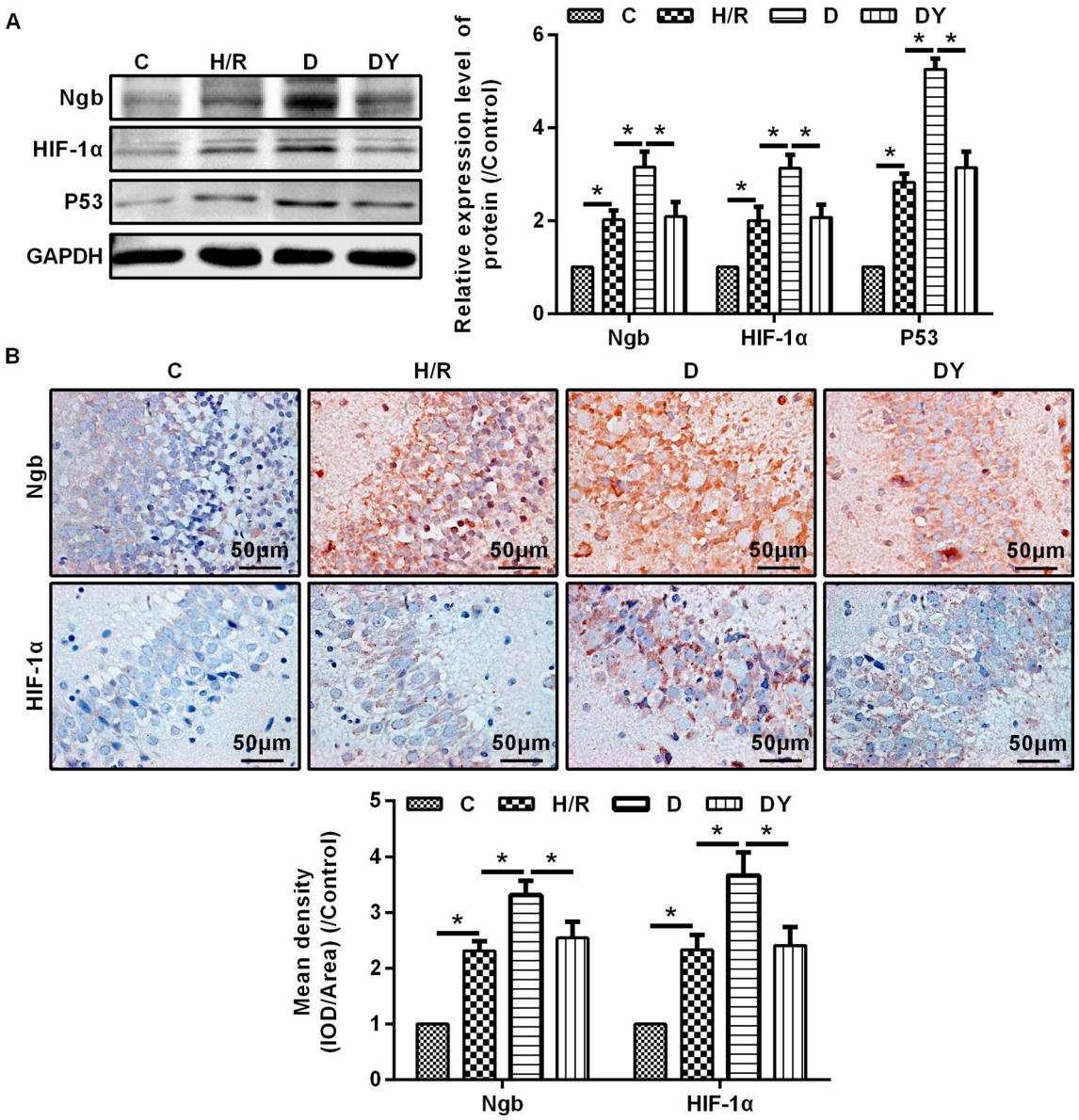


**Fig. 4.** The protein expressions changes of Ngb, HIF-1a and p53 and apoptosis of hippocampal neurons in each group. (A) Protein expressions changes of Ngb, HIF-1a and p53 were detected by Western-Blotting analysis. *Indicated H/R group compared with control group, D group compared with H/R group and DY group compared with D group. (B) Immunohistochemical analysis of Ngb and HIF-1α. *Indicated H/R group compared with control group, D group compared with H/R group and DY group compared with D group. Scale bar = 50 μm. Neuroglobin, Ngb. Hypoxia induction factor 1 alpha, HIF-1α. All data were obtained from three independent experiments.

signaling pathway [28]. Our study proved that the expression of Ngb in the H/R group was significantly increased, which was consistent with the above results. However, in D group, Ngb expressiom was higher, which has not been reported in this study, providing a new target for the clinical treatment of neonatal H/R brain injury. The results of the 28-day MWM showed that in the D group, the escape latency was significantly shortened on the fifth day, and the number of crossing platforms was significantly higher than that in the H/R group, indicating that DEX can increase the expression of Ngb to improve the ability of learning and memory in rats in the near and long term.

HIF-1α signaling pathway is the main signal transduction pathway of many factors, which is related to hypoxic-ischemic brain damage [29,30]. Cong et al. [31] found that DEX can protect rats brain from ischemia-reperfusion injury by up-regulating HIF-1α to inhibit neuronal autophagy. In this study, it was found that DEX can enhance the expression of HIF-1α, p53 and Ngb proteins related to HIF-1α/p53 pathway in H/R brain injury. The results suggested that, DEX has a protective effect to H/R brain injury and its mechanism of action may be achieved through the HIF-1α/p53 pathway. To further verify the relationship between DEX in the treatment of H/R brain injury and the HIF-1α/p53 signaling pathway, we intraperitoneally injected 2ME2, which was previously configured, to block the HIF-1α/p53 signaling pathway. Interestingly, we found that when the HIF-1α signaling pathway was inhibited, the promotion of DEX on HIF-1α, p53, and Ngb disappeared. This result further demonstrated our previous conclusion that DEX can play a role in the treatment of neonatal hypoxic encephalopathy by activating the HIF-1α/p53 signaling pathway. This provides a theoretical basis for DEX to be an effective treatment for neonatal hypoxic encephalopathy.

This study also has some limitations. DEX is usually administered by pump in clinical practice. However, the subjects of this study were 7-day-old SD rats with difficulty in caudal vein infusion and could not be completed under current conditions, so the drug was administered by single intraperitoneal injection. In addition, the results of this study

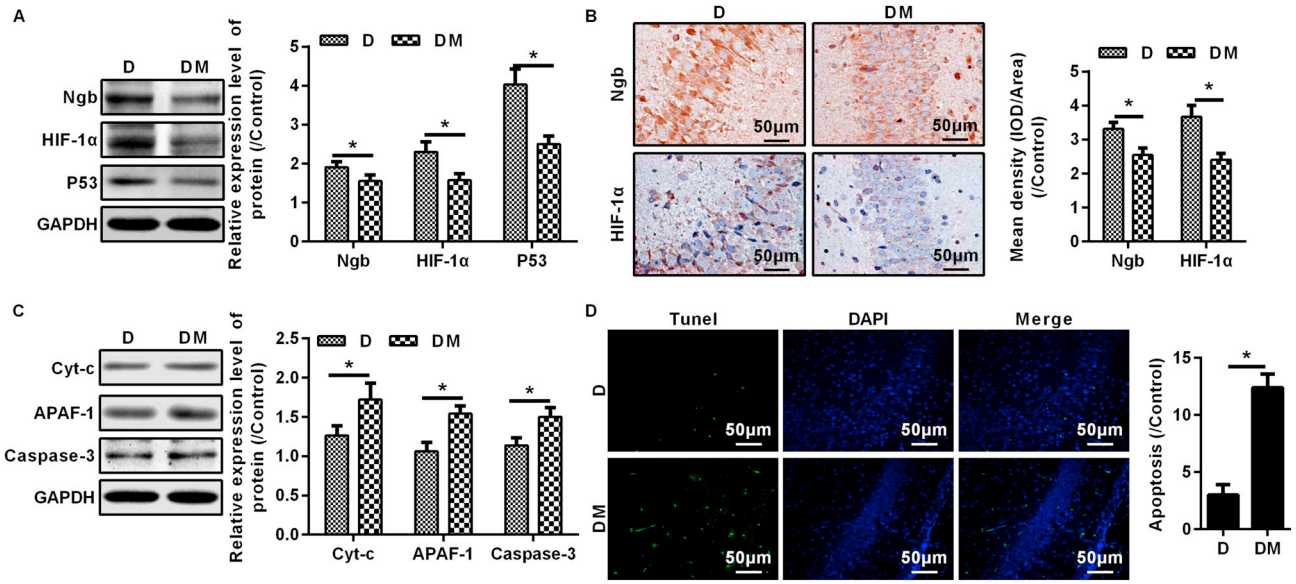


**Fig. 5.** Ngb, HIF-1α, p53, Cyt-c, APAF-1 and Caspase-3 protein expression changes after treatment with HIF-1a inhibitor-2ME2. (A) Protein expressions changes of Ngb, HIF-1α and p53 were detected by Western-Blotting analysis after treatment with HIF-1α inhibitor-2ME2. *Indicated DM group compared with D group; (B) immunohistochemical analysis of Ngb and HIF-1α after treatment with HIF-1α inhibitor-2ME2. *Indicated DM group compared with D group; scale bar = 50 μm. (C) Protein expressions changes of Cyt-c, APAF-1 and Caspase-3 were detected by Western-Blotting analysis after treatment with HIF-1α inhibitor-2ME2. *Indicated DM group compared with D group; (D) TUNEL assay detects mitochondrial apoptosis in hippocampal neurons after treatment with HIF-1a inhibitor-2ME2. *Indicated DM group compared with D group; scale bar = 50 μm Neuroglobin, Ngb. 2-methoxyestradiol, 2ME2. Hypoxia induction factor 1 alpha, HIF-1α. Cytochrome-C, Cyt-c; apoptotic protease activating factor-1, APAF-1; all data were obtained from three independent experiments.

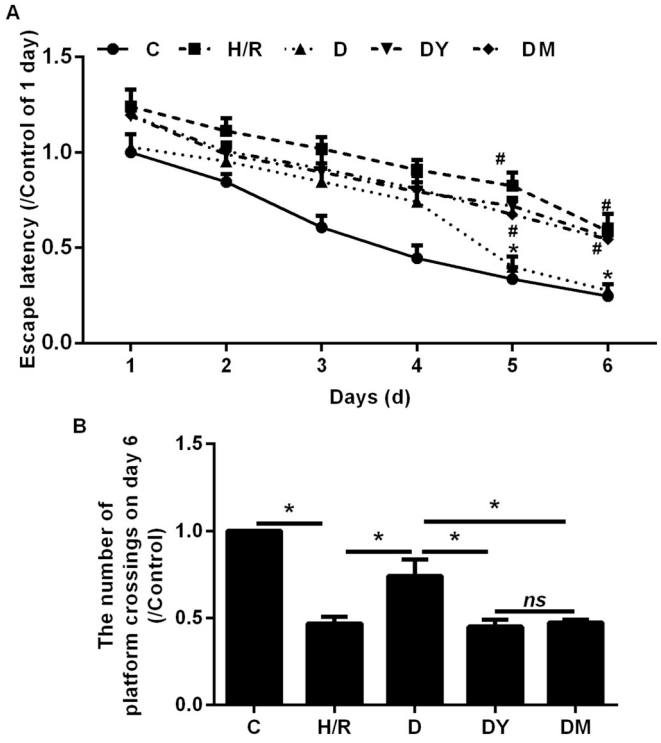


**Fig. 6.** Assessment of Morris water maze. It proved that DEX can improve the ability of learning and memory in rats in the near and long term. *Indicated H/R group compared with control group, D group compared H/R group, DY group and DM group. ns indicated DY group compared with DM group. All data were obtained from three independent experiments.

suggest that DEX can up-regulate the expression of Ngb and play a neuroprotective role in H/R injury. In view of this conclusion, we can study the regulatory effect of DEX on Ngb during monopulmonary ventilation or cardiopulmonary bypass during cardiac surgery in the future. In addition, future research should explore this effect in aged brains as patients with cardiac disease typically undergo procedures at risk for cerebral ischemia.

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## Ethics approval

All the animal experimental procedures were approved by the Experimental Animal Ethics Committee of Shengjing Hospital of China Medical University (NO. 2018PS406K). All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgements

None.

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