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Effects of long non-coding RNA NEAT1 on sepsis-induced brain injury in mice via NF-κB

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding ribonucleic acid (lncRNA) nuclear paraspeckle assembly transcript 1 (NEAT1) on sepsis-induced brain injury in mice through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

MATERIALS AND METHODS: The mouse model of sepsis was established by cecal ligation and puncture induction. The relative expression levels of NEAT1 and NF-κB in brain tissues of mice in healthy group and sepsis group were determined via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting, respectively. Subsequently, the expression of NEAT1 was silenced by transfection of small interfering RNAs (siRNAs). Meanwhile, its effect on NF-κB expression was detected. To further explore the effect of sepsis on brain injury, the content of brain water and the expression levels of apoptosis-related proteins, including B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX), in mice of healthy group, sepsis group, and sepsis + si-NEAT1 group were measured. Furthermore, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to detect the proliferation and apoptosis of nerve cells.

RESULTS: The relative expression levels of NEAT1 and NF-κB were significantly increased in the brain tissues of septic mice ($p<0.01$). Si-NEAT1 transfection significantly decreased the expressions of NEAT1 and NF-κB in brain tissues of septic mice ($p<0.05$). The content of brain water in mice of sepsis group was evidently increased ($p<0.05$). However, si-NEAT1 treatment remarkably reduced this content ($p<0.05$). In addition, sepsis markedly decreased the activity of nerve cells ($p<0.05$). However, si-NEAT1 could significantly increase the activity of nerve cells in septic mice ($p<0.05$). Moreover, si-NEAT1 notably decreased the expression of BAX ($p<0.05$), whereas it increased the expression of Bcl-2 ($p<0.05$). The results of apoptosis detection revealed that sepsis remarkably promoted the apoptosis of mouse nerve cells ($p<0.05$). In addition, si-NEAT1 transfection could evidently alleviate the apoptosis of nerve cells in septic mice ($p<0.05$).

CONCLUSIONS: LncRNA NEAT1 promotes brain injury in septic mice by positively regulating NF-κB. However, si-NEAT1 transfection can reduce this injury.

Key Words:
Long non-coding RNA NEAT1, NF-ÐB, Sepsis, Brain injury.

Introduction

Sepsis-induced brain injury refers to acute neurological dysfunction that occurs clinically. It is caused by a secondary infection *in vivo*, usually without evident nerve cell infection[1,2]. Sepsis-induced brain injury often occurs in severe patients in the intensive care unit (ICU) and those with severe systemic infection. It's reported[3] that the occurrence rate of sepsis-induced brain injury is as high as 70%. However, the pathophysiological mechanism of sepsis-induced brain injury is still unclear. It is known that brain injury induced by sepsis is primarily caused by inflammatory mediators, which induces endothelial activation. Meanwhile, it changes blood-brain barrier permeability and promotes inflammatory cell migration and neuronal apoptosis[4,5]. Some scholars believe that the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) will result in oxidative stress and the release of pro-inflammatory cytokines and chemokines. In microglia, sepsis leads to brain edema, destruction of blood-brain barrier permeability, neuronal apoptosis, and even denervation[6].

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In recent years, more and more scholars have paid attention to the role of non-coding ribonucleic acids (ncRNAs) in various biological physiological processes, such as innate immunity and apoptosis[7,8]. Long ncRNA (lncRNA) contains more than 200 nucleotides. The mechanism of lncRNAs in health and diseases has been comprehensively evaluated[9,10]. Some studies have reported differential expression of lncRNAs in human cardiomyocytes, renal tubular epithelial cells, and monocytes after exposure to lipopolysaccharide or sepsis[11]. LncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) is an important structural component of parasite plaque structure, which has recently been considered to play an important role in innate immune responses[12]. However, as far as we know, its role in sepsis-induced brain injury has not been demonstrated.

We investigated the effect of lncRNA NEAT1 on sepsis-induced brain injury in mice *via* NF-κB.

Materials and Methods

Test Reagents

Large-capacity complementary deoxyribose nucleic acid (cDNA) reverse transcription (RT) kit was purchased from TaKaRa (Dalian, China), small interfering RNA (siRNA)-NEAT1 from Ambon (Austin, TX, USA), and antibodies for Western blotting from Abcam (Cambridge, MA, USA). Polymerase Chain Reaction (PCR) primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd., and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kits were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Establishment of the Model of Sepsis In Mice

The model of sepsis was established in mice by cecal ligation and puncture induction[13]. Solid fasting was conducted one day in advance, and pentobarbital sodium was used to anesthetize mice before the operation. Subsequently, the mice were cut from the belly line to expose the cecum on a clean table. The distal part of the cecum was ligated with a 4-0 suture under the ileocecal flap, followed by pierce using a 20-gauge needle. Then the peritoneum wall and skin were sutured. After that, the mice were reared normally after waking up. This study was approved by the Animal Ethics Committee of Hangzhou Medical College.

Laboratory Design and Grouping

In this study, all experimental mice were divided into four groups, including: 1) Healthy group: no treatment was carried out. 2) Sepsis group: mice were prepared according to the method of model establishment. 3) Sepsis + si-control group: septic mice were treated with si-control. 4) Sepsis + si-NEAT1 group: septic mice were treated with si- NEAT1.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to isolate total RNA from brain tissues. Subsequently, extracted total RNA was reverse transcribed into cDNA according to the instructions of relevant kits. The expression of NEAT1 was determined using qRT-PCR. Primers used in this study were shown as follows: NEAT1: sense primer: TTGGGACAGTGTGG and antisense primer: TCAGTCCAGCAGGCA; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense primer: TGCGTGCCGCTGGAGAGAGAAGG and antisense primer: CCGGCATCGGAGAAGG.

Western Blotting Analysis

After brain tissue samples were lysed, the supernatant was separated. Protein samples were separated *via* Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 0.5% bovine serum albumin (BSA) for 1 h, the membranes were incubated with primary antibodies of NF-κB, b-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (BAX), and β-actin at 4°C overnight. After washing for three times, the membranes were incubated with goat anti-mouse IgG-HRP (Horseradish Peroxidase) for 1 h. Finally, target proteins were detected *via* diaminobenzidine (DAB; Solarbio, Beijing, China).

Brain Water Content

The brain water content was measured by the standard wet-dry method. Immediately after dissection, the wet weight of brain tissues was quantified. Subsequently, the brain samples were dried in an oven at 105°C for 48 h until the weight of the brain was constant. The water content of each brain was measured as follows: (wet weight – dry weight) / wet weight × 100%.

MTT Assay

Nerve cells were first isolated and inoculated into 96-well plates, followed by incubation for 12

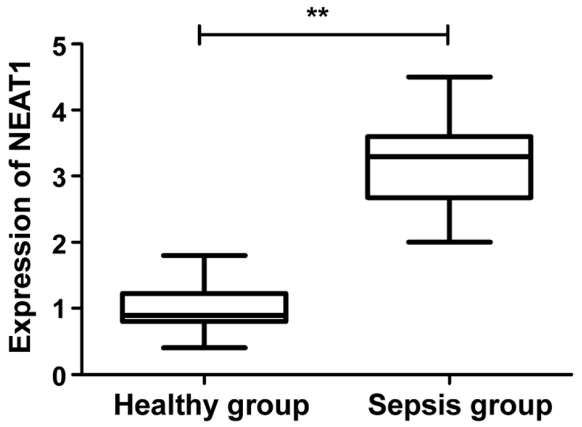

Figure 1. Expression of NEAT1 in brain tissues of mice in healthy group and sepsis group detected *via* qRT-PCR. **represents an extremely significant difference (p<0.01).

h. Subsequently, 500 μg/mL MTT solution was added to each well, followed by incubation for 3 h. After 200 μL dimethyl sulfoxide solution was added, the absorbance was measured with an enzyme reader. Finally, cell activity was calculated.

Cell Apoptosis Via TUNEL Assay

TUNEL assay was adopted to detect the apoptosis of neurons. Paraffin-removed brain slices were first sealed with 0.1% Triton X-100 at 37°C for 15 min. After washing with phosphate-buffered saline (PBS), the slices were reacted with TUNEL solution in an incubator at 37°C for 1 h in the dark. After washing with PBS, the slides were incubated with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) in an incubator at 37°C for 15 min. Then, the slices were washed again with PBS, followed by cover with coverslips for observation using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). TUNEL-positive neurons were counted in 5 randomly selected regions of the hippocampus. The apoptosis index was expressed as follows: (number of TUNEL-positive neurons/total number of DAPI-positive neurons) × 100%.

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for all statistical analysis. All experiments were conducted in triplicate. One-way analysis of variance was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). p<0.05 was considered statistically significant.

Results

Expression of NEAT1 in Brain Tissues of Septic Mice

The mouse model of sepsis was first established. Then, the relative expression level of NEAT1 in brain tissues of mice in healthy group and sepsis group was detected by qRT-PCR. The results (Figure 1) revealed that the relative expression level of NEAT1 in brain tissues of mice in sepsis group was significantly increased when compared with healthy group (p<0.01), which was nearly three times as high as that in healthy group.

Expression of NF-ĐB in Brain Tissues of Septic Mice

Subsequently, the relative expression level of NF-κB in a sepsis mouse model was determined through Western blotting. As shown in Figure 2, the results manifested that the relative protein expression level of NF-κB in brain tissues of mice in sepsis group was notably increased when compared with that of healthy group (p<0.01), which was also almost three times as high as that in healthy group.

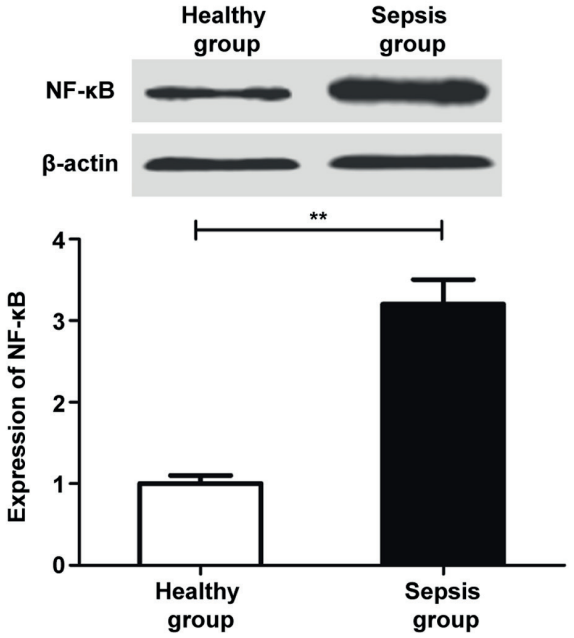

Figure 2. Expression of NF-κB in brain tissues of mice in healthy group and sepsis group detected *via* Western blotting. **represents an extremely significant difference (p<0.01).

Effect of si-NEAT1 on the Expressions of NEAT1 and NF-κB in Septic Mice

To study the effect of NEAT1 expression on NF-κB expression in septic mice, siRNAs were used to silence NEAT1 expression. Subsequently, the expression level of NF-κB was measured. As shown in Figure 3A, compared with sepsis + si-control group, the expression of NEAT1 in brain tissues of sepsis + si-NEAT1 group was significantly decreased ($p<0.01$). Meanwhile, the results in Figure 3B demonstrated that compared with sepsis + si-control group, the expression of NF-κB in brain tissues of sepsis + si-NEAT1 group was evidently decreased ($p<0.05$).

Effect of NEAT1 Inhibition on Brain Water Content in Septic Mice

To further explore the effect of sepsis on brain injury, the brain water content of mice in healthy group, sepsis group, and septic + si-NEAT1 group was measured, respectively. According to the results (Figure 4), the brain water content of mice in healthy group was within the normal range. Meanwhile, the brain water content of mice in sepsis group was significantly increased ($p<0.05$). However, the brain water content of mice in sepsis + si-NEAT1 group was remarkably reduced when compared with that of sepsis group ($p<0.05$), nearly close to normal level.

Effect of NEAT1 Inhibition on the Activity of Nerve Cells in Septic Mice

The activity of nerve cells in septic mice under different conditions was measured to further study the effect of NEAT1 on brain injury. As shown in Figure 5, the results demonstrated that compared with healthy group, the activity of nerve cells in sepsis group was notably reduced ($p<0.05$). Furthermore, compared with sepsis + si-control group, the activity of nerve cells was significantly increased in sepsis + si-NEAT1 group ($p<0.05$).

Effect of NEAT1 Inhibition on the Apoptosis of Nerve Cells in Septic Mice

Finally, the effect of NEAT1 inhibition on the apoptosis of nerve cells in septic mice was investigated after transfection of si-NEAT1 in septic

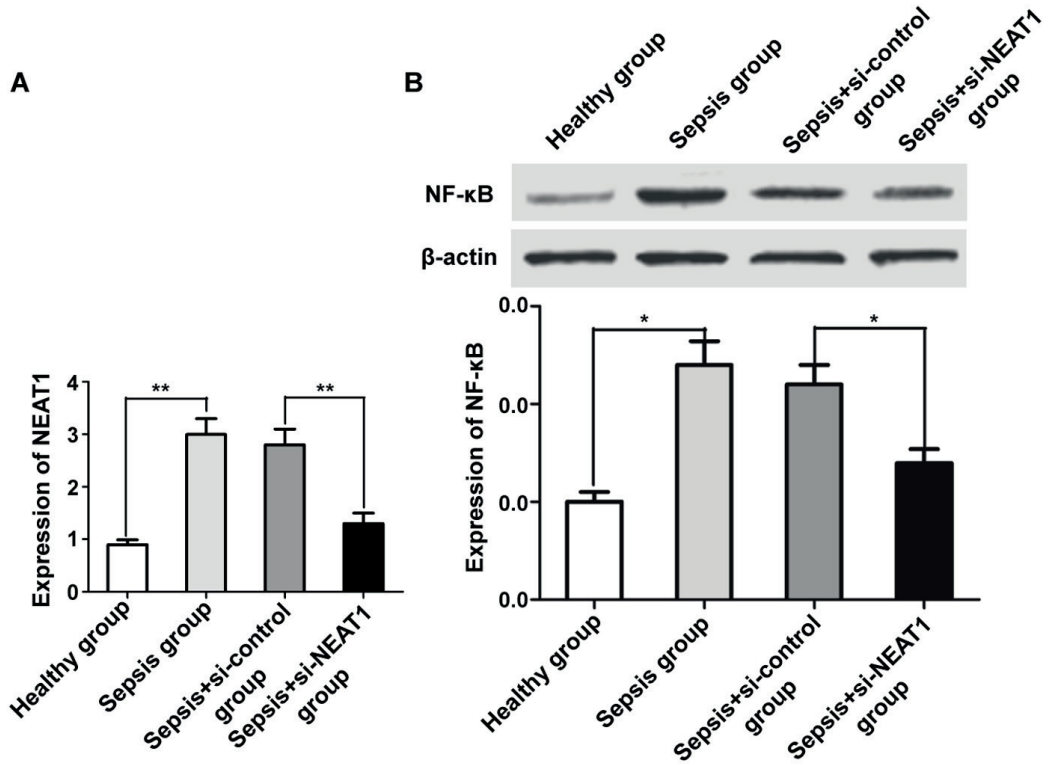

Figure 3. Effect of si-NEAT1 on the expressions of NEAT1 and NF-κB in septic mice. ***A,*** Expression of NEAT1 detected *via* qRT-PCR. **represents an extremely significant difference ($p<0.01$). ***B,*** Expression of NF-κB determined by Western blotting. *represents a significant difference ($p<0.05$).

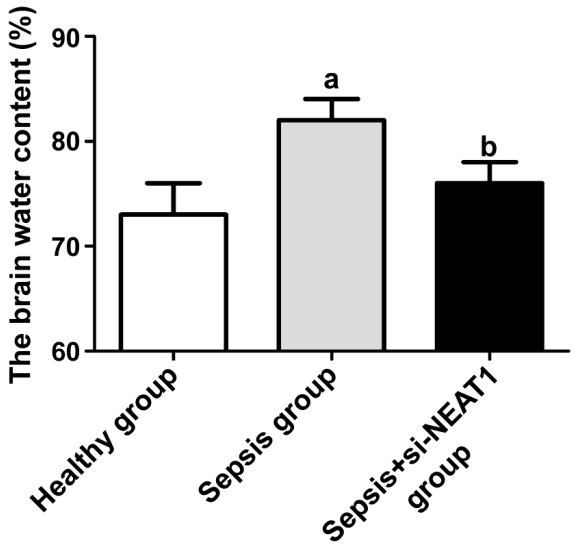

Figure 4. Effect of si-NEAT1 treatment on brain water content in septic mice. ***A,*** Compared with healthy group, brain water content in sepsis group was significantly increased ($p<0.05$). ***B,*** Compared with sepsis group, brain water content in sepsis + si-NEAT1 group was markedly decreased ($p<0.05$).

mice. Firstly, the protein expressions of BAX and Bcl-2, two molecules related to cell proliferation and apoptosis, were analyzed. Secondly, the apoptosis of nerve cells was detected by TUNEL assay. It was found that the expression of BAX in brain tissues of mice in sepsis group was significantly increased when compared with that of mice in healthy group ($p<0.05$). However, si-NEAT1 interference could significantly decrease its expression in brain tissues of septic mice ($p<0.05$). On the contrary, the expression of Bcl-2 in brain tissues of mice in sepsis group was markedly decreased when compared with that of mice in healthy group ($p<0.05$). In addition, si-NEAT1 interference remarkably increased its expression in brain tissues of septic mice ($p<0.05$) (Figure 6A-6C).

The results of apoptosis detection revealed that sepsis remarkably promoted the apoptosis of nerve cells in mice ($p<0.05$). However, si-NEAT1 transfection could evidently alleviate the apoptosis of nerve cells in septic mice ($p<0.05$) when compared with that of healthy group (Figure 6D).

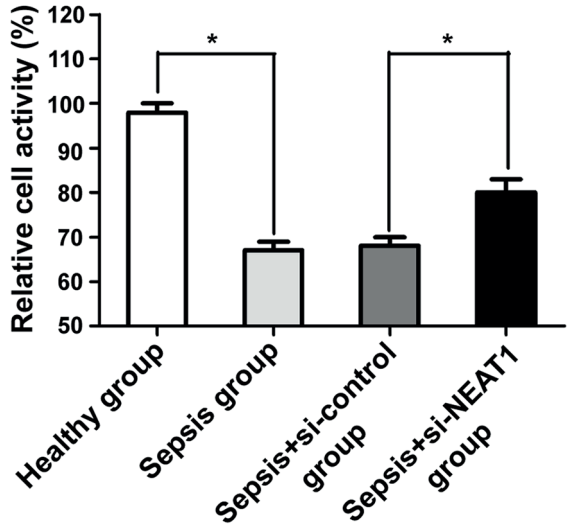

Figure 5. Nerve cell activity in different groups detected *via* MTT assay. *represents a significant difference ($p<0.05$).

Discussion

Sepsis is a common and potentially fatal systemic disease caused by microbial infection. It can lead to uncontrolled inflammation, tissue injury, and multiple organ failure[14,15]. Clinically, sepsis-induced brain injury is characterized by a series of brain dysfunction. Animal studies[16], however, provide the most information on possible causes of brain tissue injury.

We found that NEAT1 was highly expressed in mice with sepsis-induced brain injury. Meanwhile, the expression level of NF-κB was also significantly increased when compared with that of healthy controls. Si-NEAT1 could notably decrease the expressions of NEAT1 and NF-κB in brain tissues of septic mice, indicating that si-NEAT1 inhibited NF-κB pathway in septic mice. A previous study[17] has shown that NEAT1 participates in many biological processes, such as cell growth and differentiation. NEAT1 has also been shown[18] to be associated with several human cancers. This investigation revealed the function of lncRNA NEAT1 in sepsis-induced brain injury. Interestingly, the results manifested that NEAT1 was overexpressed in mice with sepsis-induced brain injury compared with that of healthy controls. Furthermore, inhibition of NEAT1 reduced brain edema injury induced by NF-κB. This indicated that NEAT1 might be involved in the progress of sepsis-induced brain injury.

Also, sepsis markedly decreased nerve cell activity. However, si-NEAT1 transfection significantly improved the activity of nerve cells, decreased the expression of BAX, and increased the expression of Bcl-2 in septic mice. The results of apoptosis detection revealed that sep-

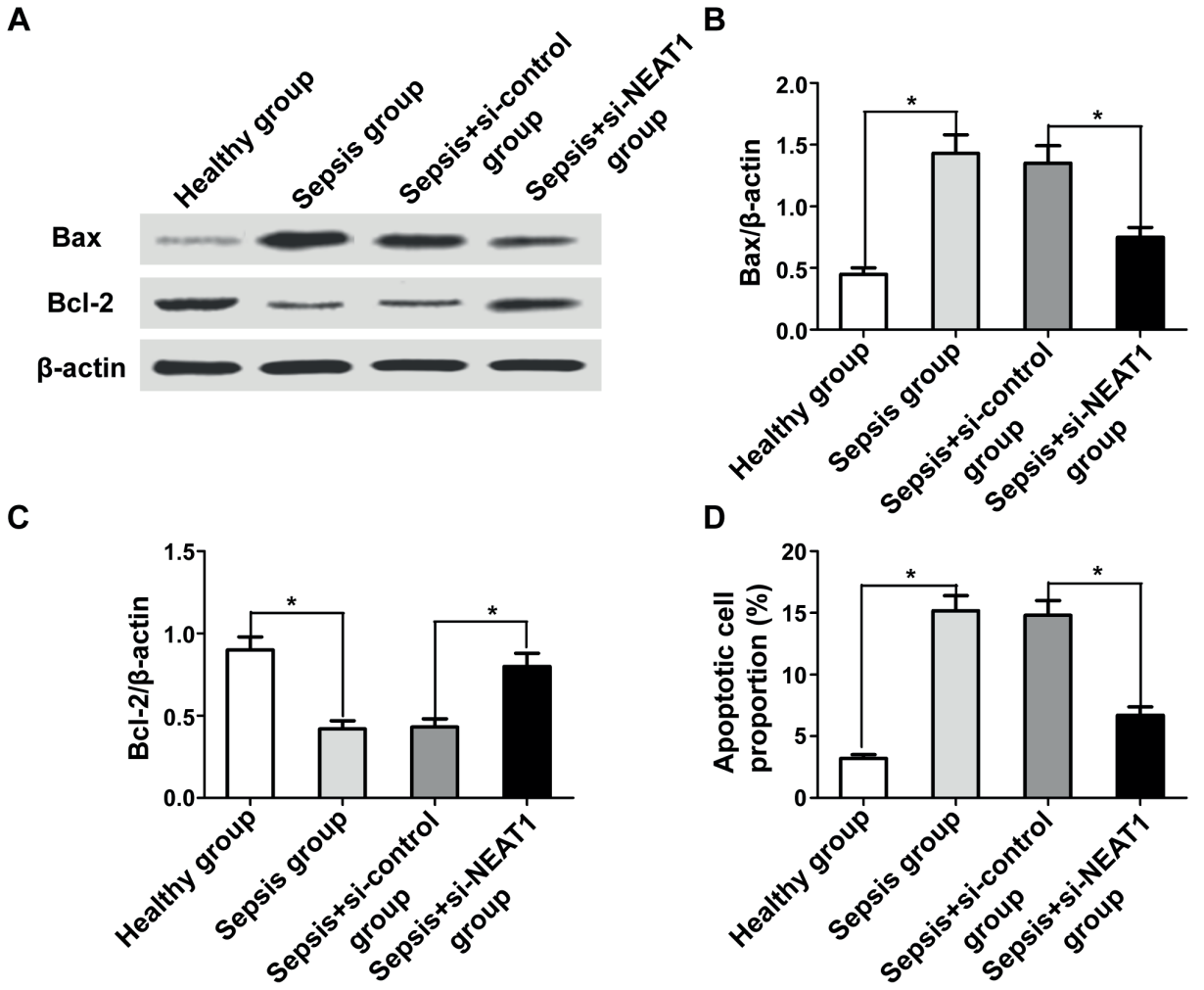

Figure 6. Effect of NEAT1 inhibition on the apoptosis of nerve cells in septic mice. ***A-C,*** Effect of si-NEAT1 on the expressions of BAX and Bcl-2 in brain tissues of septic mice detected *via* Western blotting. ***D,*** Effect of si-NEAT1 on the apoptosis of nerve cells in septic mice. *represents a significant difference ($p<0.05$).

sis remarkably promoted the apoptosis of nerve cells in mice. However, si-NEAT1 treatment evidently alleviated the apoptosis of nerve cells in septic mice. Apoptosis refers to cell suicide or programmed cell death, which is involved in the development of sepsis-induced brain injury[19]. The mitochondrial-mediated apoptosis signaling pathway has been reported[20] to be an important mechanism for regulating glucocorticoid protection in sepsis-induced brain injury. We observed that after NEAT1 down-regulation, the expression of apoptosis-related protein BAX was markedly decreased, whereas the expression of Bcl-2 was evidently increased. Some studies[21,22] have indicated that NF-κB plays a key role in inflammatory diseases. It has been proved[23] that the activation of NF-κB leads to increased expression of chemokines and cytokines, eventually causing sepsis-induced organ injury. Therefore, we suggested that the NF-κB pathway might be the downstream regulatory mechanism of NEAT1 involved in sepsis-induced brain injury. To support this view, our findings confirmed that NEAT1 inhibition reduced the protein expression of NF-κB. Based on this, it was speculated that NEAT1 might aggravate sepsis-induced brain injury by activating NF-κB pathway.

Conclusions

We found that the NEAT1 upregulation is related to sepsis-induced brain injury. NEAT1 inhibition can reduce sepsis-induced brain injury

by downregulating NF-κB pathway. Besides, NEAT1 can be used as an important diagnostic marker and therapeutic target for brain injury induced by sepsis.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Toklu HZ, Tunali AT, Velioglu-Ogunc A, Ercan F, Gedik N, Keyer-Uysal M, Sener G. Silymarin, the antioxidant component of Silybum marianum, prevents sepsis-induced acute lung and brain injury. J Surg Res 2008; 145: 214-222.
2) Polito A, Eischwald F, Maho AL, Polito A, Azabou E, Annane D, Chretien F, Stevens RD, Carlier R, Sharshar T. Pattern of brain injury in the acute setting of human septic shock. Crit Care 2013; 17: R204.
3) Ren C, Tong YL, Li JC, Dong N, Hao JW, Zhang QH, Yao YM. Early antagonism of cerebral high mobility group box-1 protein is benefit for sepsis induced brain injury. Oncotarget 2017; 8: 92578-92588.
4) Stubbs DJ, Yamamoto AK, Menon DK. Imaging in sepsis-associated encephalopathy-insights and opportunities. Nat Rev Neurol 2013; 9: 551-561.
5) Danielski LG, Giustina AD, Badawy M, Barichello T, Quevedo J, Dal-Pizzol F, Petronilho F. Brain barrier breakdown as a cause and consequence of neuroinflammation in sepsis. Mol Neurobiol 2018; 55: 1045-1053.
6) Michels M, Vieira AS, Vuolo F, Zapelini HG, Mendonca B, Mina F, Dominguini D, Steckert A, Schuck PF, Quevedo J, Petronilho F, Dal-Pizzol F. The role of microglia activation in the development of sepsis-induced long-term cognitive impairment. Brain Behav Immun 2015; 43: 54-59.
7) Zhao Y, Guo Q, Chen J, Hu J, Wang S, Sun Y. Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation. Oncol Rep 2014; 31: 358-364.
8) Shi X, Sun M, Liu H, Yao Y, Kong R, Chen F, Song Y. A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small-cell lung cancer. Mol Carcinog 2015; 54 Suppl 1: E1-E12.
9) Mohanty V, Gokmen-Polar Y, Badve S, Janga SC. Role of lncRNAs in health and disease-size and shape matter. Brief Funct Genomics 2015; 14: 115-129.
10) Li L, Wang JJ, Zhang HS. LncRNA-CARI in a rat model of myocardial infarction. Eur Rev Med Pharmacol Sci 2018; 22: 4332-4340.
11) Lorenzen JM, Schauerte C, Kolling M, Hubner A, Knapp M, Haller H, Thum T. Long noncoding RNAs in urine are detectable and may enable early detection of acute T cell-mediated rejection of renal allografts. Clin Chem 2015; 61: 1505-1514.
12) Chakravarty D, Sboner A, Nair SS, Giannopoulou E, Li R, Hennig S, Mosquera JM, Pauwels J, Park K, Kossai M, MacDonald TY, Fontugne J, Erho N, Vergara IA, Ghadessi M, Davicioni E, Jenkins RB, Palanisamy N, Chen Z, Nakagawa S, Hirose T, Bander NH, Beltran H, Fox AH, Elemento O, Rubin MA. The oestrogen receptor alpha-regulated lncRNA NEAT1 is a critical modulator of prostate cancer. Nat Commun 2014; 5: 5383.
13) Zhang H, Zhi L, Moochhala S, Moore PK, Bhatia M. Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. Am J Physiol Lung Cell Mol Physiol 2007; 292: L960-L971.
14) Fink MP, Warren HS. Strategies to improve drug development for sepsis. Nat Rev Drug Discov 2014; 13: 741-758.
15) Savioli M, Cugno M, Polli F, Taccone P, Bellani G, Spanu P, Pesenti A, Iapichino G, Gattinoni L. Tight glycemic control may favor fibrinolysis in patients with sepsis. Crit Care Med 2009; 37: 424-431.
16) Hopkins RO. Sepsis, oxidative stress, and brain injury. Crit Care Med 2007; 35: 2233-2234.
17) Hirose T, Virnicchi G, Tanigawa A, Naganuma T, Li R, Kimura H, Yokoi T, Nakagawa S, Benard M, Fox AH, Pierron G. NEAT1 long noncoding RNA regulates transcription via protein sequestration within subnuclear bodies. Mol Biol Cell 2014; 25: 169-183.
18) Choudhry H, Mole DR. Hypoxic regulation of the noncoding genome and NEAT1. Brief Funct Genomics 2016; 15: 174-185.
19) Tang G, Yang H, Chen J, Shi M, Ge L, Ge X, Zhu G. Metformin ameliorates sepsis-induced brain injury by inhibiting apoptosis, oxidative stress and neuroinflammation via the PI3K/Akt signaling pathway. Oncotarget 2017; 8: 97977-97989.
20) Luo CL, Chen XP, Yang R, Sun YX, Li QQ, Bao HJ, Cao QQ, Ni H, Qin ZH, Tao LY. Cathepsin B contributes to traumatic brain injury-induced cell death through a mitochondria-mediated apoptotic pathway. J Neurosci Res 2010; 88: 2847-2858.
21) Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001; 107: 7-11.
22) Hansdottir S, Monick MM, Lovan N, Powers L, Gerke A, Hunninghake GW. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. J Immunol 2010; 184: 965-974.
23) Villar J, Ribeiro SP, Mullen JB, Kuliszewski M, Post M, Slutsky AS. Induction of the heat shock response reduces mortality rate and organ damage in a sepsis-induced acute lung injury model. Crit Care Med 1994; 22: 914-921.