Role of Sirtuin-1 in Neonatal Hypoxic-Ischemic Encephalopathy and Its Underlying Mechanism

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Source of support: The present study was supported by the Funding Project of Bengbu Medical College of Science and Technology Development (grant no. BYKF1867) and the Key Project of Natural Science of Colleges and Universities in Anhui Province (grant no. KJ2019A0378)

Background: Neonatal hypoxic-ischemic encephalopathy (HIE) is a dreaded disease and one of the leading causes of severe neurological dysfunction in neonates. The present study explored the functions of Sirtuin-1 (SIRT1) in neonatal HIE.

Material/Methods: A HIE neonatal rat model was generated to determine SIRT1 levels in brain tissues. Cell apoptosis and cell viability were analyzed by flow cytometry and MTT assay. qRT-PCR and Western blot analysis were used to assess gene mRNA and protein levels. Subsequently, the effect of SIRT1 on HIE was investigated in vitro by constructing an oxygen-glucose deprivation (OGD) cell model.

Results: The effective construction of the HIE rat model was confirmed by the enhanced brain cell apoptosis and the increased expression of HIE-related molecular markers, including S100 calcium-binding protein B (S100B) and neuron-specific enolase (NSE). SIRT1 expression was downregulated in HIE rat brain tissues. These findings indicated that SIRT1 was downregulated in neuronal cells subjected to OGD. In addition, enhanced cell viability and reduced cell apoptosis were observed, suggesting that SIRT1 overexpression relieved OGD-induced neuronal cell injury. Transfection with SIRT1-siRNA further increased OGD-induced neuronal cell injury, evidenced by decreased cell viability and enhanced cell apoptosis. Finally, SIRT1 overexpression significantly downregulated p-p65 protein expression.

Conclusions: Our findings revealed that SIRT1 may be a novel and promising therapy target for HIE treatment.

MeSH Keywords: Hypoxia-Ischemia, Brain • Neurons • Sirtuin 1

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/924544

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Background

HIE is a common type of brain injury caused by various perinatal factors of cerebral hypoxia or ischemia [1,2]. HIE mainly causes damage to neurons and white matter, thus adversely affecting the brain of a developing baby [3]. HIE is one of the primary causes of infant mortality and the most common cause of neonatal seizures [4–6]. In addition, HIE has a high mortality rate, long-term incidence, poor prognosis, and complex pathogenesis. Previous research reported HIE incidence rates of 1.5 cases per 1000 live births in developed countries and 10–20 per 1000 live births in low- and middle-income countries [7,8]. About 24% of HIE patients die in the neonatal intensive care unit (NICU). Among the surviving newborns, cerebral palsy (10–20%), auditory and visual problems (about 40%), and motor and behavioral impairments such as overall developmental delay, epilepsy, and autism [9–11] are diagnosed. The advancement of medical standards has provided a deeper understanding of fetal pathophysiology. However, HIE remains a critical complication of mature infants, and further investigation is needed to improve HIE treatment.

Sirtuin-1 (SIRT1), a NAD-dependent lysine deacetylase, is stimulated under stress in various cell types [12]. SIRT1 protects the heart from aging, hypertrophy, and myocardial infarction [13–15]. Sun et al. indicated that SIRT1 is involved in the vascular system by regulating cell proliferation and cell cycle [16]. Nuclear factor-κB (NF-κB) is deacetylated by SIRT1. In addition, many studies have reported that SIRT1 is a vital regulator in various biological processes, including cisplatin (CDDP)-induced oxidative stress and inflammatory response via the activation of downstream genes at transcriptional levels, such as nuclear-receptor factor-2 (Nrf2) and NF-κB [17, 18]. Nevertheless, whether SIRT1 exhibits functions in neonatal HIE remains unclear.

We designed this research to assess the roles of SIRT1 in neonatal HIE and to explore the underlying mechanism.

Material and Methods

Animals

A total of 10 male Sprague-Dawley rats (7 days old, weighing 18–20 g) were provided by the Experimental Animal Center of Kunming Medical University (China). All animals were kept in a controlled environment (22–25°C, 40–50% humidity) and a 12-h light/dark cycle with ad libitum access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Bengbu Medical College and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rat HIE model conduction

We anesthetized 7-day-old rats with isoflurane (4% induction and 1.5% maintenance) before surgery. The left common carotid artery was ligated at both ends using a 6-0 silk surgical suture, and cut along the middle. Following 1.5 h of recovery, the rats were placed in an anoxic sealed chamber (N₂ and 8% O₂ equilibrated) at 37°C for 2.5 h. The sham-operated rats were anesthetized and the left common carotid arteries were exposed, but not ligated. Rats were anesthetized with propylthiouracil injection (40 mg/kg) and then killed by cervical vertebra dislocation (rats with cardiac and respiratory arrest were confirmed as dead) at 72 h following hypoxia for further experiments. All efforts were made to alleviate the pain of the rats throughout the experiment. In addition, all experiments were stopped when the rats lost more than 15% of their body weight.

Cell culture and transfection

Primary neuronal cells were isolated from newborn rats. Briefly, the rats were anesthetized and then killed by cervical dislocation. The hippocampal tissue was collected, digested with 0.05% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and then centrifuged. Subsequently, the deposits were re-suspended in DMEM medium (Gibco; Thermo Fisher Scientific, Inc) supplemented with 5% serum (Gibco; Thermo Fisher Scientific, Inc.). Primary rat neurons were transfected with 0.2 μM SIRT1-siRNA, 0.2 μM control-siRNA, 1 μg SIRT1-plasmid, or 1 μg control-plasmid for 48 h using lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc) in accordance with the manufacturer’s instructions. Transfection efficiency was determined using qRT-PCR and Western blot assay 48 h following cell transfection. Experiments were repeated 3 times.

OGD model establishment

Primary rat neurons were grown in glucose-free DMEM (without FBS and glucose) and then cultured in a humidified atmosphere containing 95% N₂ and 5% CO₂ at 37°C for 2 h. Subsequently, the cell culture medium was replaced with complete medium prior the completion of the OGD. Cells were then placed into a normal incubator for an additional 24 h. The normal medium under normoxia served as the control.

MTT assay

Primary rat neurons were transfected with SIRT1-siRNA, control-siRNA, SIRT1-plasmid, or control-plasmid for 48 h and subsequently underwent OGD treatment. Next, cells were stimulated with 20 μl MTT reagent (Solarbio) and incubated for an additional 4 h at 37°C. DMSO (150 μl) was added into each
well and shaken for 15 min. The optical density (OD) was detected at 570 nm by a micro-plate reader. Experiments were repeated 3 times.

**Cell apoptosis assay**

Neuronal cell apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (MultiSciences Biotech Co.) according to the manufacturer’s instructions. In brief, Annexin V-FITC and PI were used to dye neuronal cells for 30 min in the dark. The apoptotic cells were measured using flow cytometry (BD Biosciences) and the data were analyzed using WinMDI software (version 2.5; Purdue University Cytometry Laboratories). Experiments were repeated 3 times.

Rat brain tissues were mechanically dissociated into single-cell suspension using a 200-mesh sieve to investigate cell apoptosis in HIE rats. Subsequently, the cell suspension was centrifuged (670.8×g) at 4°C for 5 min and the supernatant was discarded. The cell apoptosis was analyzed as previously described.

**qRT-PCR assay**

Total RNA from brain tissue and neuronal cells were treated with Trizol (Takara Biotechnology Co.) following the manufacturer’s protocol. Then, cDNA was synthesized from total RNA using a reverse transcription kit (Vazyme) according to the manufacturer’s protocol. The reaction volume was 20 μl and the reverse transcription reaction conditions were 70°C for 5 min, 37°C for 5 min, and 42°C for 60 min. qRT-PCR was performed using a SYBR kit (Vazyme). The qPCR reaction was conducted with the following parameters: 95°C for 3 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Gene expressions were performed by 2^ΔΔCt method.

**Western blot analysis**

RIPA lysis buffer (Beyotime, China) and protease inhibitors were used to isolate total proteins from neuronal cells or brain tissues. Cells were incubated for 30 min and then re-suspended with lysis. Cells suspension was centrifuged and the supernatants containing proteins were stored prior to use. The protein concentration was determined by a BCA assay kit. Enhanced chemiluminescence method (ECL, EMD Millipore). GAPDH protein expression levels served as the loading control for normalization.

Primary antibodies anti-Bcl-2, anti-Bax, anti-SIRT1, anti-p65, anti-p-p65, anti-GAPDH and the secondary goat anti-rabbit antibody were obtained from Cell Signaling Technology.

**Statistical analysis**

The results are depicted as the mean±SD from triplicate experiments. GraphPad 6.0 software was used for the statistical analysis. The t test and one-way ANOVA followed by Tukey’s test were performed to assess statistically significant differences between 2 and multiple groups, respectively. P<0.05 indicated a statistically significant difference.

**Results**

**Expression level of SIRT1 in HIE rats brain tissues.**

Three days after establishing the neonatal HIE rat model, the brain tissue was isolated and the brain cell apoptosis was assessed using flow cytometry. We found more apoptotic cells in rat brain tissues of the HIE group than in the control group (Figure 1A, 1B). Subsequently, the levels of related molecular markers S100B and NSE, which participate in the progression of HIE [19–22], were detected. Western blot assay revealed that S100B and NSE protein levels were significantly higher in the HIE group than in the control group (Figure 1C). Western blot assay and qRT-PCR were conducted to detect the expression of SIRT1 in HIE neonatal rats brain tissues. As indicated in Figure 1D and 1E, the levels of SIRT1 were distinctly lower in the rat brain tissue of the HIE group compared to the control group.

**Levels of SIRT1 in the OGD model**

The OGD cell model was established in vitro. Primary rat neuronal cells were exposed to OGD (95% N₂ and 5% CO₂) for 48 h. Western blot assay showed that OGD treatment markedly enhanced S100B and NSE protein levels in cultured neurons (Figure 2A). Western blot assay and qRT-PCR were performed to investigate the SIRT1 expression levels. SIRT1 mRNA levels and protein expression were obviously lower in the OGD group than in the control group (Figure 2B, 2C).

**Transfection efficiency of SIRT1-plasmid/SIRT1-siRNA in primary rat neuronal cells**

To assess the roles of SIRT1 in HIE, we first determined the transfection efficiency of SIRT1 overexpression/silencing in primary rat neuronal cells. SIRT1-siRNA, control-siRNA,
SIRT1-plasmid, or control-plasmid were transfected in primary rat neuronal cells for 48 h and SIRT1 levels were analyzed using qRT-PCR and Western blot assays. The results showed that transfection of the primary rat neuronal cells with the SIRT1-plasmid (Figure 3A, 3B) and SIRT1-siRNA (Figure 3C, 3D) increased and decreased the expression levels of SIRT1, respectively, compared with the control group.

SIRT1 overexpression relieved OGD-treated neuronal cell damage

To further explore the effect of SIRT1 overexpression (SIRT1-plasmid) on OGD-induced neuronal cell injury, MTT and flow cytometry were performed to evaluate cell viability and apoptosis, respectively. Our data demonstrated that cell viability in
the OGD group was notably lower than in the control group. In addition, SIRT1 overexpression increased neuronal cells viability compared with the OGD group (Figure 4A). Compared with the control group, cell apoptosis in the OGD group was higher, whereas SIRT1 overexpression decreased neuronal cell apoptosis (Figure 4B, 4C). In addition, Western blot assay showed that Bax and Bcl-2 expression levels were increased and decreased in the OGD group, respectively. Compared with the OGD group, SIRT1 overexpression inhibited Bax and induced Bcl-2 protein expression (Figure 4D–4F). These results indicated that SIRT1 overexpression reduced the OGD-induced cell injury.

SIRT1 silencing further aggravated OGD-induced neuronal cell injury

Subsequently, the effect of SIRT1 silencing (SIRT1-siRNA) on OGD-induced neuronal cell injury was investigated. MTT assay revealed a decreased cell viability rate in the OGD group compared to the control (Figure 5A). Compared with the OGD group, Western blot assay showed that Bax and Bcl-2 expression levels were increased and decreased in the OGD group, respectively. SIRT1 downregulation further promoted neuronal cell apoptosis (Figure 5B, 5C). Finally, Western blot assay showed that Bax expression was increased and Bcl-2 expression was decreased in the OGD group. However, SIRT1 silencing obviously increased and reduced the expression of Bax and Bcl-2, respectively (Figure 5D). These results indicated that SIRT1 downregulation further aggravated OGD-induced neuronal cell injury.

The effect of SIRT1 expression on NF-κB signaling pathway in OGD-induced neuronal cells

Finally, we investigated the specific potential mechanisms of SIRT1 on OGD-induced neuronal cells. NF-κB signaling pathway activity was analyzed by measuring the protein levels of p-p65 and p65. Compared with the control group, p-p65 protein expression and the ratio of p-p65/p65 were significantly increased in the OGD group. In addition, SIRT1 overexpression (Figure 6A, 6B) and silencing (Figure 6C, 6D) significantly decreased and increased, respectively, the p-p65 protein expression and the p-p65/p65 ratio.

Discussion

In this research we constructed a neonatal HIE rat model to determine SIRT1 expression. On the third day, the rats were killed and brain tissue was collected. The results revealed that

Figure 3. Transfection efficiency of SIRT1 overexpression (SIRT1-plasmid) or silencing (SIRT1-siRNA) in primary rat neuronal cells. SIRT1 protein levels and mRNA gene expression in primary rat neuronal cells transfected with SIRT1-plasmid or control-plasmid for 48 h were determined by (A) Western blot and (B) qRT-PCR assays, respectively. SIRT1 protein levels and mRNA gene expression in primary rat neuronal cells transfected with SIRT1-siRNA or control-siRNA for 48 h were determined by (C) Western blot and (D) qRT-PCR assays, respectively. Control – cells without any treatment; Control-plasmid: cells were transfected with control-plasmid; SIRT1-plasmid: cells were transfected with SIRT1-plasmid; Control-siRNA: cells were transfected with control-siRNA; SIRT1-siRNA: cells were transfected with SIRT1-siRNA. The results showed as the mean±SD. ** P<0.01 vs. control group. SIRT1 – sirtuin-1.
Figure 4. SIRT1 overexpression increased cell viability and decreased cell apoptosis in OGD-induced primary rat neuronal cells. Primary rat neuronal cells were transfected with SIRT1-plasmid or control-plasmid for 48 h, then the cells were exposed to OGD for 48 h. (A) Cell proliferation was assessed using MTT assay. (B) Flow cytometry was used to determine apoptotic cells. (C) Quantification of apoptotic cells. (D) Bax and Bcl-2 protein levels were determined by Western blot assay. (E, F) The ratio of Bcl-2/GAPDH and Bax/GAPDH was calculated and presented. Control: cells without any treatment; OGD: cells were subjected to OGD treatment; OGD+control-plasmid: cells were transfected with control-plasmid and then subjected to OGD treatment; OGD+SIRT1-plasmid: cells were transfected with SIRT1-plasmid and then subjected to OGD treatment. The results showed as the mean±SD. ** P<0.01 vs. control group; ## P<0.01 vs. OGD group. OGD – oxygen and glucose deprivation; SIRT1 – sirtuin-1.
the cell apoptosis rate and the expression of S100B and NSE were remarkably amplified, whereas SIRT1 expression was obviously reduced in the HIE group compared to the control group. Subsequently, an OGD cell model was established to elucidate the effect of SIRT1 expression on HIE in vitro. The results suggested that SIRT1 was downregulated in the OGD-induced cells. In addition, SIRT1 overexpression and silencing alleviated and aggravated, respectively, the OGD-induced neuronal cell injury. Levene et al. demonstrated that 3–5/1000 newborn babies have HIE [23]. Previous studies suggested that HIE is the main cause of high morbidity and mortality [24]. The pathophysiology of HIE involves several different events (mitochondrial dysfunction, calcium surge, excitotoxicity, reactive oxygen species accumulation, and inflammation) that are strictly interlinked [25]. In the pathogenesis of hypoxia-ischemia, time of injury and timing of treatment play critical roles [26]. Therapeutic hypothermia (TH) has become the standard treatment method of HIE, resulting in reduced infant mortality and neurodevelopmental disability [27–31]. Previous reports have shown that TH can promote the survival and reduce the disability rate of neonates with HIE, but the mortality and disability of HIE...
The expression levels of the related molecular markers S100B and NSE during the development of the HIE were measured. ** P<0.01 vs. control group; # P<0.05, ## P<0.01 vs. OGD group. OGD – oxygen and glucose deprivation; SIRT1 – sirtuin-1.

Figure 6. Effect of SIRT1 on NF-κB signaling pathway in OGD-induced neuronal cells. Primary rat neuronal cells were transfected with SIRT1-plasmid/SIRT1-siRNA or control-plasmid/control-siRNA for 48 h, then the cells were treated with OGD for 48 h. (A) p-p65 and p65 protein levels in neurons transfected with SIRT1-plasmid or control-plasmid for 48 h were determined by Western blot assay. (B) The ratio of p-p65/p65 in neurons transfected with SIRT1-plasmid or control-plasmid for 48 h was calculated and presented. (C) p-p65 and p65 protein levels in neurons transfected with SIRT1-siRNA or control-siRNA for 48 h were determined by Western blot assay. (D) The ratio of p-p65/p65 in neurons transfected with SIRT1-siRNA or control-siRNA for 48 h was calculated and presented. Control: cells without any treatment; OGD: cells were subjected to OGD treatment; OGD+control-plasmid: cells were transfected with control-plasmid and then subjected to OGD treatment; OGD+SIRT1-plasmid: cells were transfected with SIRT1-plasmid and then subjected to OGD treatment; OGD+control-siRNA: cells were transfected with control-siRNA and then subjected to OGD treatment; OGD+SIRT1-siRNA: cells were transfected with SIRT1-siRNA and then subjected to OGD treatment. The results are presented as the mean±SD. ** P<0.01 vs. control group; # P<0.05, ## P<0.01 vs. OGD group. OGD – oxygen and glucose deprivation; SIRT1 – sirtuin-1.

SIRTs are class III histone deacetylase (HDAC), including SIRT1-SIRT7. SIRT depends on NAD+, indicating that sirtuin activity acts as a regulator for the solute ratio of NAD+/NADH cells, thus directly linking sirtuin activity to cellular metabolism and energy status [41,42]. SIRT1, a member of the silent information regulator 2 (Sir2) family, was reported to be involved in the occurrence and development of tumors [43,44]. In addition, whether SIRT1 acts as a tumor-suppressor or a tumor-promoter protein depends on cell type [45]. Increasing evidence shows that SIRT1 is related to hypoxia. SIRT1-regulated HMGB1 production is related to TLR4 signaling and is a possible anti-inflammatory mechanism of resveratrol in neonatal hypoxic-ischemic brain damage [46]. SIRT1 was reported to be involved in the role of ghrelin in modulating oxidative stress and neuronal apoptosis in a neonatal HIE rat model [47]. Licochalcone A has neuroprotective effects on OGD/R-treated rat cortical neurons through the SIRT1/Nrf2 pathway [48]. In this research, we generated a HIE rat model to investigate the roles of SIRT1 in the neonatal HIE and to illustrate the underlying mechanisms. However, our study has certain limitations. The expression levels of the related molecular markers S100B and NSE during the development of the HIE were measured.
confirmed by Western blot assay, but results from HE staining would be more convincing. The levels of SIRT1 in the brain tissue of HIE rats was assessed using Western blot and qRT-PCR assay, but not IHC.

We found that SIRT1 was downregulated in the HIE group. Laemmle et al. indicated that SIRT1 was obviously upregulated in hypoxic cells [48]. Furthermore, it has been reported that SIRT1 is overexpressed in various tumor cells and tissues [49–51]. Thereafter, in vitro experiments were performed. Similarly, SIRT1 was downregulated in OGD-induced cells. SIRT1 overexpression and silencing alleviated and aggravated, respectively, the OGD-induced neuronal cell injury. A previous study indicated that SIRT1 regulates endothelin-type B receptors via the NF-κB signaling pathway [52]. SIRT1 overexpression and silencing protected mouse osteoblasts from TNF-α-induced injury in vitro by inhibiting the NF-κB signaling pathway [53]. In this report, our findings indicated that SIRT1 affected the activation of NF-κB signaling pathway in OGD-induced neurons.

Conclusions

SIRT1 can protect neurons from ischemia and hypoxia by inhibiting activation of the NF-κB signaling pathway, thus exhibiting a protective effect on HIE. Our observations indicate that SIRT1 may serve as a novel and effective therapeutic target for HIE treatment. However, this research is only a preliminary study of the role of SIRT1 in HIE. To clarify the role and mechanism of SIRT1 in HIE, further in-depth investigations are needed. For example, whether SIRT1 directly or indirectly regulates NF-κB in hypoxic-ischemic encephalopathy has not been studied to date and needs further investigation. The expression of SIRT1 in HIE patients needs to be confirmed, and the relationship between SIRT1 expression and clinicopathological features of patients with HIE should be investigated. We plan to address all of these topics in the future.

Conflict of interests

None.

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