

Original Article

Protective against ischemia-reperfusion injury of rat hippocampal neurons and expression of GluR2/NR2B by dexmedetomidine by inhibition miR-199a-SIRT1 signal pathway

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Abstract: Cerebral ischemia is one severe condition for perioperative patients. Dexmedetomidine (Dex) is one novel high selective α_2 -adrenergic agonist and is one common auxiliary medicine in anesthetized surgery. miR-199a is expressed in hippocampal tissues, and related with impaired neurites, regeneration and synaptic plasticity. However, whether miR-199a-SIRT1 signal pathway is involved in expression of GluR2 or NR2B expression in hippocampal neurons after ischemia-reperfusion (I/R) injury with Dex pretreatment has not been reported. This study aimed to investigate protective role of Dex on neuron apoptosis in I/R rats and possible mechanisms. This study utilized rat cerebral I/R model, on which Western blot was used to detect protein expression. MiR-199a expression and cell apoptosis were measured by real-time quantitative PCR (RT-qPCR) and TUNEL assay. MiR-199a inhibitor of SIRT1-knockdown lentivirus was applied via intracerebral ventricular (ICV) injection for investigating related mechanisms. In cerebral I/R model, miR-199a expression was significantly elevated whilst SIRT1 protein was down-regulated. GluR2 and NR2B expression levels were remarkably decreased ($P < 0.05$ comparing to sham group). Dex treatment reversed such changes to certain extents and meanwhile alleviated hippocampal neuron apoptosis caused by I/R injury. After transfecting miR-199a or SIRT1-knockdown lentivirus, inhibitory effects of Dex on cell apoptosis were largely abolished. In conclusion, our study demonstrated that Dex can alleviate I/R induced neuron apoptosis in rats possibly via suppressing miR-199a-SIRT1 signal pathway, providing novel theory for treating cerebral I/R injury.

Keywords: Dexmedetomidine, miR-199a, cerebral ischemia reperfusion

Introduction

Cerebral ischemia resides in pathological processes of various neurologic diseases such as cerebrovascular disorders or brain tumors, as well as in systemic conditions including heart arrest and shock. Brain ischemia can be displayed as focal or diffused, or be divided into permeant or temporal sub-types. Perioperative brain ischemia is one major form of cerebral ischemia injury, and may cause severe consequences [1]. Dexmedetomidine (Dex) is one novel high selective α_2 -adrenergic agonist. It has sedative, hypnosis, painkiller and antianxiety functions, and has certain protective effects including suppressing sympathetic nerve activ-

ity, stabilizing hemodynamics, and inhibiting stress response, and thus has become common auxiliary medicine in anesthetized operation [2, 3]. However, whether pre-treatment of Dex has effects on GluR2 and NR2B expression in hippocampal neurons on I/R rats, and whether miR-199a/SIRT1 signal pathway is involved are still unreported yet.

SIRT1 is one nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, and belongs to type 3 histone deacetylase. SIRT1 plays critical roles in various cellular processes including cell apoptosis and survival, endocrine signal transduction, chromatin remodeling and gene transcription [4, 5]. SIRT1 is commonly believed to

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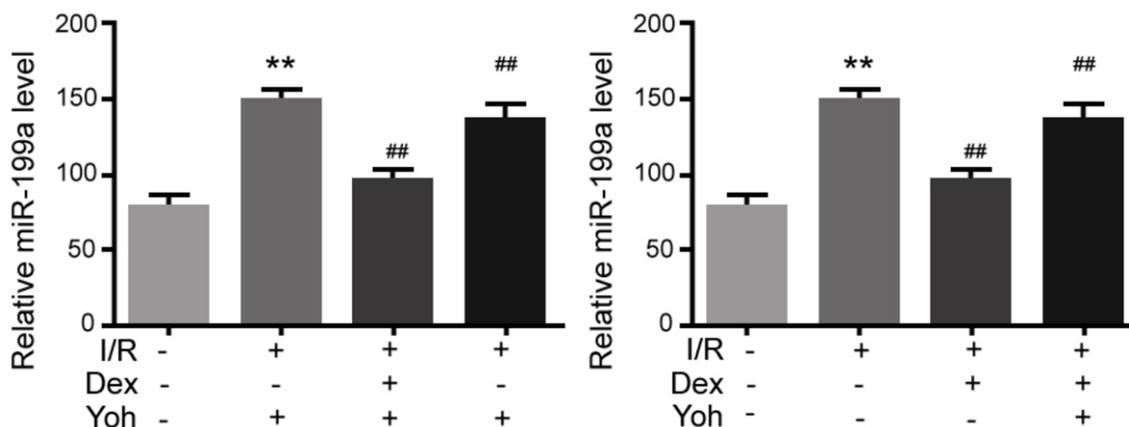


Figure 1. Hippocampal miR-199a expression level in I/R rats. **, $P < 0.05$ compared to sham group; ##, $P < 0.05$ compared to I/R group.

mediate cell apoptosis via modulating acetylation of target proteins [6]. In rat liver tissues, SIRT1 can regulate apoptosis via deacetylation on p53 [7]. Recent studies identified SIRT1 as one important endogenous apoptotic inhibitor in neurodegenerative disease [8]. Other studies showed critical roles of SIRT1 in facilitating axonal regeneration and synaptic growth of mammalian species [9]. Induced cell apoptosis is one important reason for ischemia-reperfusion (I/R) injury [10]. SIRT1 has been shown to be down-regulated in various I/R models on kidney [10], liver [11] and intestine [12], while the up-regulation of SIRT1 has protective effects on multiple I/R injuries.

Recent studies showed critical roles of microRNA (miR) in onset and progression of multiple diseases. MiR is one group of non-coding small molecule single stranded RNA with 18~24 bp length, and can bind with 3'-untranslated region (3'UTR) of target mRNA via completely or incompletely complementary binding. Under the direction of RNA exonuclease, it can achieve selective degradation of mRNA, thus inhibiting or activating downstream genes [13]. MiR mainly regulates downstream genes via suppressing mRNA transcription. Meanwhile, mRNA degradation or activation modulation can affect cellular functions or oncogene activation via down-regulating certain protein levels [14].

Some evidences showed that miR might be critical for pathogenesis of various neurologic diseases, and is novel treatment targets for

neurodegenerative diseases such as epilepsy [15, 16]. Previous studies showed specific expression of miR-199a in hippocampal tissues, and was related with impaired neurites, regeneration and synaptic plasticity [17]. Moreover, recent studies found that miR-199a directly functions on miRNA of SIRT1 [18]. However, the exact role of miR-199a in I/R remains poorly understood.

This study aimed to generate a rat cerebral I/R model, on which miR-199a agonist or inhibitor was applied to investigate the effect of Dex on rat cerebral I/R induced hippocampal neuron injury and expression of GluR2 or NR2B, along with the regulatory function of miR-199a-SIRT1 signal pathway.

Materials and methods

Major reagents

Dex was purchased from Enhua Pharma Corp (China). Yohimbine (Yoh) was purchased from Yuancheng Saichuang Tech (China). Endogenous reference antibody β -actin was purchased from Kangcheng Bio (China). P53 antibody was purchased from Abcam (Hong Kong, China). Rabbit anti-mouse IgG (H+L), rabbit anti-mouse IgG (H+L) were purchased from Proteintech (China). SYBR Green PCR Master Mix was purchased from Toyobo (China). MiR-199a inhibitor, mimics and negative control (NC) sequences were purchased from Gimma Corp (China). ECL chromogenic substrate was purchased from Beyotime (China). TUNEL apoptosis assay kit was purchased from Beyotime

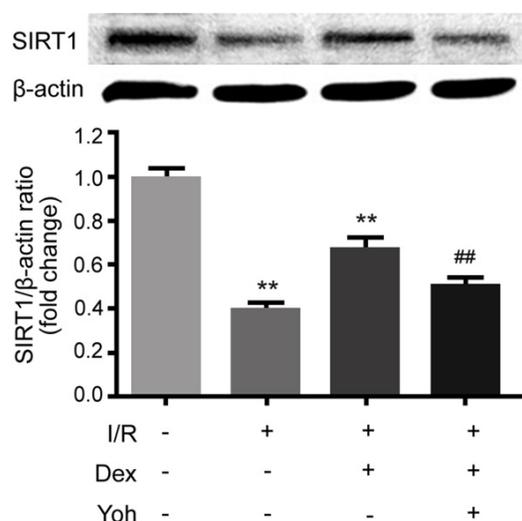


Figure 2. SIRT1 expression level in I/R rats. **, $P < 0.05$ compared to sham group; ##, $P < 0.05$ compared to I/R group.

(China). MiR-199a agonist and SIRT1 knock-down lentivirus were provided by Gimma Biotech (China).

Major equipment

Stereotaxic apparatus was purchased from Ruanlong Tech Corp (China). Gel imaging system UVP Multispectral (CA, US); PS-9 semi-dry transferring electrophoresis apparatus was purchased from Jingmai Tech (China). Model Thermo-354 microplate reader was purchased from Thermo Fisher (NY, US).

Experimental animal

Male SD rats (2 months age, $N=60$) were provided by Laboratory Animal Center, The Second Military Medical University.

Model preparation

Whole-brain I/R model was prepared as previous documentation [19]. Anesthetized rats were fixed on the stage of stereotaxic apparatus. The head skin was prepared for a middle incision after iodine sterilization to expose bilateral cervical vertebra hole. Coagulating electrode was inserted into bilateral holes to permanently block bilateral vertebral artery. The animal was then placed in a supine position, and a middle incision was made to expose bilateral common carotid artery. 24 h later, rats

were re-anesthetized and the frontier cervical incision was opened to clamp bilateral common carotid artery for making whole brain ischemia. 5 min later, bilateral artery clips were removed to re-perfuse the brain.

Drug delivery

Dex (100 $\mu\text{g}/\text{kg}$) and yohimbine (100 $\mu\text{g}/\text{kg}$) was applied 30 min before ischemia. 50 ng/kg miR-199a inhibitor or 20 ng/kg lentivirus vector with SIRT1 knockdown was applied into the ventricles 24 h before ischemia.

Extraction of hippocampal tissue protein

Rat hippocampal tissues were rinsed in PBS, and excess water was removed by filter paper. 10 μL 100 mM PMSF was added into 1 mL lysate. 1000 μL lysate solution was mixed with 100 mg tissues for homogenization, followed by iced incubation for 5~10 min. The mixture was centrifuged at 4°C for 12000 rpm for 5 min. The supernatant was saved as total protein solution.

Western blot

Total protein solution was quantified by BCA approach and was equalized to identical concentrations by saline. After adding the loading buffer, proteins were denatures in boiled water for 5 min, and was loaded into 10% SDS-PAGE for electrophoresis separation of targeted protein and proteins with similar molecular weight. Proteins were then transferred to PVDF membrane under 300 mA for 1 h. Anti-rabbit SIRT1 antibody (1:1000) was added for 4°C overnight incubation. After three times of PBST rinsing, secondary antibody (1:1000) was added for 37°C incubation for 2 h. Chemiluminescence approach was used to visualize the protein band.

Total RNA extraction

1 mL Trizol was mixed with 100 mg hippocampal tissue samples for 5 min iced incubation. After mixture in pipetting, lysate was saved and mixed with 200 μL chloroform, and was processed by 15 s vigorous shaking and 3 min room temperature incubation. With 4°C centrifugation at 12000 g for 10 min, the supernatant was discarded and 1 ml ethanol was added for three times of rinsing. The supernatant was

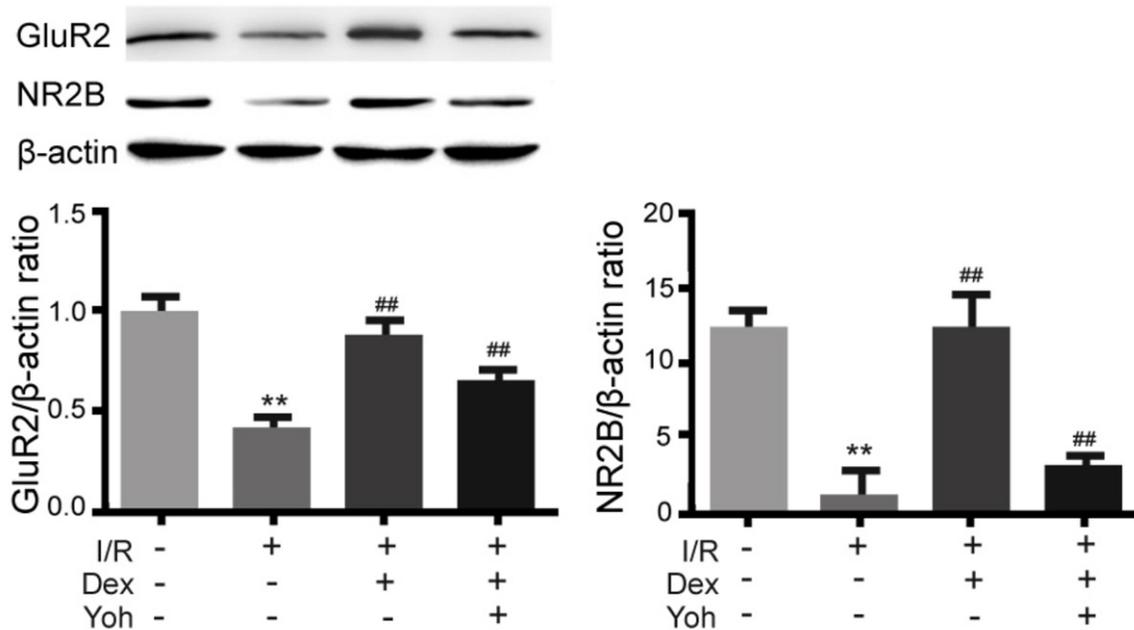


Figure 3. Hippocampal expression of GluR2 and NR2B in ischemia rat hippocampal tissues. **, $P < 0.05$ compared to sham group; ##, $P < 0.05$ compared to I/R group.

carefully removed and mRNA was dissolved in 20 μ L DEPC water.

Real-time quantitative PCR

MiR-199a primers were designed and synthesized by Sigma using the following sequence [20]: forward, 5'-TTGAA TTCTA ACACC TTCGT GGCTA CAGAG-3'; reverse, 5'-TTAGA TCTCA TTTAT CGAGG GAAGG ATTG-3'. U6 RNA was used as the internal reference using forward primer (5'-CTCGC TTCGG CAGCA CA-3') and reverse primer (5'-AACGC TTCAC GAATT TGCCT-3'). PCR was performed in a 50 μ l system following the manual instruction using the program: 50°C for 30 min, followed by 95°C for 5 min, and 40 cycles each containing 95°C for 30 s, 55°C for 30 s and 72°C for 50 s, and ended with 72°C for 5 min. After reaction, amplification curve and melting curve were confirmed. Relative expression level was calculated by comparing Ct values of target gene against internal reference gene, and gene expression was quantified by 2- $\Delta\Delta$ Ct approach.

TUNEL staining

Hippocampal tissues from all groups of rats were prepared in cyro-sections, and were fixed

in paraformaldehyde. Slices were rinsed in 3% H₂O₂-methanol solution for 10 min, and were incubated in 0.2% Triton for 5 min. With twice PBS rinsing, 50 μ L TUNEL reaction solution was added into each well for 37°C dark incubation for 1 h. With three times of PBS rinsing, images were captured under a fluorescent microscope. Five fields were randomly selected from each well and the number of positive staining cells was recorded for statistical analysis.

Statistical methods

SPSS10.0 software was used for processing all experimental data, which were presented as mean \pm standard deviation (SD). For between-group comparison, one-way analysis of variance (ANOVA) was performed and further paired comparison was performed by SNK-Q test.

Results

MiR-199a expression in cerebral ischemia rats

We measured hippocampal miR-199a expression and as shown in **Figure 1**, I/R group showed significantly elevated miR-199a expression ($P < 0.05$ comparing to sham group). After

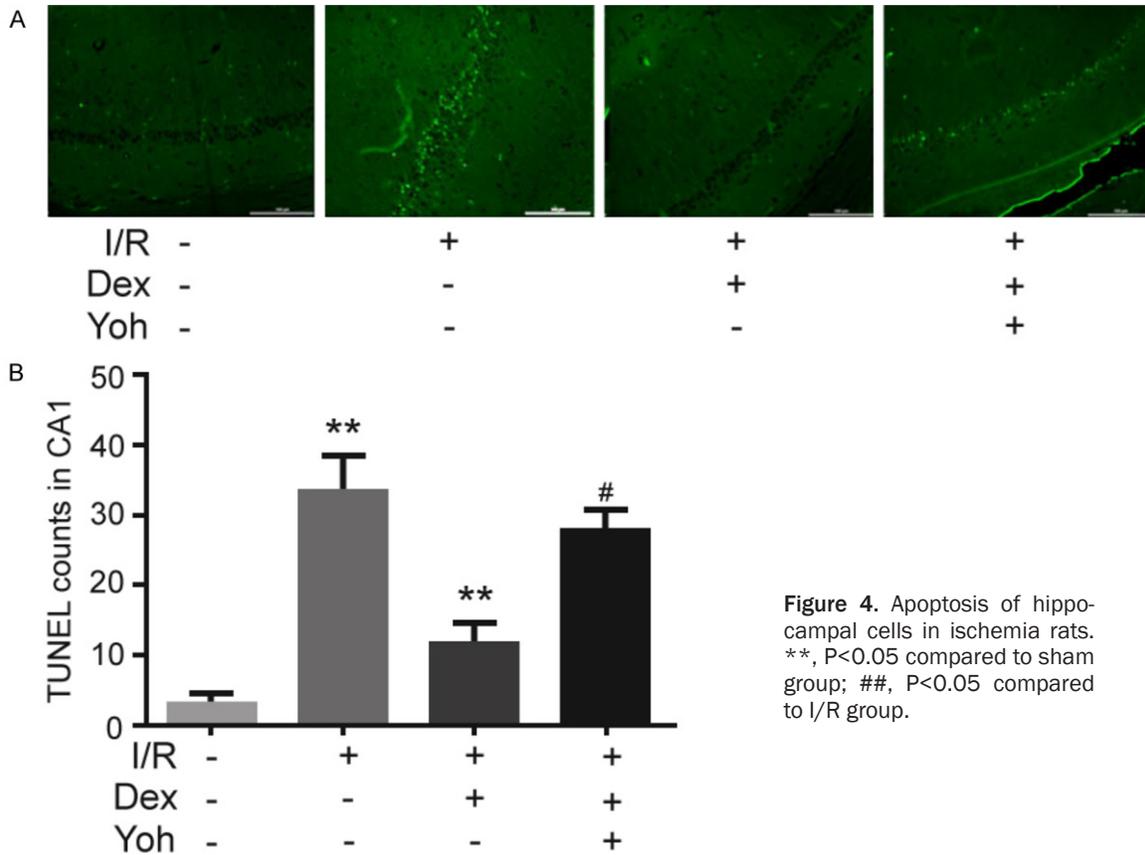


Figure 4. Apoptosis of hippocampal cells in ischemia rats. **, P<0.05 compared to sham group; ##, P<0.05 compared to I/R group.

Dex treatment, I/R-induced miR-199a over-expression was significantly depressed. The treatment of Yohimbine significantly suppressed inhibitor effect of Dex on miR-199a. These results suggested that Dex treatment could suppress miR-199a expression.

SIRT expression level in ischemia rats

With reference to database including Targetscan or miRNA, we proposed that SIRT1 might be the target gene of miR-199a. Meantime SIRT1 plays a protective role against I/R injury of liver or intestinal tissues [18]. Therefore in this study, we measured SIRT1 expression. As shown in **Figure 2**, compared to sham group, I/R significantly decreased SIRT1 expression, and Dex treatment remarkably elevated SIRT1 expression. Comparing to single usage of Dex, Yohimbine can inhibit the up-regulation effect on SIRT1 by Dex to certain extents. These results showed that Dex could up-regulate SIRT1 expression. Based on literatures, we proposed that Dex could up-regulate SIRT1 probably related with inhibiting miR-199a expression.

GluR2 and NR2B expression level in ischemia rat hippocampal tissues

We measured expression level of GluR2 and NR2B proteins. As shown in **Figure 3**, I/R group showed significantly depressed GluR2 or NR2B protein expression. Dex treatment can reverse I/R induced down-regulation of GluR2 or NR2B to certain extents. Yohimbine treatment inhibited up-regulatory effect on GluR2 and NR2B proteins by Dex. All these results showed that Dex could protect I/R injury via up-regulating GluR2 and NR2B protein expression.

Apoptosis of hippocampal cells in ischemia rats

Cell apoptosis is one important index reflecting I/R injury severity. TUNEL approach was used to measure cell apoptosis. As shown in **Figure 4**, there are significantly more TUNEL-positive cells in I/R group. Dex treatment significantly decreased the number of TUNEL-positive cells, whilst Yohimbine treatment could decrease Dex-induced inhibition on hippocampal neuron apoptosis.

miR in I/R injury

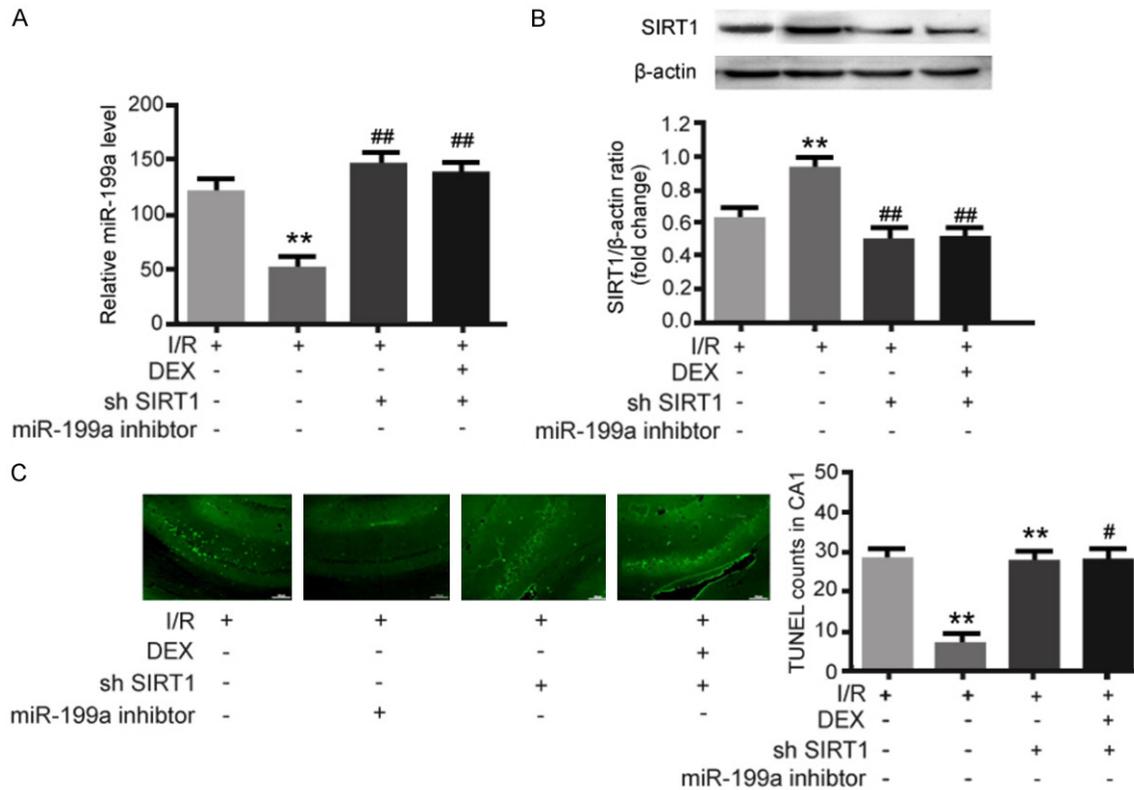


Figure 5. Dex suppressed hippocampal neuron apoptosis via miR-199a inhibition and SIRT1 up-regulation. **, P<0.05 compared to sham group; ##, P<0.05 compared to I/R group.

Dex inhibited miR-199a to up-regulate SIRT1 expression for suppressing hippocampal neuron apoptosis

These abovementioned results showed that Dex could up-regulate GluR2 and NR2B expression level in ischemia rat hippocampal tissues to suppress neuron apoptosis. In the meanwhile, Dex could up-regulate SIRT1 level via inhibiting miR-199a. We thus speculated that Dex probably protected cerebral I/R injury via inhibiting miR-199a/SIRT1 signal pathway. We thus utilized miR-199a or lentiviral vector with SIRT1 knockdown to investigate its mechanisms. As shown in **Figure 5C**, lentivirus-mediated SIRT1 knockdown significantly enhanced the number of apoptotic neurons, and Dex-mediated inhibitory effects on apoptosis were significantly compromised. These data suggested that Dex-mediated apoptotic inhibition on hippocampal neurons was dependent on SIRT1. We then transfected miR-199 inhibitor, which was found to have similar effects as those of Dex, as they all inhibited miR-199a level (**Figure 5A**), thus up-regulating SIRT1

(**Figure 5B**) to suppress cell apoptosis. These data indicated that Dex has protective roles on hippocampal neurons probably via inhibiting miR-199a and further up-regulation of SIRT1 expression.

Discussion

Cerebral I/R model can mimic the clinical conditions such as hypotension shock or cardiac arrest with high efficiency. Neuronal death during cerebral I/R is one dynamic process, whose mechanism has not been fully illustrated. Several schools including energy failure, excitotoxicity, calcium overloading or oxygen free radicals have been proposed, and calcium overloading may play indispensable roles in I/R injury as the last pathway for neuron death [21].

As one important subunit of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), GluR2 plays a critical role in modifying permeability of calcium ion channels [22]. Study showed that severe secondary injury may enhance calcium ion permeability of AMPAR via

suppressing GluR2 subunit expression level, thus aggravating neuron damage. Meanwhile large amounts of calcium inward are one critical factor inducing neuron death [23]. In this study, we identified decreased GluR2 down-regulation, which was accompanied with large amounts of neuron apoptosis. Dex, on the other hand, can improve down-regulation of GluR2 to certain extents, and inhibit neuron apoptosis.

N-methyl-D-aspartate (NMDA) receptor, especially its NR2B subunit, plays a crucial role in synaptic plasticity and learning or memory process [24]. Early study utilized NR2B over-expressing transgenic mice and showed that elongated opening of hippocampal NMDA receptor resulted in improved learning and memory performance in behavior tasks including water maze, tone- or contextual-specific conditional fear learning. These data suggested that NR2B played an important role in hippocampal synaptic plasticity and learning/memory function. In this study, we found significantly decreased NR2B expression in I/R rats, and Dex could potentiate NR2B expression level to certain extents. These results suggested that Dex played certain roles in alleviating learning or memory deficit in I/R rats.

As one histone deacetylase, SIRT1 expression is critical for animal survival. Studies in sarcoma showed that suppression of SIRT1 expression could alleviate cell autophagy process and induced tumor cell death [25]. SIRT1 has been widely studied in heart, kidney and Alzheimer's disease models, in which SIRT1 inactivation and NAD consumption are critical factors inducing epilepsy induced neuron death [26]. In the meanwhile, some studies pointed that SIRT1 agonist resveratrol could protect neurons and relieve epilepsy via up-regulating SIRT1. Similar findings can be found in I/R models of intestine or liver models. This study revealed similar functions between Dex and resveratrol, as both of which could potentiate SIRT1 expression. These data suggested that Dex protected cerebral I/R injury probably via up-regulating SIRT1. However, as a histone deacetylase, whether SIRT1 ameliorates cerebral I/R injury through acting on histone remains unclear and requires further investigation.

Recent studies showed that the inhibition of expression of certain miRNA molecules including miR-34a, miR-132 and miR-134 could exert

direct or indirect neuroprotective functions, but leaving its mechanism unexplored. These data suggested that miRNA played a crucial role in modulating neuroprotection, and their involvement in I/R injury [13]. In this study, miR-199a is one upstream regulatory target of SIRT1 and has been found to be remarkably up-regulated in I/R model, which also had prominent inhibition of SIRT1. However, Dex could inhibit over-expression of miR-199a in I/R model to certain extents. We thus speculated that Dex achieved its protective role against I/R injury probably via suppressing miR-199a signaling pathway. Via intraventricular drug delivery, we successfully knocked-down SIRT1 expression level and suppressed miR-199a simultaneously, proving that Dex did exert its neuroprotective functions against I/R injury via suppressing miR-199a expression and consequent up-regulation of SIRT1.

In this study, we proved that Dex had protective role against cerebral I/R injury on rats. Via intra-ventricular drug deliver, we demonstrated that Dex exerted such protective roles via inhibiting miR-199a-SIRT1 signaling pathway, whilst Yohimbine could block Dex effects to certain extents, suggesting the involvement of alpha-receptor in such regulation. However, the mechanism of coupling between miR-199a-SIRT1 signaling pathway and Dex requires further experiments to validate.

Conclusion

Our study demonstrated that Dex has protective functions against cerebral I/R injury, via inhibiting miR-199a-SIRT1 signal pathway. This study provides novel targets for cerebral I/R treatment.

Disclosure of conflict of interest

None.

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