Neuroprotective effects of NEP1-40 and fasudil on Nogo-A expression in neonatal rats with hypoxic-ischemic brain damage

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ABSTRACT. The hypoxic-ischemic encephalopathy caused by peripartum asphyxia is a serious disease in newborn infants, and effective therapies need to be developed to reduce injury-related disorders. We evaluated the effects of NEP1-40 and fasudil on Nogo-A expression in neonatal hypoxic-ischemic brain damage (HIBD) rats. Seven-day-old Wistar rats were randomly divided into control, HIBD, NEP1-40, and fasudil groups. NEP1-40 and fasudil groups were injected intraperitoneally with these compounds. Rat brains at 6, 24, 72 h, and 7 days after HIBD were collected to determine histopathological damage and the expression levels of Nogo-A. Histopathological damage was reduced in NEP1-40 and fasudil groups compared with the untreated HIBD group. The expression of Nogo-A in the HIBD group was significantly higher than that in control, NEP1-40 and fasudil groups at the same times. Compared with the fasudil group, the expression levels of Nogo-A were significantly reduced in the NEP1-40 group. We

conclude that NPE1-40 and fasudil have potential for neuroprotective effects in the neonatal rat HIBD model, mediated by inhibiting Nogo-A/ Rho pathways.

Key words: Hypoxic-ischemic brain damage; NEP1-40; Fasudil; Nogo-A

INTRODUCTION

Perinatal hypoxic-ischemic (HI) is a major cause of brain injury in the newborn, with a high disability and mortality rate (Hossain, 2008). Lack of regenerative ability in the central nervous system after injury is considered to be the fundamental cause (Walmsley and Mir, 2007). Traditional symptomatic intervention cannot effectively improve the prognosis. However, in recent years many studies have revealed that there are at least three myelin-associated neurite outgrowth inhibitory factors that exert an inhibitory effect on the elongation of central nerve fibers and neural regeneration and plasticity through the Nogo receptor (Nogo-R), including Nogo-A (Savio and Schwab, 1990; Prinjha et al., 2000; Oertle et al., 2003), myelin-associated glycoprotein (MAG; McKerracher et al., 1994; Mukhopadhyay et al., 1994) and oligodendrocyte-myelin glycoprotein (OMgp; Kottis et al., 2002; Wong et al., 2002). These axonal growth inhibitors transmit inhibitory signals through common intracellular molecules such as RhoA and its effector Rho kinases (ROCK; Kubo and Yamashita, 2007). Blocking one of the above inhibitory proteins has induced a degree of axonal regeneration (GrandPre et al., 2002; Goldshmit et al., 2004). Suppressing RhoA or ROCK with pharmacological inhibitors has stimulated axonal growth and improved behavioral recovery after spinal cord lesion in rodents (Fournier et al., 2003; Bertrand et al., 2005).

NEP1-40, the competitive antagonist of Nogo-R for sequences of Nogo-66 amino, prevents the combination of central nervous system inhibitors with Nogo-R and contributes to nerve regeneration (Cao et al., 2008). Fasudil, the sole clinically available ROCK inhibitor, is already in use to treat cerebral vasospasm, and its anti-angina effect has also been confirmed in humans (Vicari et al., 2005). In this study, we explored the possible neuroprotective effect of NEP1-40 and fasudil and Nogo-A expression in neonatal rats with hypoxic-ischemic brain damage (HIBD), by HE staining, transmission electron microscopy, *in situ* hybridization, and immunochemical staining.

MATERIAL AND METHODS

Subjects

A total of 128 healthy Wistar rats aged 7 days were purchased from the Shandong University Laboratory Animal Center, weighing 12~18 g, male and female open. They were randomly divided into 4 groups on average: 1) the control group: intraperitoneally injected with normal saline; 2) HIBD group: intraperitoneally injected with normal saline; 3) NEP1-40 group: intraperitoneally injected with NEP1-40 (American Diagnostica Inc.) at a dose of 12.5 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (Cao et al., 2008); 4) fasudil group: intraperitoneally injected with fasudil (American Diagnostica Inc.) at a dose of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (Vicari et al., 2005). In addition, rats of each group were randomly divided

into 4 groups on average by observation time of 6, 24, 72 h, and 7 days (Guo et al., 2008). All rats of the HIBD, NEP1-40 and fasudil groups were performed strictly according to the Rice procedure (Rice et al., 1981). All experiments conformed to the Guidelines for Animal Experimentation at School of Medicine, Shandong University, on the ethical use of animals, and all efforts were made to minimize the number of animals used and their suffering.

Hypoxia-ischemia model

HIBD was induced by a modification of the method originally reported by Rice et al. (1981). The pups were anesthetized with 10% chloral hydrate (3.0 mL/kg). The neck was incised at the midline, and the left carotid artery was permanently ligated with 5-0 surgical silk. The duration of the anesthesia and surgery did not exceed 5 min per pup. A heat lamp was used during the procedure to maintain body temperature at 37°C. Following a 2-h recovery and feeding period, the animals were exposed to 120 min of hypoxia (8% O_2 and 92% N_2) by placing them in airtight containers partially submerged in a 37°C-water bath to maintain a constant thermal environment. In the control operation group, the left common carotid artery was exposed but was not ligated, and the animals were not submitted to hypoxia.

Sample preparation and morphological analyses

Seven days after HI injury, rats were anesthetized with an overdose of 10% chloral hydrate. The thorax was opened, and the left ventricle was perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS. After perfusion, the brains were removed and post-fixed in the same fixative for 3 days. Frozen sections of the brains were prepared. The brains were sectioned coronally into sequential 20-μm slices, which were stored at -4°C until used. Sections were chosen for *in situ* hybridization and immunohistochemical staining to detect Nogo-A-positive cells, and some for hematoxylin and eosin (HE) staining to observe pathological changes. In addition, parts of the brain tissues were reserved for observation of histological changes by transmission electron microscopy.

In situ hybridization

After being warmed to room temperature, slide-mounted sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 15 min (all steps were performed at room temperature unless otherwise indicated) and rinsed three times (5 min each) in 4X SSC (standard saline citrate, pH 7.2) (1X SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate). The slides were incubated with prehybridization solution (50% formamide, 50% 4X SSC) for 2 h at 37°C. The probe was added to each tissue section at a concentration of 1 μg/mL and hybridized overnight at 42°C. Hybridization was performed by incubating the sections with buffer (4X SSC, 50% deionized formamide, 0.12 M phosphate buffer, pH 7.2, Denhardt's solution, 2.5% tRNA, 10% dextran sulfate). After hybridization, the sections were rinsed in 1X SSC, pH 7.2, for 10 min, followed by rinsing three times in 1X SSC at 55°C for 20 min each time. After high-stringency washing (2X SSC twice, 1X SSC twice, 0.5X SSC twice at 37°C), sections were incubated with labeled-detection probe. Brown indicated strong

hybridization. As negative controls, sense probes were used in all hybridization and no positive signals were observed.

Immunohistochemistry

Sections were incubated in 3% hydrogen peroxide (H_2O_2) for 20 min to prevent reaction with endogenous peroxidases. After microwave antigen retrieval and blocking with goat serum for 30 min at 37°C, sections were subsequently incubated with the primary antibody, rabbit anti-rat Nogo-A (Wuhan Boster Biological Technology, Ltd., China) (1:200) at 4°C overnight. After rinsing with PBS, the sections were incubated with goat anti-rabbit IgG as the secondary antibody (Beijing Zhongshan Biotechnology Co. Ltd., China) for 30 min and then placed in a horseradish peroxidase complex solution for 30 min at 37°C. Peroxidase activity was revealed by immersing the sections in a mixture containing 0.05% 3-3'-diaminobenzidine (DAB) (Beijing Zhongshan Biotechnology Co. Ltd.) and 0.03% H_2O_2 for 5 min. Sections were then air-dried, dehydrated and coverslipped. Images of the slides were captured using a computer-assisted image analyzer system (Olympus, Japan). The application of a control serum, instead of the primary antibody, on other sections of the same brain samples provided a negative control for each staining.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software. Results are reported as means ± SD. A value of $P < 0.05$ indicated a statistically significant difference.

RESULTS

Morphological outcome

The gross appearance of rat brains was observed at 6, 24, 72 h, and 7 days among the groups. Brain edema and congestion occurred in the HIBD group, especially in the left hemisphere. Edema and congestion was more evident at 7 days in the HIBD group than in the control group (Figure 1A,B). With the administration of NEP1-40 and fasudil, edema and congestion were alleviated at every time compared with the HIBD group (Figure 1C,D). The alleviation was more evident in the NEP1-40 group than in the fasudil group.

Histological change

The left hemisphere showed evidence of cellular shrinkage and darkening, extending throughout the ischemic cortex following HIBD. Injured neurons and cell loss were noted in both the hippocampus and cerebral cortex. Conventional stains such as HE showed cerebral edema, eosinophilic staining, homogeneous change, and shrinkage in neurons under the light microscope. Moderate edema of cells and diffuse neuronal degeneration were the most abundant changes in the HIBD group at 7 days (Figure 2B). Similar changes were much reduced in the NEP1-40 and fasudil groups (Figure 2C,D). In each treatment group, however, numerous damaged neurons were observed as compared to the control group. There was no neuronal damage or edema in the control group (Figure 2A).
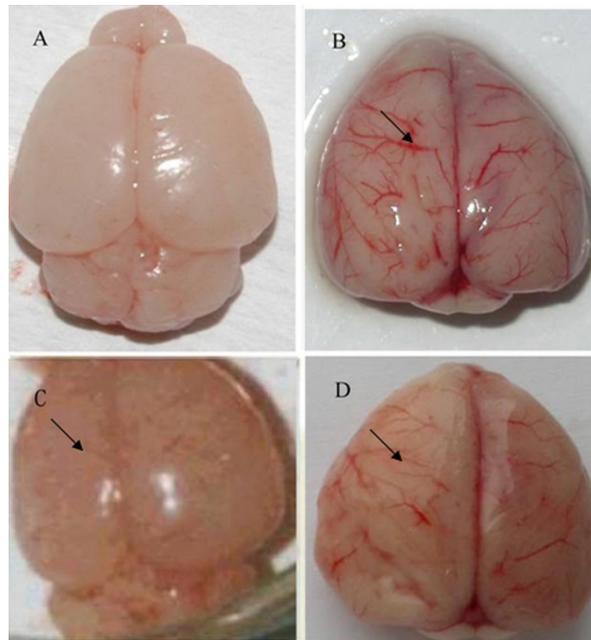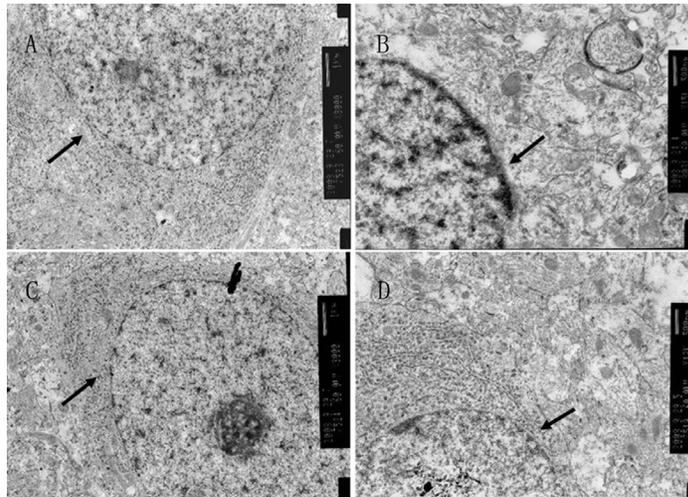
Figure 1. Gross appearance of rat brain 7 days after hypoxic-ischemic brain damage (HIBD) **A.** Control group. **B.** HIBD group. **C.** NEP1-40 group. **D.** Fasudil group. Arrows indicate edema and congestion.
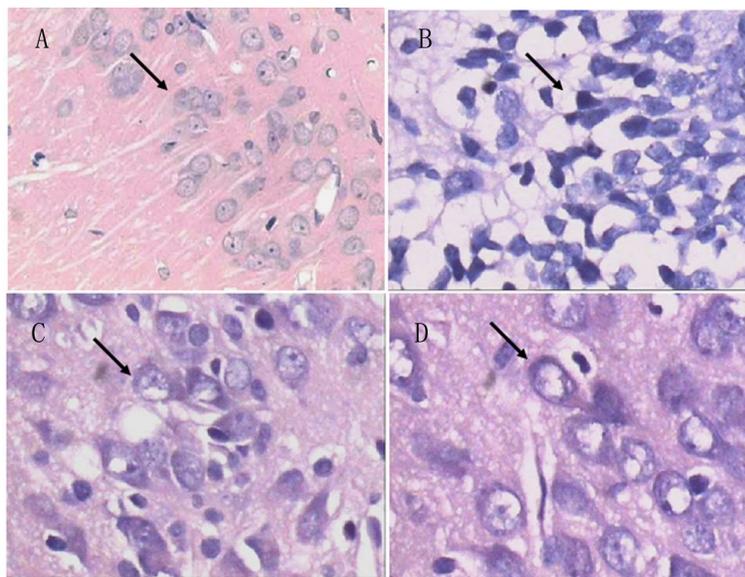
Figure 2. Pathological changes in nerve cells 7 days after hypoxic-ischemic brain damage (HIBD) in neonatal rats (400X). **A.** Control group. **B.** HIBD group. **C.** NEP1-40 group. **D.** Fasudil group. Arrows indicate shrunken nucleus and loss of cytoplasm. These changes were much reduced in each treatment group. There was no neuronal damage or edema in controls.

Under electron microscope, there were obvious changes among the different groups. Differences between the control and HIBD groups were evident with time. There were neuron shrinkage, mitochondria swelling, nuclei shrinkage, nuclear membrane incisure, chromatin under nuclear membrane, rupture and dissolution of cristae, vacuoles and capsules in HIBD (Figure 3B), unlike in the control group (Figure 3A). Such changes were much reduced in NEP1-40 and fasudil groups (Figure 3C,D).

Figure 3. Ultrastructural changes in nerve cells 7 days after hypoxic-ischemic brain damage (HIBD) in neonatal rats (EM _ 8000). **A.** Control group. **B.** HIBD group. **C.** NEP1-40 group. **D.** Fasudil group. Arrows indicate neuron shrinkage, nuclear shrinkage, nuclear membrane incisure, chromatin under nuclear membrane, rupture/dissolution of cristae. These changes were much reduced in NEP1-40 and fasudil groups.

Nogo-A gene expression

Nogo-A mRNA expression was observed in glial cells and the cytoplasm of neurons in newborn rat brain cortex and hippocampus. The expression of Nogo-A mRNA in the HIBD group increased significantly with time and was much higher than in control, NEP1-40 and fasudil groups at the same times (Figure 4A-D). Nogo-A mRNA expression in the NEP1-40 group decreased significantly with time (Table 1, $P < 0.05$), while in the fasudil group, the expression of Nogo-A mRNA increased at 6 and 24 h and then decreased at 72 h and 7 days. There was a significant decrease between 6 h and 72 days (Table 1, $P < 0.05$).

Table 1. Expression of Nogo-A mRNA in brain tissue.

Group	N	Nogo-A mRNA			
		6 h	24 h	72 h	7 days
Control	32	10.25 ± 1.83	10.62 ± 1.69	11.50 ± 1.60	11.06 ± 2.03
HIBD	29	17.87 ± 1.25^a	21.50 ± 2.00^a	30.50 ± 1.62^a	35.50 ± 5.60^a
NEP1-40	30	13.50 ± 1.60^{ab}	7.75 ± 1.02^{ab}	6.05 ± 1.04^{ab}	5.12 ± 0.99^{ab}
Fasudil	31	17.63 ± 1.56^{ac}	25.75 ± 1.49^{abc}	17.00 ± 1.69^{ab}	14.25 ± 1.49^{abc}

Data are reported as means ± SD in percentage. HIBD = hypoxic-ischemic brain damage. [a]$P < 0.05$ versus control group; [b]$P < 0.05$ versus HIBD group; [c]$P < 0.05$ versus NEP1-40 group.

Nogo-A protein expression

The average optical density of Nogo-A in the hippocampus and in the cerebral cortex was determined. The expression of Nogo-A protein in the HIBD group increased in the cerebral cortex and hippocampus with time and was much higher than in control, NEP1-40 and Fasudil groups at the same times (Figure 5A-D). The expression of Nogo-A protein was correlated with the expression of Nogo-A mRNA. Nogo-A protein expression was significantly reduced in NEP1-40 and Fasudil groups compared to the HIBD group (data not shown).
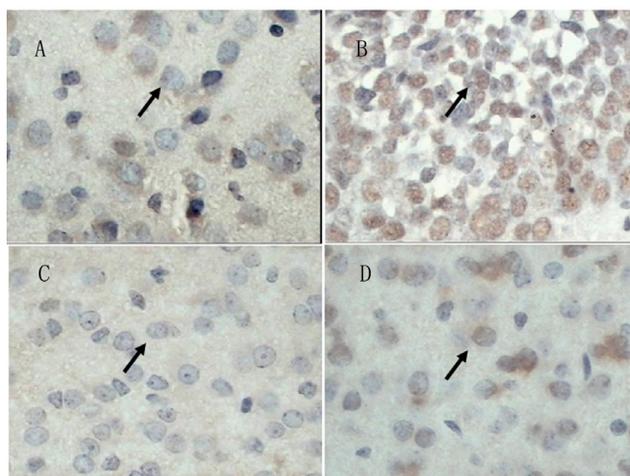
Figure 4. Brain tissues 7 days after hypoxic-ischemic brain damage (HIBD) in neonatal rats by *in situ* hybridization (400X). **A.** Control group. **B.** HIBD group. **C.** NEP1-40 group. **D.** Fasudil group. Arrows indicate brown dots in the nuclei. The number of positive cells in the HIBD group was increased compared to control, NEP1-40 and fasudil groups.
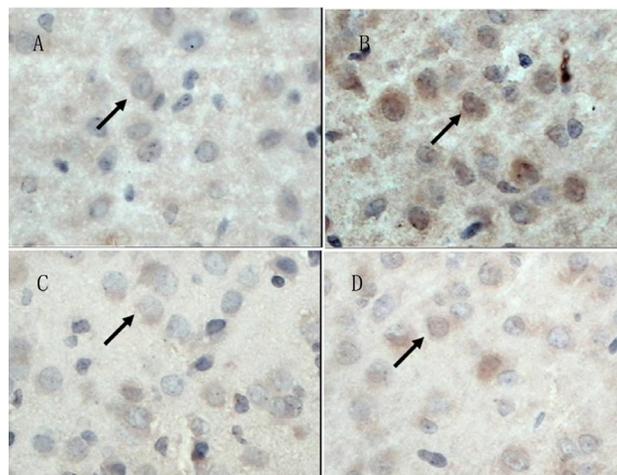
Figure 5. Brain tissues 7 days after hypoxic-ischemic brain damage (HIBD) in neonatal rats by immunohistochemistry (400X). **A.** Control group. **B.** HIBD group. **C.** NEP1-40 group. **D.** Fasudil group. Arrows indicate brown dots in the cytoplasm. The number of positive cells in the HIBD group was increased compared to control, NEP1-40 and fasudil groups.

DISCUSSION

Hypoxic-ischemic encephalopathy in peripartum results in severe brain damage in neonates, including necrosis and apoptosis of brain tissues, and leads to neurological and neurobehavioral deficit (Perlman, 2006). Studies have demonstrated that the 7-day-old rodent has the analogous brain maturity to model a third-trimester human fetus (Kim et al., 2008; Yesilirmak et al., 2008). Despite the fact that perinatal asphyxia closely corresponds to experimental models of cerebral HI, no reliable treatment strategies have been introduced to mitigate neurological injury and the resulting impairments in the clinical setting. In this study, we observed that NEP1-40 and fasudil reduced pathological damage and antagonized the inhibitory effect of Nogo-A, and thus, they may play a vital role in nerve regeneration after HIBD.

Nogo, a member of the reticulon family of membrane-associated molecules, has been identified in myelin of the central nervous system as an inhibitor of axonal outgrowth (Schnaar and Lopez, 2009). An enhanced activity of the Nogo-A pathways may interfere with central nervous system plasticity and hamper neurological functional improvement. Wang et al. (2006) reported that Nogo-A is upregulated in developing rat brain following HIBD. The upregulation of Nogo-A in developing rat brain following HIBD is correlated with the appearance of pathological damage, and thus, these proteins may play a role in events following neonatal HIBD. In our study, Nogo-A was found to be upregulated and there were pathological damages in the ischemic cortex after HIBD, which were consistent with the earlier reports. Importantly, the application of NEP1-40 and fasudil can induce axonal regeneration and reduce the expression of Nogo-A and thus improve synaptic plasticity. The mechanism through which NEP1-40 and fasudil exert a neuroprotective effect is still unknown. One possible explanation is that the Nogo-A/ROCK pathway may be involved in synaptic plasticity.

Cerebral HIBD has been reported to impair memory because hippocampal neurons are susceptible to the deleterious effects of HI, and because the hippocampus is involved in the regulation of memory (Gillani et al., 2010). We found that NEP1-40 and fasudil resulted in a considerable reduction in HI-induced brain injury, when measured in the terms of the response capacity, food response and crawling. Compared to the HIBD group, the response capacity, food response and crawling were improved in NPE1-40 and fasudil groups. NEP1-40 and fasudil groups exhibited a protective effect in the neonatal rat with HIBD.

In conclusion, both NEP1-40 and fasudil treatments after HIBD are capable of improving some of neurological performance, reducing the expression of Nogo-A, and alleviating brain damage. NEP1-40 and fasudil may have a neuroprotective effect in the neonatal rat HIBD model mediated by inhibiting Nogo-A/ROCK pathways. The precise molecular and cellular mechanism of NEP1-40 and fasudil and their potential implications for improvement of behavior following HIBD require further investigation.

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