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Neuronal nitric oxide synthase inhibition reduces brain damage by promoting collateral recruitment in a cerebral hypoxia-ischemia mice model

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Abstract. – OBJECTIVE: The collateral circulation development is considered as a compensatory inherent mechanism to restore damaged blood perfusion after ischemia. We aimed to detect the collateral flow and the mean blood-flow velocities (mBFVs) level in the basilar trunk during or after cerebral hypoxia-ischemia in the mice brain and explore the effect of neuronal nitric oxide synthase (nNOS) inhibition on the collateral flow.

MATERIALS AND METHODS: C57BL/6J mice and the nNOS knockout (KO) mice were randomly divided into a sham-operated group (control) and the hypoxia-ischemia (HI) groups that were treated with the phosphate buffered solution (PBS) control or 7-nitroindazole (7-NI). Cortexes were harvested after the HI treatment for analysis of nNOS expression using Western blot and reverse transcription-polymerase chain reaction (RT-PCR). Ultrasound imaging experiments were performed to detect the collateral flow and the mBFVs level in the basilar trunk.

RESULTS: After cerebral HI, the cortical nNOS mRNA and protein levels increased markedly compared with the sham-operated control mice. Besides, 7-NI treatment had no effect on the blood flow in the sham-operated control mice. What's more, either the 7-NI pretreatment or the nNOS gene knockdown before the HI procedure could attenuate the brain injury by the increased collateral flow and the decreased mBFVs level in the basilar trunk.

CONCLUSIONS: nNOS inhibition protected hypoxic-ischemic-induced mice brain damage by the increased collateral flow and the decreased mBFVs level in the basilar trunk. Therefore, the 7-NI administration may have potential utility for the treatment of HI injury in human beings.

Key Words:
Hypoxic-ischemic brain damage, Neuronal nitric oxide synthase, Collateral flow, Cortex, Mice.

Introduction

Hypoxia-ischemia (HI) is one common contributor to brain damage and leads to learning disabilities, cerebral palsy, and epilepsy[1]. Recent data[2] have suggested a higher occurence of focal ischemia-reperfusion resulting in stroke, in addition to the global cerebral ischemia arising from the systemic asphyxia. A study[3] verified that the collateral recruitment establishment or not, revealed by the blood velocities changes in the basilar trunk, determined the extension degree of the lesion.

Nitric oxide (NO), as one of the vasoactive molecules that can regulate the collateral recruitment, is a special molecule. NO is a reactive, highly diffusible and small molecule produced by the three kinds of NO synthases (NOS) and usually released from the perivascular nitrergic neurons and varieties of endothelial cells[4,5]. NO plays an important role in the vasodilation, the increase of the local blood flow, and the decrease of vascular resistance in the cerebral circulation[6,7]. Studies of hypoxia ischemic models in neonatal cerebral rat concerning the NOS inhibitors have provided similar results for NO effects on the lesion size and the cerebral blood flow (CBF)[8], in consistent with many other researches demonstrating the beneficial impact of NO and NOS inhibition on perinatal hypoxia ischemia[9]. However, no direct evidence has been provided for its participation in the modulation of CBF changes during injury in the adult cerebral rat.

In this work, we investigated the modulation of arterial recruitment by nNOS inhibition before the onset of hypoxia-ischemia in the C57BL/6J mice and the neuronal nitric oxide synthase knock-down (nNOS KO) mice and explored the

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level of cortical nNOS expression after cerebral hypoxia-ischemia. Besides, the role of selective nNOS inhibitor was also elucidated on the regulation of the collateral flow and the mean blood-flow velocities (mBFVs) level in the basilar trunk during or after cerebral hypoxia-ischemia.

Materials and methods

Animal Preparation

C57BL/6J male mice were used as control mice throughout the study weighted between 25-30 g, obtained from the Nanjing Medical Animal Research Center. The neuronal nitric oxide synthase knock-down (nNOS KO) male mice (2-3 months of age) and their wild-type littermates (WT mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The Animal Care and Use Committee approved all experiments carried in the investigation according to the Declaration of the National Institutes of Health Guide for Use and Care of Laboratory Animals.

Experimental Hypoxia-Ischemia

Cerebral hypoxia-ischemia experiment was performed by the middle cerebral artery (MCAO) occlusion on the left side on the basis of the published methods previously[10,11]. Briefly, mice were anesthetized with 1.5% to 2% isoflurane inhalation driven by the 100% oxygen flow. Then, the trachea was carefully intubated and the lungs were mechanically ventilated (110 breaths/min, tidal volume: 0.5 ml). Using a temperature control system, the Body was modulated at 37.0°C. After the incision of the skin, a coated 6-0 filament (Doccol Corp., Redlands, CA, USA) was inserted to the internal carotid artery (ICA) through the external carotid artery (ECA) and advanced 11 mm from the carotid bifurcation, the cerebral ischemia began. After 60 minutes ischemic induction, the filament was quickly collected and the reperfusion then started.

Drug Treatment

The cell-permeable, reversible and competitive selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI, Calbiochem, 25 mg/kg, NJ, USA) was injected intraperitoneally (i.p.) 15 min before surgery, and/or immediately at the reperfusion onset. Vehicle groups received phosphate-buffered saline (PBS) i.p. The calculation of equal doses was carried out on a surface area basis[12]; the dose used in mice was about twice than that used in rats. This dose has been chosen according to previous data demonstrating an effect in the ischemic rat[9].

Ultrasound Imaging Measurements

Thermo-regulated mice were subjected to ultrasound inspection with 0.5% isoflurane anesthesia by the echocardiograph (Vivid 7, GE Medical Systems ultrasound®, Horten, Norway) equipped with the 12-MHz linear transducer (12 L)[13]. The time-average mean blood-flow velocities (mBFVs) were measured in the basilar trunk (BT) at three-time points: before the surgery, during the ischemia (at 40 min), and 15 min after the removal of the Common Carotid Artery (CCA) occlusion.

Western Blot

For biochemical analysis, animals were sacrificed by decapitation, and the cortexes were stored at −80°C until used. After treatment, the cortex was rinsed with ice-cold PBS and subjective to total protein extraction with RIPA (radioimmunoprecipitation assay) lysis buffer. The protein concentrations were quantified by the bicinchoninic acid (BCA) method. 30 ug protein samples were run on 10% gels and then transferred to the PVDF (polyvinylidene difluoride) membrane. After 1 hour of blocking with the 5% non-fat milk, the membranes were incubated with the primary mouse anti-nNOS (1:1000; Chemicon, IL, USA), the rabbit anti-GAPDH antibody (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. After washing in Tris-Buffered Saline and Tween 20 (TBST) for three times, the membranes were then incubated with a peroxidase (HRP) labeled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The bands were washed again, were enhanced with chemiluminescence reagents and visualized with the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

After treatment, the cortex was collected to determine the expression of nNOS mRNA by qRT-PCR. Total RNA was extracted by the TRIzol RNA extraction reagent (Invitrogen, Carlsbad, CA, USA) following the protocols and quantified by the spectrophotometer method. Purified RNA with equal volume was reverse transcribed (RevertAid First Strand cDNA Synthesis Ki, Thermo, K1622, Waltham, MA, USA). The cDNA products were subjected to Real-time quantification on an ABI PRISM 7000 Sequence Detection System (TaKaRa Biotechnology, Dalian, China).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM) and were analyzed using ANOVA followed by Bonferroni-Dunn correction. Statistical analysis was performed using the SPSS software, version 20.0 (SPSS IBM, Armonk, NY, USA). $p<0.05$ was considered statistically significant.

Results

The Level of Cortical nNOS Increases After HI Treatment

After treatment of cerebral hypoxia-ischemia, the cortical nNOS levels increased markedly compared with the sham-operated control mice. Quantitative RT-PCR analysis revealed that the nNOS mRNA level significantly up-regulated in the HI treated group (1.85-fold higher than that of the control, n=8, $p=0.008$, Figure 1A). The relative nNOS protein level was 1.53-fold higher than that of the control mice (n=8, $p=0.013$, Figure 1B) using the Western blot analysis.

7-NI Treatment Had no Effect on the Blood Flow in the Sham-Operated Control Mice

We disposed 10 mice to 7-NI 15 min before the period of cerebral sham-operated hypoxia-ischemia and compared them with 10 animals subjected to the same procedure and treated with the same volume of PBS. Compared to the PBS-treated mice, the mBFVs level in the basilar trunk did not significantly change in the 7-NI treated animals during or after cerebral sham-operated HI treatment ($p=0.464$, $p=0.675$, respectively, Figure 2A, B).

Pretreatment of 7-NI Increases Collateral Recruitment After HI Treatment

10 animals were treated with 7-NI 15 min before the period of cerebral hypoxia-ischemia, and were compared with those subjected to the same volume of PBS. In contrast to the result obtained in the sham-operated mice, the 7-NI treatment in the HI group significantly decreased the mBFVs level in the basilar trunk and increased the collateral flow compared with the PBS treated HI mice during or after cerebral hypoxia-ischemia treatment ($p=0.009$, $p=0.014$, respectively, Figure 3A, B).

The nNOS KO Mice Increase the Blood Flow After HI Treatment

To further confirm the participation of nNOS in the regulation of the mBFVs level in the basilar trunk during or after cerebral hypoxia-ischemia, we performed the next experiment in the nNOS KO mice and their wild-type control. Consistent with the 7-NI pretreatment before the HI, the nNOS KO mice markedly decreased

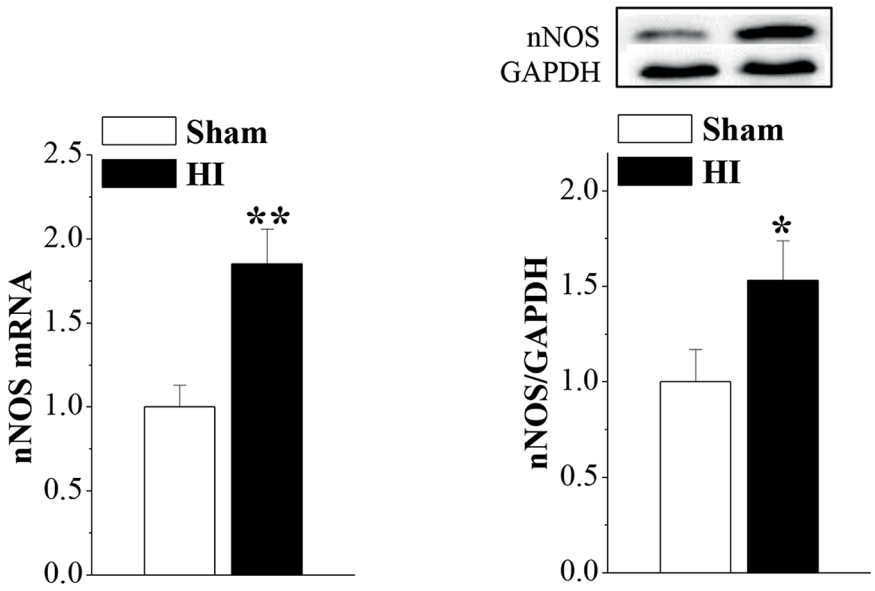

Figure 1. The level of cortical nNOS after HI treatment. The nNOS mRNA level by Quantitative RT-PCR analysis ***(A)*** and the relative nNOS protein level ***(B)*** using the Western blot analysis (compared with the sham-operated mice, *$p<0.05$, **$p<0.01$).

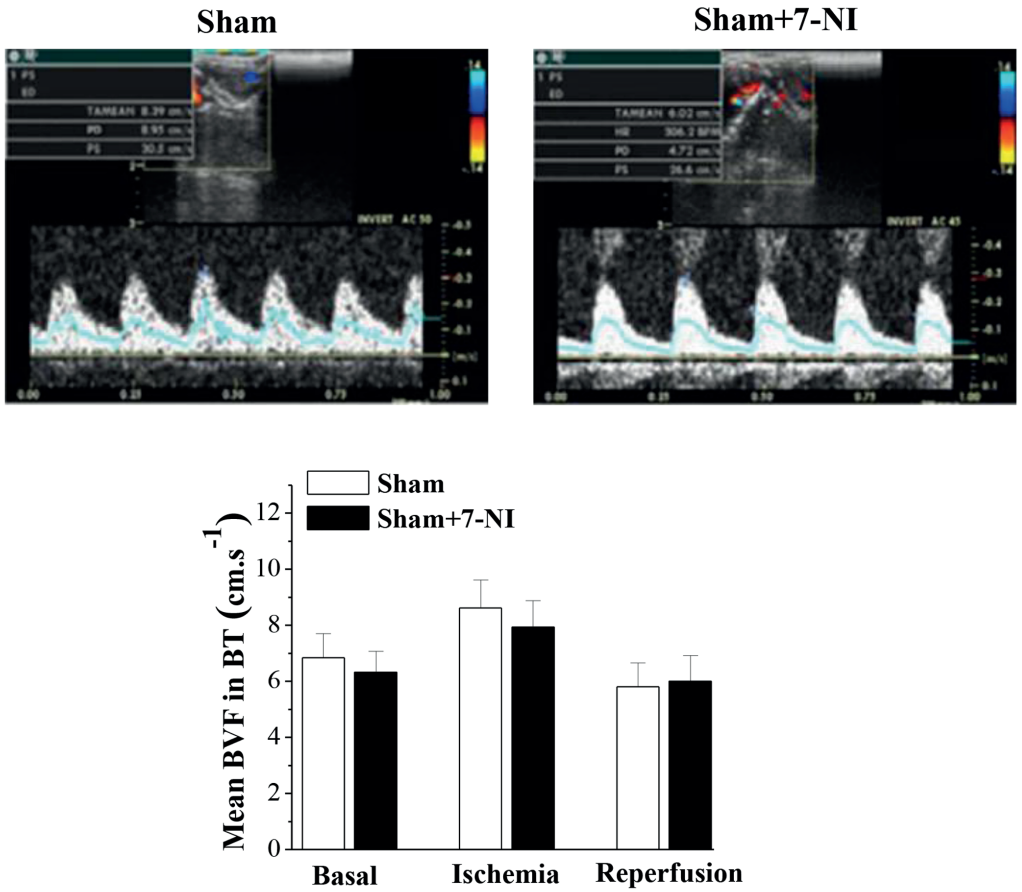

Figure 2. 7-NI treatment on the blood flow in the sham-operated control mice. The mBFVs in the basilar trunk by the method of ultrasound imaging ***(A)*** and the relative mBFVs levels in BT ***(B)*** were calculated using the SPSS software (compared with the PBS treated sham-operated mice, *p<0.05, **p<0.01).

the mBFVs level in the basilar trunk and increased the collateral flow compared with the WT mice during or after the same cerebral hypoxia-ischemia treatment (p=0.022, p=0.006, respectively, Figure 4A, B).

Discussion

In the present study, the results showed that the nNOS level may play an important role in the modulation of the collateral flow and the mBFVs level in the basilar trunk during or after cerebral hypoxia-ischemia. The cortical nNOS mRNA and protein levels increased significantly in the HI group compared with the sham-operated mice. Moreover, either the 7-NI pretreatment or the nNOS gene knock-down before the HI procedure could attenuate the brain injury by the increased collateral flow and the decreased mBFVs level in the basilar trunk. However, the 7-NI pretreatment had no effect on the sham-operated group, which may suggest that the nNOS beneficial function on attenuating the brain injury occur only followed the cerebral hypoxia-ischemia onset instead of the basic condition.

After the cerebral ischemia onset, multifaceted inflammatory reactions quickly emerge in the next few hours. The reactions induced plenties of inflammatory mediators, such as chemokines and cytokines, which then result in many further inflammatory mediators release strengthing the inflammatory reaction[14,15]. NO is a kind of physiological gaseous messenger in the central nervous system[16,17] and synthesized by the nNO synthase-catalyzed reactions. The activation of nNOS and L-citrulline from L-arginine[18] participates in the transduction

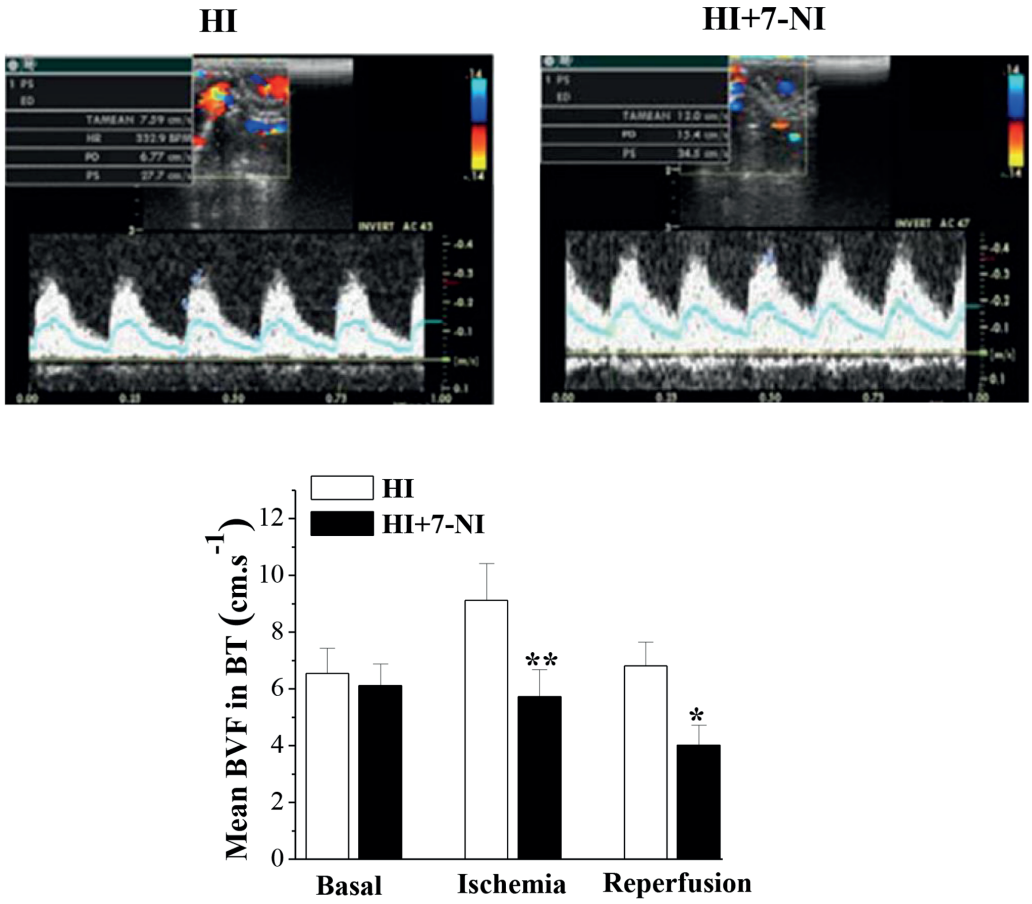

Figure 3. Pretreatment of 7-NI on the blood flow after HI treatment. The mBFVs in the basilar trunk by the method of Ultrasound imaging ***(A)*** and the relative mBFVs levels in BT ***(B)*** were calculated using the SPSS software (compared with the PBS treated hypoxia-ischemia mice, *$p<0.05$, **$p<0.01$).

pathways leading to the intracellular cyclic guanosine monophosphate (GMP) levels elevations[19,20]. Meanwhile, the precise function of the nNOS production remains to be further elucidated. It looks like that this enzyme is involved in many important physiological functions, for example, nNOS may participate in the regulation of the memory or CBF[21,22]. The biosynthesis of nNOS in the brain has increased from the freezing tissue samples of the bilateral carotid occlusion and the focal ischemia of the rat's brain. The nNOS-NO signaling pathway up-regulated according to the ischemic damage degree[23] and during the period of reperfusion if ischemia time was longer than 15 min[24] but disappeared after the nNOS inhibitor 7-nitroindazole (7-NI) administration[25], which may support our present results.

Conclusions

We showed that the 7-NI pretreatment or the nNOS gene knock-down before the HI procedure could attenuate the brain injury by the increased collateral flow and the decreased mBFVs level in the basilar trunk. The close correlation between the brain damage and the 7-NI administration may provide some experimental evidence for the possible effect of the 7-NI against cerebral hypoxia-ischemia. The clear mechanisms underlying the nNOS action and its utility for the treatment of HI injury in human still need to be investigated further.

Conflict of Interest

The Authors declare that they have no conflict of interest.

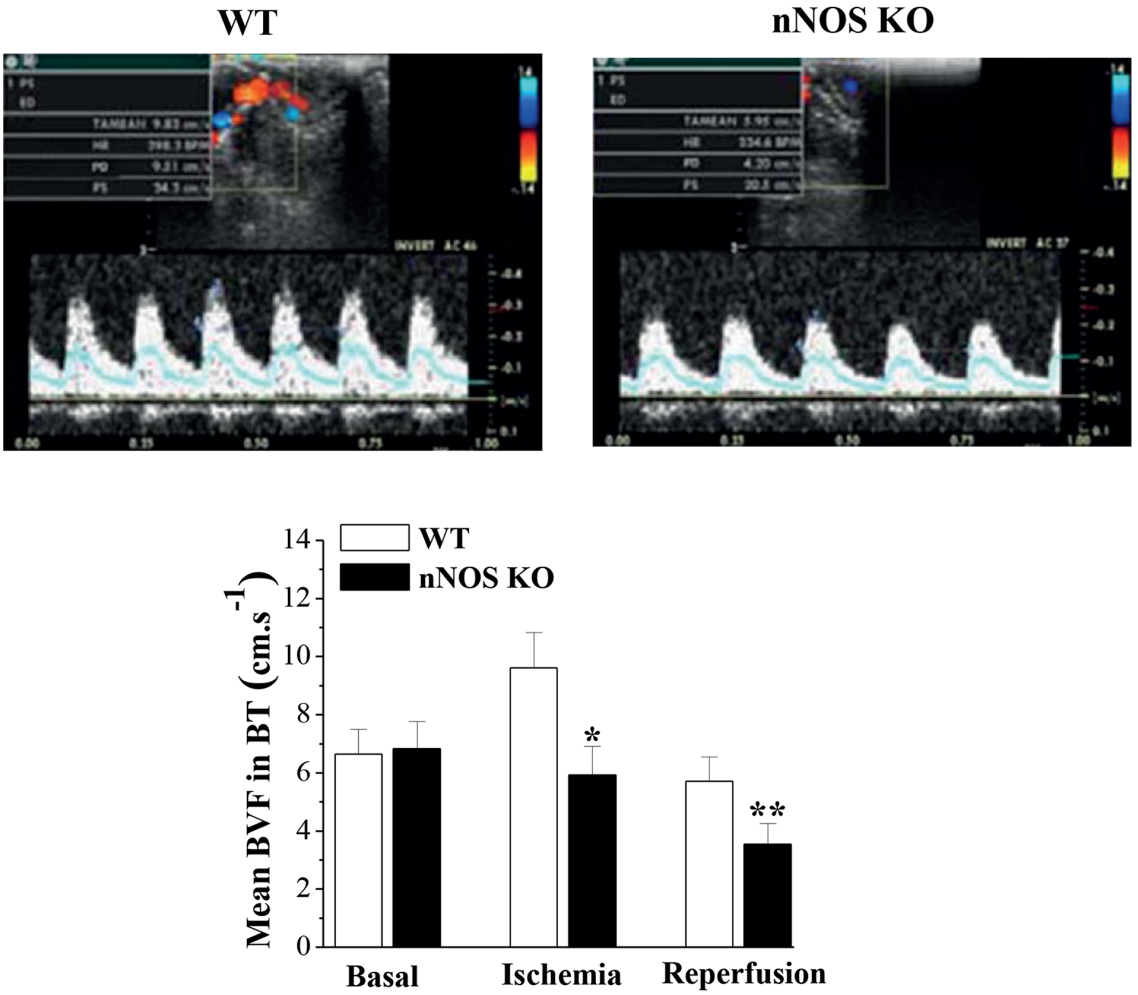

Figure 4. The blood flow of the nNOS KO mice after HI treatment. The mBFVs in the basilar trunk by the method of ultrasound imaging ***(A)*** and the relative mBFVs levels in BT ***(B)*** were calculated using the SPSS software (compared with the hypoxia-ischemia WT mice, *$p<0.05$, **$p<0.01$).

References

1) Charriaut-Marlangue C, Bonnin P, Gharib A, Leger PL, Villapol S, Pocard M, Gressens P, Renolleau S, Baud O. Inhaled nitric oxide reduces brain damage by collateral recruitment in a neonatal stroke model. Stroke 2012; 43: 3078-3084.
2) Golomb MR, Garg BP, Saha C, Azzouz F, Williams LS. Cerebral palsy after perinatal arterial ischemic stroke. J Child Neurol 2008; 23: 279-286.
3) Bonnin P, Leger PL, Deroide N, Fau S, Baud O, Pocard M, Charriaut-Marlangue C, Renolleau S. Impact of intracranial blood-flow redistribution on stroke size during ischemia-reperfusion in 7-day-old rats. J Neurosci Methods 2011; 198: 103-109.
4) Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109-142.
5) Miao R, Leng D, Liu M, Huang XX, Li JF, Gong JN, Liang Y, Zhai ZG, Yang YH, Wang Y, Wan J. Alteration of endothelial nitric oxide synthase expression in acute pulmonary embolism: a study from bench to bioinformatics. Eur Rev Med Pharmacol Sci 2017; 21: 827-836.
6) Pinard E, Engrand N, Seylaz J. Dynamic cerebral microcirculatory changes in transient forebrain ischemia in rats: involvement of type I nitric oxide synthase. J Cereb Blood Flow Metab 2000; 20: 1648-1658.
7) Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: Recent advances. Pharmacol Rev 2009; 61: 62-97.
8) Willmot M, Gibson C, Gray L, Murphy S, Bath P. Nitric oxide synthase inhibitors in experimental ischemic stroke and their effects on infarct size and cerebral blood flow: a systematic review. Free Radic Biol Med 2005; 39: 412-425.
9) Leger PL, Bonnin P, Moretti R, Tanaka S, Duranteau J, Renolleau S, Baud O, Charriaut-Marlangue C. Early recruitment of cerebral microcirculation by neuronal nitric oxide synthase inhibition in a juvenile ischemic rat model. Cerebrovasc Dis 2016; 41: 40-49.
10) Hua F, Ma J, Ha T, Kelley JL, Kao RL, Schweitzer JB, Kalbfleisch JH, Williams DL, Li C. Differential roles of TLR2 and TLR4 in acute focal cerebral ischemia/reperfusion injury in mice. Brain Res 2009; 1262: 100-108.
11) Hua F, Wang J, Sayeed I, Ishrat T, Atif F, Stein DG. The TRIF-dependent signaling pathway is not required for acute cerebral ischemia/reperfusion injury in mice. Biochem Biophys Res Commun 2009; 390: 678-683.
12) Voisin EM, Ruthsatz M, Collins JM, Hoyle PC. Extrapolation of animal toxicity to humans: Interspecies comparisons in drug development. Regul Toxicol Pharmacol 1990; 12: 107-116.
13) Bonnin P, Leger PL, Villapol S, Deroide N, Gressens P, Pocard M, Renolleau S, Baud O, Charriaut-Marlangue C. Dual action of NO synthases on blood flow and infarct volume consecutive to neonatal focal cerebral ischemia. Exp Neurol 2012; 236: 50-57.
14) Thakkar R, Wang R, Sareddy G, Wang J, Thiruvaiyaru D, Vadlamudi R, Zhang Q, Brann D. NLRP3 inflammasome activation in the brain after global cerebral ischemia and regulation by 17beta-Estradiol. Oxid Med Cell Longev 2016; 2016: 8309031.
15) Chevin M, Guiraut C, Maurice-Gelinas C, Deslauriers J, Grignon S, Sebire G. Neuroprotective effects of hypothermia in inflammatory-sensitized hypoxic-ischemic encephalopathy. Int J Dev Neurosci 2016; 55: 1-8.
16) Garthwaite J, Boulton CL. Nitric oxide signaling in the central nervous system. Annu Rev Physiol 1995; 57: 683-706.
17) Stojanovic M, Scepanovic L, Hrncic D, Rasic-Markovic A, Djuric D, Stanojlovic O. Multidisciplinary approach to nitric oxide signaling: focus on the gastrointestinal and the central nervous system. Vojnosanit Pregl 2015; 72: 619-624.
18) Morishita T, Tsutsui M, Shimokawa H, Horiuchi M, Tanimoto A, Suda O, Tasaki H, Huang PL, Sasaguri Y, Yanagihara N, Nakashima Y. Vasculoprotective roles of neuronal nitric oxide synthase. FASEB J 2002; 16: 1994-1996.
19) Lu Y, Hu J, Zhang Y, Dong CS, Wong GT. Remote intrathecal morphine preconditioning confers cardioprotection via spinal cord nitric oxide/cyclic guanosine monophosphate/protein kinase G pathway. J Surg Res 2015; 193: 43-51.
20) Chalimoniuk M, Chrapusta SJ, Lukacova N, Langfort J. Endurance training upregulates the nitric oxide/soluble guanylyl cyclase/cyclic guanosine 3',5'-monophosphate pathway in the striatum, midbrain and cerebellum of male rats. Brain Res 2015; 1618: 29-40.
21) Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat Rev Neurosci 2004; 5: 347-360.
22) Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 1994; 63: 175-195.
23) Sato S, Tominaga T, Ohnishi T, Ohnishi ST. EPR spin-trapping study of nitric oxide formation during bilateral carotid occlusion in the rat. Biochim Biophys Acta 1993; 1181: 195-197.
24) Sato S, Tominaga T, Ohnishi T, Ohnishi ST. Electron paramagnetic resonance study on nitric oxide production during brain focal ischemia and reperfusion in the rat. Brain Res 1994; 647: 91-96.
25) Muramatsu K, Sheldon RA, Black SM, Tauber M, Ferriero DM. Nitric oxide synthase activity and inhibition after neonatal hypoxia ischemia in the mouse brain. Brain Res Dev Brain Res 2000; 123: 119-127.