



# Recombinant Human Erythropoietin Exerts Neuroprotective Effects via Modulation of Nitric Oxide Synthase on Hypoxic-Ischemic Brain Injury in Neonatal Rats

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**Objective:** Erythropoietin (EPO) has neuroprotective effects in many animal models of brain injury including hypoxic-ischemic (HI) encephalopathy, trauma- and excitotoxicity. Current studies have demonstrated the neuroprotective effects of EPO, but there are limited the same consequences occurring during neonatal periods. Here, we investigated whether recombinant human EPO (rHuEPO) can protect the developing rat brain from HI injury via the modulation of nitric oxide synthase.

**Methods:** The *in vitro* model involved culturing embryonic cortical neuronal cells of Sprague-Dawley (SD) rats at 19 days gestation. The cultured cells were divided into five groups: normoxia (N), hypoxia (H), 1, 10 and 100 IU/mL rHuEPO-treated (H+E1, H+E10, and H+E100). In the *in vivo* model, left carotid artery ligation was performed in 7-day-old SD rat pups. The animals were divided into six groups: normoxia control, normoxia Sham-operated, hypoxia only (H), hypoxia+vehicle, hypoxia+rHuEPO before a hypoxic insult (HE-B), and hypoxia+rHuEPO after a hypoxic insult (HE-A). Western blotting and real-time polymerase chain reaction using antibodies and primers of inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) were performed.

**Results:** The rHuEPO-treated group in the *in vitro* model showed increased expressions of iNOS and eNOS and decreased expression of nNOS compared to those of the H group. In the HE-A and HE-B groups of the *in vivo* model of the, the results were similar as aforementioned.

**Conclusion:** rHuEPO exerts neuroprotective properties over perinatal HI brain injuries through the modulation of nitric oxide synthase.

**Key Words:** Erythropoietin, Nitric oxide synthase, Hypoxia-ischemia, Brain

## Introduction

Nitric oxide (NO) overproduction by NO synthase (NOS) has been implicated in the pathogenesis of hypoxic-ischemic (HI) brain damage in neonatal rats.<sup>1-3</sup> There are three isoforms of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS) and an inducible isoform of NOS (iNOS), all of which share approximately 50% or more amino acid homology.<sup>4,5</sup> Among them, nNOS and eNOS are calcium-independent and produce low levels of NO under physiological conditions, while iNOS is not calcium-dependent and induced by inflammatory cytokines.<sup>4,6</sup> Under physiological conditions, iNOS is expressed in both the endothelial layers of large vessels and the choroid plexus to modulate basal brain perfusion. However, during pathological conditions such as hypoxic-ischemic injuries, iNOS is progressively overexpressed in the injured microvasculatures.<sup>5</sup> Studies have shown that the activation of nNOS from neuronal cells and iNOS from both inflammatory and endo-

thelial cells were mainly involved in the production of high NO levels resulting from neuronal damage under various pathological conditions.<sup>1,2,7,8</sup> NOS inhibitors and other agents that reduce toxic NO formation have been shown to decrease neuronal damage triggered by HI injuries by targeting nNOS or iNOS.<sup>2,3</sup>

Recent studies suggest that erythropoietin (EPO) can influence NO synthesis.<sup>9,10</sup> EPO, a 34-kDa glycoprotein, is the principal hematopoietic hormone synthesized by the kidney.<sup>11,12</sup> EPO and its receptors (erythropoietin receptor, EPO-R) are also expressed in nonhematopoietic tissues such as, the nervous system.<sup>11</sup> EPO has been identified as a potential neuroprotective agent in a wide variety of experimental paradigms, from neuronal cell culture to *in vivo* models of the brain.<sup>13-15</sup> There has also been several clinical trials for the use of EPO as a neuroprotective agents in humans.<sup>16-18</sup> Potential mechanisms proposed for the neuroprotective effects of EPO include anti-apoptotic, anti-oxidative and anti-inflammatory activities.<sup>12,19</sup> However, the exact mechanisms of the neuroprotective action of EPO remain elusive. Particularly, there are limited studies that focus on the possible direct modulatory effects of EPO on the expression of discrete NOS isoforms in a neonatal HI injury model.

Therefore, the aim of this study was to investigate the expression of three NOS subtypes in the neonatal HI rat model and determine whether systemically administering recombinant human EPO (rHuEPO) can modulate the expression of each NOS isoform and protect the developing rat brain from HI injuries via the NO-mediated mechanism.

## Methods

### 1. Embryonic cortical neuronal cell culture

This study was performed in accordance with the approved animal use guidelines of the Catholic University of Daegu. The cortical neuronal cell culture of rat embryos based on the Brewer<sup>20</sup> method was performed. Sprague-Dawley (SD) rats pregnant for 19 days were anesthetized with Zoletil® 50 (10 mg/kg intraperitoneally; Vibac Laboratories, Carros, France) and the uterus was removed. The dissected brain cortical tissues were then placed in 2 mL trypsin for 1 minute. Trypsin

(Gibco®BRL, Invitrogen, Grand Island, NY, USA) was removed by washing with Hanks' balanced salt solution (Gibco®BRL). The cells were moved, passed 6-7 times and dispersed. The cell suspension was centrifuged, and the pellets were washed.

After cell counting, they have inoculated in plating Neurobasal media (Gibco®BRL) with about  $2 \times 10^6$  cells/mm<sup>2</sup> in each dish. The cell cultures were incubated in a CO<sub>2</sub> chamber. A fifth of the culture solutions was changed every three days and was replaced.

The cultured cells were divided into five groups: normoxia group (N), hypoxia group (H), and hypoxia+ erythropoietin groups (1, 10, 100 IU/mL) (H+ E1, H+ E10, H+ E100). The N group was prepared in 5% CO<sub>2</sub> incubators while the H group and H+ E groups (before a hypoxia injury) in 1% O<sub>2</sub> incubators (94% N<sub>2</sub>, 5% CO<sub>2</sub>) for 6 hours. The doses used in the *in vitro* experiments were based on the previous study that reduced cytotoxicity of oligodendrocytes by inflammatory stimuli.<sup>21</sup> The experiments were repeated three times. rHuEPO was purchased from CJ Cheiljedang Corporation (Seoul, Korea).

### 2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To determine the protective effects of rHuEPO in the cultured cortical neuronal cells before a hypoxic insult, the relative cell viability was reported.<sup>22</sup> MTT purchased from Ducheфа (Haarlem, The Netherlands) was used to evaluate cell viability following the manufacturer's instructions. Briefly, Cells were plated in 96-well plates. All wells were treated with 1:10-diluted MTT stock solution (5 mg/mL), mixed, and incubated at 37°C for 3 hours. The solution was discarded by gently inverting the plates, and the wells were filled with 100 µL of the lysing buffer (Dimethyl sulfoxide: 95% ethanol=1:1). Finally, the absorbance of the samples was detected using a microtiter plate enzyme-linked immunosorbent assay reader at a wavelength of 540 nm.

### 3. Animal protocols and drug administration

The neonatal rat HI procedure was performed as described by Rice et al.<sup>23</sup> We chose 7-day-old SD rat pups weighing between 12 and 16 grams as HI brain injuries in 7-day-old rats can be considered similar to perinatal asphyxia in full-term infants. The rat pups were anesthetized with isoflurane. The

left common carotid artery (CCA) was exposed, isolated and permanently doubly ligated with 5-0 surgical silk. The incision was sutured, and the animal was allowed to recover in a warm environment. The whole surgical procedure was completed in less than 5 minutes. Before induction of hypoxia, the rat pups were placed in plastic chambers that, were partially submerged in a 37°C water bath. Subjection to systemic hypoxia was performed in such plastic chambers with humidified 8% O<sub>2</sub> and 92% N<sub>2</sub> for 2 hours. After this hypoxic exposure, the pups were returned to their dams for the indicated time. rHuEPO was prepared in phosphate-buffered saline (PBS) and injected intraperitoneally at a dose of 1,000 IU/kg either 30 minutes before or after the hypoxic exposure. The dosage used in the *in vivo* experiments was based on previous studies that showed neuroprotective effect in neonatal hypoxic-ischemic rat models.<sup>3,24</sup>

Sham-operated control animals had their left CCA separated from the vagal nerve; however, no ligation ensued prior to their subjection to hypoxia. Following hypoxic treatment the animals were left in the jars for 15 minutes at normoxia, after which they were returned to their cages. The animals (n=68) were divided into six groups. In group 1 (Normoxia control [NC] n=6), none were exposed to hypoxia. In group 2 (Normoxia Sham-operated [NS] n=6), the animals were subjected to open-close surgery without CCA ligation. In group 3 (hypoxia only [H] n=14, number of death=1), animals were subjected to hypoxia without rHuEPO injection. In group 4 (hypoxia+ vehicle [HV] n=14, number of death=5), the animals were injected with PBS (the same volume as that of rHuEPO). In group 5 (hypoxia+ rHuEPO "before a hypoxic insult" [HE-B] n=14, number of death=2), pups were injected with 1,000 IU/kg of rHuEPO 30 minutes before a hypoxic insult. In group 6 (hypoxia+ rHuEPO "after a hypoxic insult" [HE-A] n=14, number of death=4), pups were injected with the same dose of rHuEPO 30 minutes after a hypoxic insult. Pups from each litter were randomly assigned and marked to an NC, NS, H, HV, HE-B or HE-A group.

#### 4. Brain extraction and protein isolation

Pups were killed at 7 days after the hypoxic insult. Left cerebral hemispheres from rat brains were immediately removed, frozen in liquid nitrogen and stored at -70°C until use. Frozen tissues were homogenized in protein lysis buffer containing

complete™ protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), 1 M Tris-HCl (pH 8.0), 5 M NaCl, 10% Nonidet™ P-40 and 1 M 1,4-dithio-DL-threitol (DTT). After incubation for 20 minutes on ice, the samples were centrifuged at 12,000 rpm at 4°C for 30 minutes, and the supernatant was transferred to a new tube. Total protein was measured with a Bio-Rad Bradford kit (Bio-Rad Laboratories, Hercules, CA, USA).

#### 5. Hematoxylin and eosin (H&E) stain

Histological studies were performed 7 days after the hypoxic insult. After exposing their chest cavities, rats were transcardially perfused with 20 mL of ice-cold saline followed by 20 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) under anesthesia. The brain was removed, fixed in 4% PFA, embedded in paraffin and prepared for light microscopy. Serial 4 µm thick, coronal sections (containing hippocampus/thalamus/hypothalam Coronal Section, five slices per each group) were cut on a cryostat and touch-mounted on slides. Sections were deparaffinized in xylene for 30 minutes and serially treated with 100% (5 minutes), 96% (10 minutes), and 70% (10 minutes) ethanol. Slides were stained with hematoxylin, rinsed for a few seconds with water to remove excess stain, placed in 1% Eosin, then again rinsed briefly in water to remove the acid and washed through a successive series of 70%, 95%, and 100% ethanol at 5 minutes each. Finally, the slides were transferred to xylene for clearing, mounted and covered with cover slips. The brain areas were measured using a densitometer (Multi Gauge Software; Fuji Photofilm, Tokyo, Japan) and was calculated as the ratio of the signal intensity in the ischemic (left) hemisphere compared to the contralateral (right) hemisphere.

#### 6. Western blotting

Samples of equal amounts of proteins (30 µg) were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then they were denaturated in sodium dodecyl sulfate gel-loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 20% glycerol, 4% SDS, and 0.2% bromophenol blue) in boiling water for 10 minutes. After electrophoresis proteins were electrotransferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at a con-

stant voltage of 10 V for 30 minutes. After transfer, the membrane was washed twice with 1x tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST pH 7.4) and then pre-incubated with a blocking buffer (5% nonfat dry milk in TBST) at room temperature for 1 hour. The membrane was then incubated with the primary antibodies 1:1,000 dilutions in TBST at 4°C overnight. After washing, the blots were incubated with the secondary antibody (1:2,000) at room temperature for 1 hour. Finally, the membrane was washed and developed with an enhanced chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences Corp., Piscataway, NJ, USA) or SUPREX (Neuronex, Pohang, Korea). Primary antibodies included iNOS, eNOS, and nNOS were purchased from Stressgen Bioreagents Corporation (Ann Arbor, MI, USA). Secondary antibodies used were goat anti-mouse or rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

## 7. Semi-quantitation of the western blots

The intensity of the corresponding western blot band was measured by using a densitometer (Multi Gauge Software; Fuji Photofilm) and was calculated as the ratio of the signal intensity in the ischemic hemisphere compared to the contralateral hemisphere.

## 8. RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA extraction was carried out with TRIzol® (Invitrogen, Carlsbad, CA, USA). Briefly, a piece of tissue was homogenized in 1 mL of TRIzol® reagent. Total RNA was separated from DNA and proteins by adding chloroform and was precipitated using isopropanol. The precipitate was washed twice with 100% ethanol, air-dried and re-diluted in diethylpyrocabonate-treated distilled water. The amount and purity of extracted RNA were quantitated by GeneQuant™ 1300 spectrophotometer (GE Healthcare Life Sciences, Logan, UT, USA). The RNA was then stored at -70°C before further processing. For real-time PCR (for reverse transcription), total RNA (1 µg) was reverse transcribed for 1 hour at 37°C in a reaction mixture containing 20 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTP (Promega), 0.5 ng Oligo (dT) 15 primer (Promega), 1x RT buffer and 200 U M-MLV reverse transcriptase (Promega). The reaction mixture was then incubated at 95°C for 5

minutes to stop the reaction. The cDNA was then stored at -20°C before further processing.

Real-time PCR was performed in 48 well PCR plates (Mini Opticon™ Real-Time PCR System; Bio-Rad Laboratories) using DyNAmo™ SYBR® green qPCR kit (FINNZYMES, Espoo, Finland) according to the manufacturer's instructions. Amplification conditions are shown in Table 1. The thermal profile was 95°C for 15 minutes followed by 40 cycles of denaturation, annealing, and extension as indicated in Table 1. Real-time PCR data were analyzed with LightCycler software (Bio-Rad Laboratories). Each sample was conducted in triplicate.

## 9. Statistics analysis

The data were analyzed using the SPSS version 22.0 statistical analysis package (IBM SPSS, Armonk, NY, USA). Examined data were assessed using the *t*-test, general lineal model, and analysis of variance. In each test, the data were expressed as the mean±standard deviation, and *P*<0.05 was accepted as statistically significant.

# Results

## 1. Microscopic images of the cultured neuronal cells (*in vitro*)

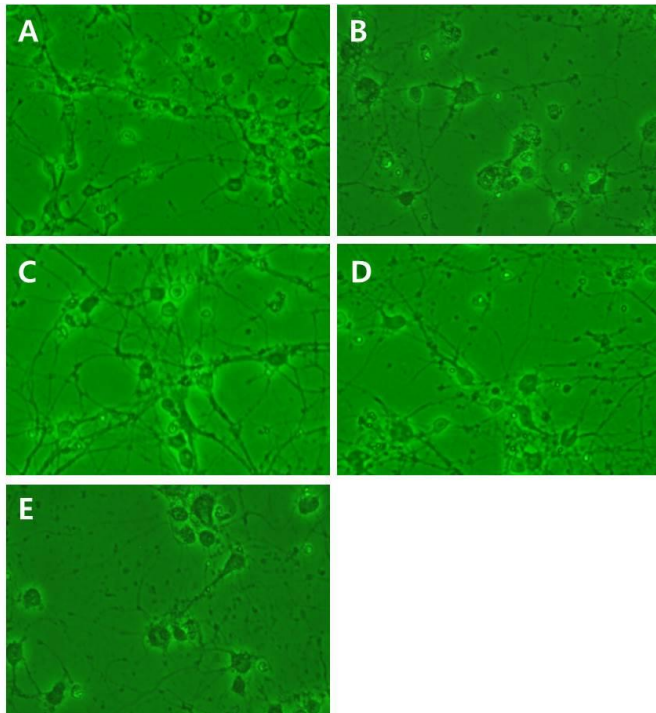
The cortical neuronal cells were observed using an inverted microscope (TS 100-F; Nikon Instruments Inc., Garden City, NY, USA) under high magnification (×200). Compared to those in the N group (Fig. 1A), the neuronal cells in the H group (Fig. 1B) were reduced in number, appeared swollen and with less number of processes, potentially indicating cell damage. After rHuEPO treatment, the cellular pattern in the H+E1 (Fig. 1C)

**Table 1.** Primer Pairs and Annealing Temperature for Real-Time PCR

Name	Primer sequence (5'-3')	Annealing
iNOS	F:AGGCTTGGGTCTTGTAGCCTAGT	55°C
	R:ATTCTGTGCAGTCCCAGTGAGGAA	
eNOS	F:GGATTCTGGCAAGACCGATTAC	57°C
	R:GGTGAGGACTTGTCACAACT	
nNOS	F:CCTTCCGAAGCTTCTGGCAACAGC	59°C
	R:TGGACTCAGATCTAAGCGGTGG	

Abbreviations: PCR, polymerase chain reaction; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.



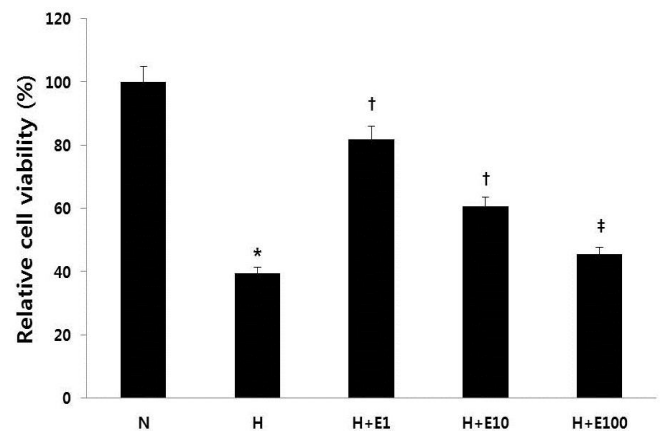


**Fig. 1.** High magnification ( $\times 200$ ) photomicrographs of brain cortical cell culture from a pregnant 19 days Sprague-Dawley rat were revealed. The recombinant Human Erythropoietin (rHuEPO) was administered at 1, 10, and 100 IU/mL. (A) Normoxia group (N), (B) hypoxia group (H), (C) hypoxia+1 IU/mL rHuEPO treated group (H+E1), (D) hypoxia+10 IU/mL rHuEPO treated group (H+E10), (E) hypoxia+100 IU/mL rHuEPO treated group (H+E100).

and H+ E10 (Fig. 1D) groups was similar to that of the N group, suggesting that rHuEPO was able to decrease hypoxia-induced cell damage. The amount and morphology of neuronal cells in the H+ E100 group (Fig. 1E) was similar to that in the H group, suggesting that rHuEPO at this concentration did not attenuate processes involved in cellular damage.

## 2. MTT assay of the cultured neuronal cells (*in vitro*)

To determine the protective effects of rHuEPO in cultured neuronal cells, relative cell viability in the H and rHuEPO-treated groups were determined by the MTT assay, with viability expressed as a percentage (%) relative to that of the N (Fig. 2). The relative cell viability ( $39.4 \pm 5.4\%$ ) of the H group ( $P < 0.01$ ) was significantly reduced compared to that ( $100 \pm 5.0\%$ ) of the N group, while those in the H+ E1 and H+ E10 groups were  $81.8 \pm 5.5\%$  and  $60.6 \pm 5.2\%$ , respectively, both higher than for the H group. However, the relative cell viability in the H+ E100 group was  $45.4 \pm 5.0\%$ , with less extensive im-



**Fig. 2.** The recombinant Human Erythropoietin (rHuEPO) attenuates hypoxic injury of rat brain. Cultured embryonic cortical neuronal cells were prepared with rHuEPO for 30 min before a hypoxic insult. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. N, normoxia group; H, hypoxia group; H+E1, hypoxia+1 IU/mL rHuEPO-treated group; H+E10, hypoxia+10 IU/mL rHuEPO-treated group; H+E100, hypoxia+100 IU/mL rHuEPO-treated group. \* $P < 0.01$  for the H group compared to the N group.  $^{\dagger}P < 0.01$  for the H+E1 and H+E10 groups compared to the H group.  $^{\ddagger}P < 0.05$  for the H+E100 group compared to the H group.

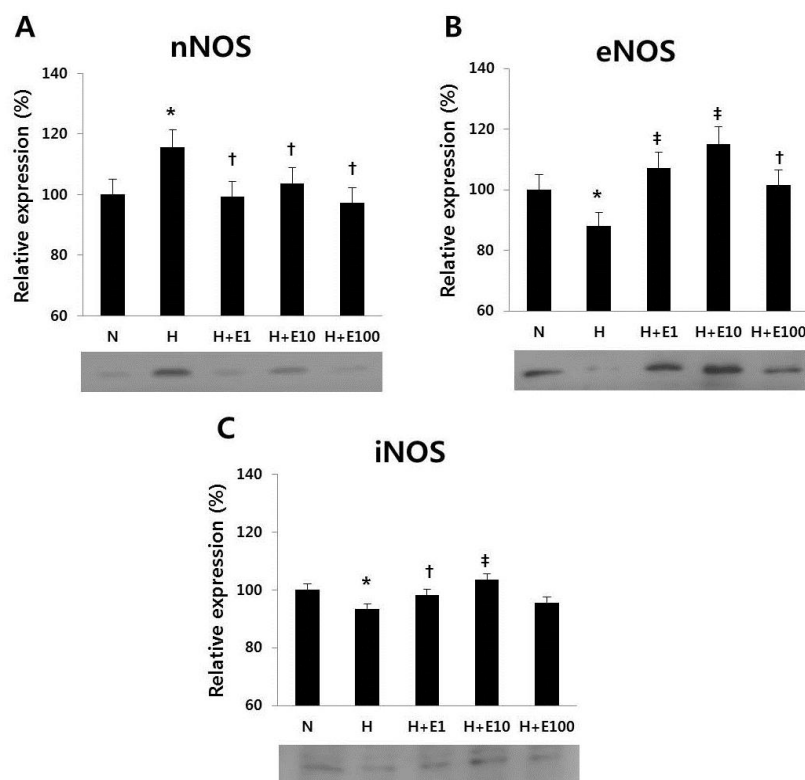
provement than other rHuEPO-treated groups.

## 3. Expressions of nNOS, eNOS, and iNOS by Western blot (*in vitro*)

The protein expression of nNOS in the H group was higher compared to the N group; however, with rHuEPO treatment, expression was observed to decrease to the level of the N group ( $P < 0.05$ ) (Fig. 3A). The protein expression of eNOS ( $P < 0.05$ ) (Fig. 3B) was reduced in the H group, but with rHuEPO treatment, levels were seen to increase in the H+ E1 and H+ E10 groups. In the H+ E100 group, expression of eNOS was increased to a lesser extent than the H+ E1 or H+ E10 groups. The protein expression pattern of iNOS ( $P < 0.05$ ) (Fig. 3C) was similar to eNOS; in that it was reduced in the H group compared to that in the N group and increased in the H+ E1 and H+ E10 groups. But, the response of rHuEPO treatment was more prominent in eNOS than iNOS. In the H+ E100 group, rHuEPO did not induce significant changes in expression of iNOS.

## 4. Expressions of iNOS, eNOS, and nNOS mRNA by real-time PCR (*in vitro*)

The mRNA expression of nNOS in the H group was higher



**Fig. 3.** Western blotting of (A) nNOS, (B) eNOS, and (C) iNOS in cultured cortical neuronal cells from 19-day-old rat embryos was revealed. The recombinant Human Erythropoietin (rHuEPO) was administered at 1, 10, 100 IU/mL. N, normoxia group; H, hypoxia group; H+E1, hypoxia+1 IU/mL rHuEPO treated group; H+E10, hypoxia+10 IU/mL rHuEPO treated group; H+E100, hypoxia+100 IU/mL rHuEPO treated group. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase. \* $P < 0.05$  for the H group compared to the N group. † $P < 0.05$ , ‡ $P < 0.05$  for the H+E1, H+E10, and H+E100 groups compared to the H group.

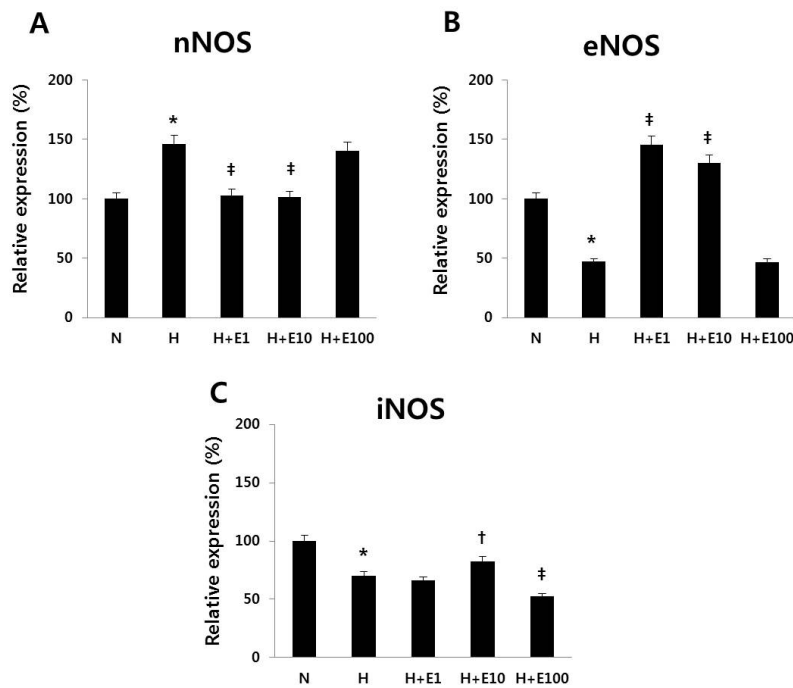
compared to the N group. However, with rHuEPO treatment, expression was observed to decrease to the level of the N group ( $P < 0.05$ ) (Fig. 4A). In the H+E100 group, rHuEPO did not significantly attenuate expression of nNOS.

The mRNA expression of eNOS ( $P < 0.05$ ) (Fig. 4B) was reduced in the H group, but with rHuEPO treatment, levels were seen to increase in the H+E1 and H+E10 groups. In the H+E100 group, rHuEPO fail to upregulate eNOS expression.

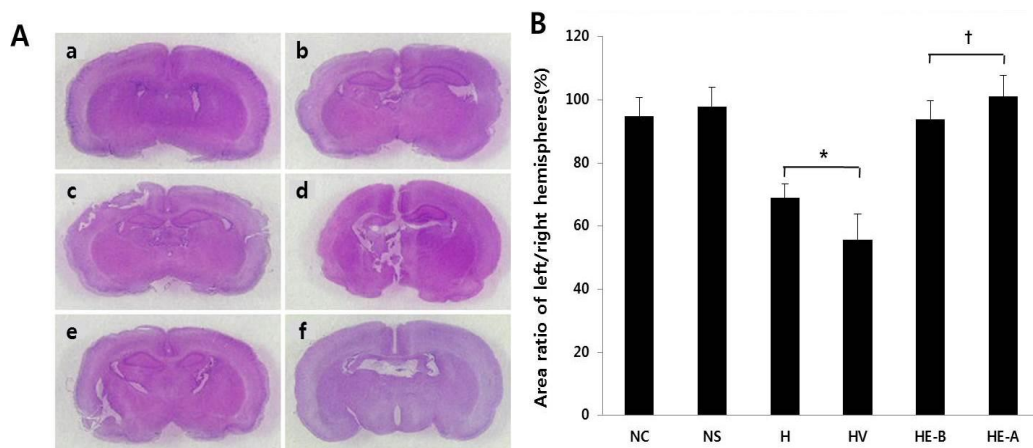
The mRNA expression of iNOS ( $P < 0.05$ ) (Fig. 4C) was reduced in the H group compared to that in the N group. Administration of rHuEPO did not increased iNOS expression to the level of the N group while eNOS showed higher expression than the N group in the H+E1 and H+E10 groups.

## 5. Gross morphology of rat brain stained with H&E (*in vivo*)

The severity of brain damage was assessed by comparing the cerebral hemisphere areas that were ipsilateral and contralateral to the carotid ligation for the calculation of percent damage. The histologic findings and the area ratio of left/right hemispheres in each group were shown in Fig. 5A and 5B, respectively. There are no significant changes in the area between both hemispheres in the NC (Fig. 5A-a) and NS (Fig. 5A-b) groups. H&E stain of 7-day-old rat brains after HI insult revealed that unilateral HI injuries (Fig. 5A-c and 5A-d) destroyed affected brain cortex and reduced the area of the ischemic hemisphere, in comparison to those of the contralateral hemisphere. Rat brains were more preserved in the rHuEPO-treated group (Fig. 5A-e and 5A-f) than in the vehicle-treated group (Fig. 5A-d), suggesting that rHuEPO may provide signi-



**Fig. 4.** Real-time PCR of (A) nNOS, (B) eNOS, and (C) iNOS in cultured cortical neuronal cells from 19-day-old rat embryos was revealed. The recombinant Human Erythropoietin (rHuEPO) was administered at 1, 10, 100 IU/mL. N, normoxia group; H, hypoxia group; H+E1, hypoxia+1 IU/mL rHuEPO treated group; H+E10, hypoxia+10 IU/mL rHuEPO treated group; H+E100, hypoxia+100 IU/mL rHuEPO treated group. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase. \* $P < 0.01$  for the H group compared to the N group. † $P < 0.05$ , ‡ $P < 0.01$  for the H+E1, H+E10, and H+E100 groups compared to the H group.



**Fig. 5.** (A) H&E staining. (a) normoxia control group, (b) normoxia sham-operated group, (c) hypoxia group, (d) hypoxia+vehicle group, (e) hypoxia+recombinant Human Erythropoietin (rHuEPO)-treated group before a hypoxic insult, (f) hypoxia+rHuEPO-treated group after a hypoxic insult ( $n=5$  in each group). (B) Area ratio of left/right hemispheres. NC, normoxia control group; NS, normoxia sham-operated group; H, hypoxia group; HV, hypoxia+vehicle group; HE-B, hypoxia+rHuEPO group treated before a hypoxic insult; HE-A, hypoxia+rHuEPO group treated after a hypoxic insult. \* $P < 0.01$  for the H, HV groups compared to the NC, NS groups. † $P < 0.01$  for the HE-B, HE-A groups compared to the H, HV groups.

ficant protection against cerebral HI injuries.

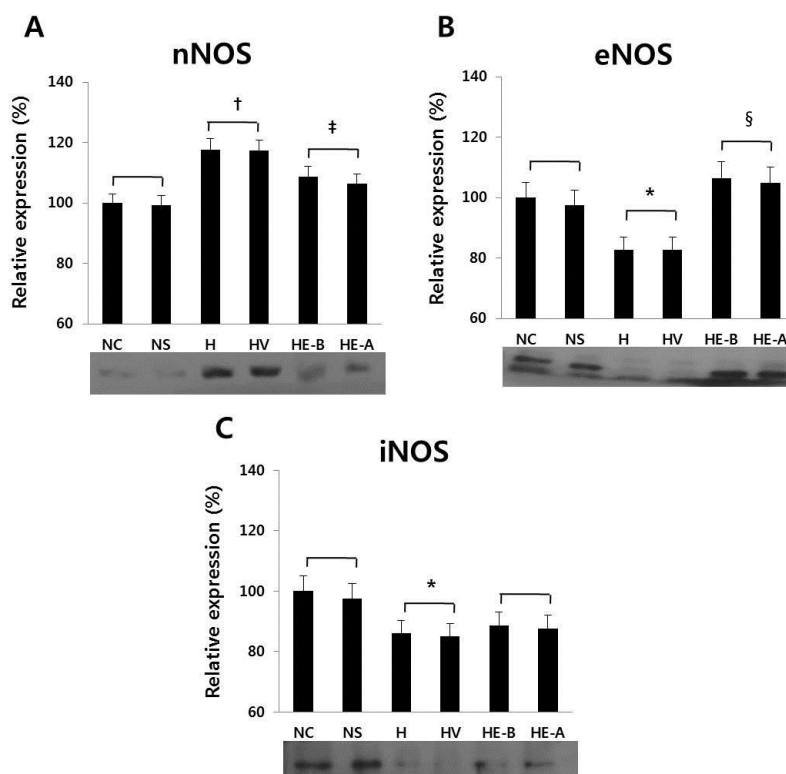
#### 6. Expressions of nNOS, eNOS, and iNOS by Western blot (*in vivo*)

The expression pattern of nNOS was opposite to that of eNOS. The protein expression of nNOS ( $P<0.05$ ) (Fig. 6A) increased and that of eNOS ( $P<0.05$ ) (Fig. 6B) decreased in the H and HV groups compared with the NC and NS groups. As for the rHuEPO-treated groups, increased expression of nNOS after HI injury was attenuated, while expression of eNOS was increased as compared with that of the H or HV groups. The protein expression of iNOS ( $P<0.05$ ) (Fig. 6C) was decreased in both H and HV groups, while in the HE-B and HE-A groups, rHuEPO did not significantly affect the expression of iNOS.

#### 7. Expressions of iNOS, eNOS, and nNOS mRNA by real-time PCR (*in vivo*)

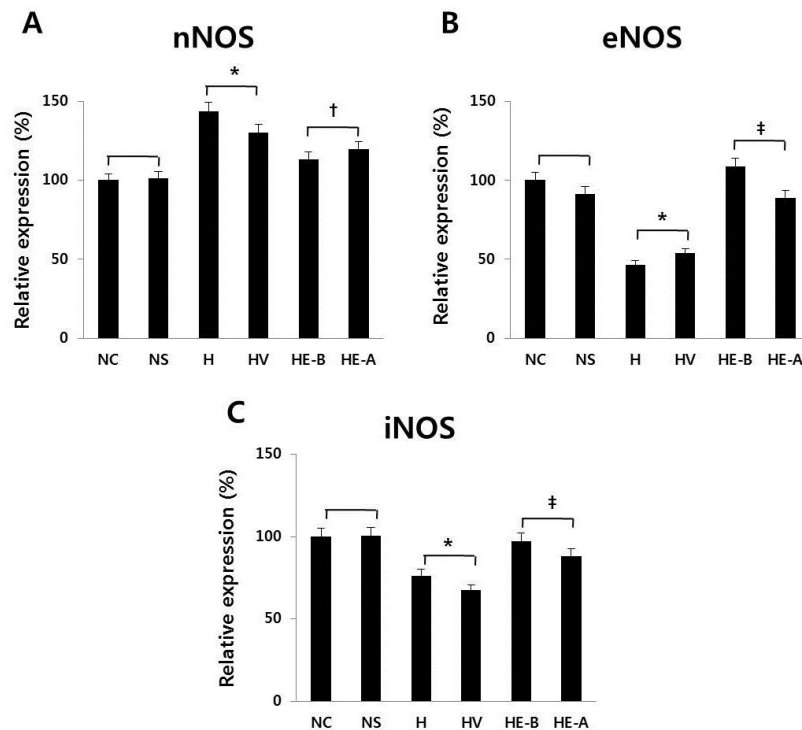
The mRNA expression pattern between nNOS and eNOS was also opposite. The mRNA expression of nNOS ( $P<0.05$ ) (Fig. 7A) increased and that of eNOS ( $P<0.05$ ) (Fig. 7B) decreased in the H and HV groups compared with the NC and NS groups. As for the rHuEPO-treated groups, increased expression of nNOS after HI injury was attenuated, while expression of eNOS was increased as compared with that of the H or HV groups. The mRNA expression of iNOS ( $P<0.05$ ) (Fig. 7C) was decreased in both H and HV groups, while in the HE-B and HE-A groups, rHuEPO increased mRNA expression of iNOS with a lesser degree compared with eNOS.

## Discussion



**Fig. 6.** Western blotting of (A) nNOS, (B) eNOS, and (C) iNOS at 7 days after a hypoxic injury was revealed. The recombinant Human Erythropoietin (rHuEPO) was administered at 1,000 IU/kg. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase. NC, normoxia control group; NS, normoxia sham-operated group; H, hypoxia group; HV, hypoxia+vehicle group; HE-B, hypoxia+rHuEPO group treated before a hypoxic insult; HE-A, hypoxia+rHuEPO group treated after a hypoxic insult. <sup>\*</sup> $P<0.05$ , <sup>†</sup> $P<0.01$  for the H, HV groups compared to the NC, NS groups; <sup>‡</sup> $P<0.05$ , <sup>§</sup> $P<0.01$  for the HE-B, HE-A groups compared to the H, HV groups.





**Fig. 7.** Real-time PCR of (A) nNOS, (B) eNOS, and (C) iNOS at 7 days after a hypoxic injury was revealed. The recombinant Human Erythropoietin (rHuEPO) was administered at 1,000 IU/kg. nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NC, normoxia control group; NS, normoxia sham-operated group; H, hypoxia group; HV, hypoxia+vehicle group; HE-B, hypoxia+rHuEPO group treated before a hypoxic insult; HE-A, hypoxia+rHuEPO group treated after a hypoxic insult. \* $P < 0.01$  for the H, HV groups compared to the NC, NS groups. † $P < 0.05$ . ‡ $P < 0.01$  for the HE-B, HE-A groups compared to the H, HV groups.

Excessive NO production is one of the most important downstream mechanisms of neuronal injury after HI insult as NOS inhibition reduces neuronal cell damage in adult or neonatal ischemic rat models.<sup>2,25</sup> In addition, a significant increase of NO metabolites was observed only on the ligated side of the cortex in ischemic rat models.<sup>1</sup> Administration of exogenous rHuEPO has been shown to reduce endogenously induced NO production.<sup>3</sup> In this study, rHuEPO significantly led to increasing cell viability against hypoxic injury in the *in vitro* model and attenuated tissue loss *in vivo* model, indicating that rHuEPO provides neuronal cell protection post-HI brain injury. However, the effect of rHuEPO on each isoform has not yet been fully elucidated.

Even though each isoform of NOS has both toxic and protective roles, several studies have demonstrated increased NO

production by nNOS or iNOS in brains suffering from damage after hypoxic or HI insults.<sup>26,27</sup> During the acute phase of ischemic insults, extracellular glutamate release and calcium influxes through N-methyl-D-aspartate (NMDA) receptor activation activates calcium-dependent nNOS and eNOS.<sup>7,26</sup> Initial activation of nNOS and eNOS was primarily demonstrated in several ischemic rat models.<sup>1,27-29</sup> However, NO production by iNOS is delayed compared to those of nNOS or eNOS because iNOS is induced by inflammatory cytokines released from damaged brain tissues.<sup>1,8</sup> It has been shown that there were 2 peaks of NO production during the hypoxic and reoxygenation periods in the neonatal HI rat model.<sup>1</sup> They demonstrated that 7-nitronidazole, a selective nNOS inhibitor, attenuates both peaks while aminoguanine, a selective iNOS inhibitor, partially attenuates the latter peak. These suggest that nNOS play an important role in NO overproduction during

both the hypoxic and reoxygenation phases of HI injury, and pathways involving nNOS may affect the later activation of iNOS. Thus, nNOS inhibition can also ablate the ensuing NO overproduction by iNOS.

There are several evidences supporting that nNOS activation contributes to neuronal cell death after HI injury in the neonatal brain, and that its inhibition results in neuroprotection.<sup>1,2,27,30</sup> Higher expression of nNOS has been noted in regions of the developing brain that are selectively vulnerable to HI injuries.<sup>7</sup> Although nNOS-positive neurons were observed to decrease 7 days after MCA occlusion,<sup>4</sup> another study showed that nNOS containing neurons were densely present at the margins of the necrotic cavitation at 7 days post-insult.<sup>7</sup> Due to the lesser vulnerability of nNOS-containing neurons against HI injuries, and a selective increase of nNOS fibers after an HI insult, these neurons may participate in sustained NO production.<sup>27,30</sup> The present study showed that there was indeed increased expressions of nNOS protein and mRNA at 7 days post-HI insult, suggesting that nNOS may potentially produce NO constantly. Taking these data into consideration, intervention targeting nNOS may exert neuroprotection by attenuating constant NO production against HI injuries. This study demonstrated that exogenous rHuEPO administration attenuated nNOS activation and increased eNOS activation after an HI insult with gross or microscopic evidence of tissue or cell protection.

Although eNOS has been shown to be calcium-dependent and thus upregulated after HI insults,<sup>28,29</sup> the expression of eNOS was observed to decrease 7 days post-H or HI. This isoform of NOS protects against ischemic brain injuries through vasodilation and the maintenance of cerebral blood flow.<sup>2</sup> The increased expression of eNOS was observed in rHuEPO-treated groups which resulted in less damage. The study by Niwa et al.<sup>4</sup> identified the expression of iNOS and eNOS were higher than the baseline level. In the same study, eNOS was mainly expressed in the endothelium, while iNOS is found in both endothelial and inflammatory cells.<sup>7</sup> In another study, subacute increases in iNOS mRNA expression was observed at 2 days after induction of focal ischemia, after which decrease was observed at 7 days post-HI.<sup>31</sup> Our *in vitro* study of cortical neurons revealed that neuronal cells can induce also expression of eNOS and iNOS as a protective response against HI injuries. Administration of rHuEPO led to improve neuronal cell viability

and increase neuronal eNOS and iNOS, which may induce vasodilation or regional cerebral blood flow increase, and further provide the additive effect of neuroprotection. Interestingly, the expression patterns of iNOS and eNOS after HI insults and rHuEPO administration *in vivo* were similar to the *in vitro* studies. Considering the results from our *in vitro* studies, the decreased expressions of eNOS or iNOS suggest that neuronal cells containing these isoforms may be more vulnerable than those bearing nNOS. Since eNOS and iNOS were measured at 7 days post-HI insult, the profound neuronal damages have already occurred, thereby resulting in significant neuronal, glial and endothelial cell loss. Increased expressions of eNOS or iNOS after rHuEPO administration may thus act as a neuroprotection by increasing local cerebral blood flow and modulating gene expression.<sup>28,29</sup>

Reports regarding the effects of rHuEPO on iNOS have been controversial. A study implicated that EPO reduced cytotoxicity as well as NO production resulted from damaged inflammatory oligodendrocytes.<sup>21</sup> In contrast, there is also evidence opposing the role of EPO in the prevention of cytokine-induced increases in NO production, showing significant reductions in neuronal apoptosis despite a cytokine-induced NO insult.<sup>21,25</sup> The disputes in these studies suggest that the effect of EPO on iNOS may be cell type-specific. In the present study, the degree of change in iNOS expression upon rHuEPO treatment was observed to a lesser extent than eNOS expression. EPO has been reported to have little effect on cytokine-induced NO production, suggesting that EPO may have less consequences on iNOS expression.<sup>25</sup> In addition, the time course of iNOS showed that its expression usually peaks at 24-48 hours and returns to the baseline at approximately 7 days. This observation may contribute to the minimal effect of rHuEPO on iNOS expression in the present study. Although the expression of iNOS was heightened to a certain extent, it might not have been enough to offset the positive effects of suppressing nNOS and increasing eNOS expressions.

EPO prevents apoptosis induced by NMDA or NO.<sup>13,25,32</sup> Although EPO did not attenuate NO production by iNOS, its anti-inflammatory action may indirectly attenuate iNOS activation. EPO exerts its neuroprotective properties by reducing the NO formation and antagonizing the toxicity of free radicals mediated by NO.<sup>16,19</sup> In addition, EPO exerts its physiological functions

through EPO-R, which serves to link intracellular signaling pathways including the antiapoptotic machineries.<sup>33</sup> Moreover, EPO has long-term protective effects such as involvement in neurogenesis, oligodendrogenesis, angiogenesis and tissue remodeling.<sup>14,15,33-36</sup> Glutamate-induced overactivation of NMDA receptors on neurons leads to excessive production of NO via calcium-dependent nNOS.<sup>7</sup> Therefore, EPO may indirectly reduce excessive NO production by reducing the activation of NMDA receptors. Although immature brains may have less capacity to activate NOS in response to HI injury,<sup>26</sup> injuries induced by NMDA are enhanced in immature brains, showing susceptibility to excitotoxicity.<sup>2</sup> Additionally, nNOS has been shown to co-localize with NMDA receptors at the synapse.<sup>7</sup> In summary, the effects of rHuEPO on NO production may potentially be primarily modulated by nNOS.

In our *in vivo* studies, rHuEPO showed a neuroprotective effect in both HE-B and HE-A groups with an attenuation of nNOS activation. A previous study showed that selective nNOS inhibitors did not exert neuroprotective effects when administered after a cerebral injury in contrast to being administered prior to the injury.<sup>7</sup> However, in this study, rHuEPO decreased neuronal damage irrespective of the timing of rHuEPO administration, which was accompanied by both nNOS attenuation and eNOS activation. These results suggest that there may be other potential protective mechanisms besides nNOS inhibition. In addition, the protective effect of selective NOS inhibitors was dependent on the ability to suppress NO production for prolonged periods after the initial insult.<sup>2</sup> In the present study, rHuEPO attenuates nNOS activation even at 7 days after HI, indicating that rHuEPO can suppress NO production for a prolonged time, thereby improving cell survival after insult. With regards to dosage, our *in vitro* study demonstrated that, as the dose of rHuEPO increases, fewer cells remain viable. A previous study reported the adverse effects of high EPO doses, which included increased thrombosis and decreased cell viability.<sup>37</sup> According to another study, 1 IU/mL of EPO cerebrospinal fluid levels showed neuroprotection without adverse complications such as erythropoiesis or thrombosis.<sup>17</sup> There is evidence that extreme doses of EPO do not provide significant tissue protection,<sup>15</sup> which is consistent with the present study. EPO-induced erythropoiesis might increase the thrombotic effect. Therefore, a higher dose may result in adverse outcomes on cell

viability. The mRNA expression of iNOS, eNOS, and nNOS did not differ between the H group and the H+ E100 group. Moreover, among the rHuEPO-treated groups in our *in vitro* study, the number of viable cells was the lowest in the H+ E100 group.

While selective NOS inhibitors have been reported to exhibit neuroprotective effects, other studies have demonstrated contradicting results with non-selective NOS inhibitors.<sup>2,6,38</sup> Because these contradictions are thought to be consequences of the inhibition of eNOS at higher doses of non-selective NOS inhibitors, the development of drugs that inhibit nNOS without suppressing eNOS is warranted.<sup>38,39</sup> In this study, rHuEPO not only inhibited prolonged activation of nNOS but also increased eNOS expression, lead to decreasing infarct size and cytoprotection. Therefore, EPO is considered as a potential neuroprotective candidate. Furthermore, since it has been used in clinical practice to treat anemia, it has advantages in clinical applications than other agents. However, it has been reported that high doses of rHuEPO can increase mortality due to thrombosis or hemorrhage, thus it is necessary to find the optimal dose and timing of administration.<sup>37</sup>

A limitation of this study is that each isoform's expression was only investigated at 7 days after the insult and failed to reflect the time-course expression of each NOS isoforms. It has also been reported earlier that EPO treatment decreases infarct size while later EPO treatment can influence neurogenesis.<sup>15,34-36</sup> Therefore, further studies will be needed to evaluate optimal dosage, timing and the need for multiple doses.

In conclusion, our experiments demonstrate that rHuEPO can prevent the degeneration of neonatal cerebral neuronal cells caused by hypoxic insult. In addition, neuroprotective effects of rHuEPO on HI brain injury in neonatal rats may involve NO-mediated mechanisms. EPO may be useful for further developments of clinical therapies related to perinatal HI encephalopathy.

## References

- 1) Higuchi Y, Hattori H, Kume T, Tsuji M, Akaike A, Furusho K. Increase in nitric oxide in the hypoxic-ischemic neonatal rat brain and suppression by 7-nitroindazole and aminoguanidine. *Eur J Pharmacol* 1998;342:47-9.
- 2) Ishida A, Trescher WH, Lange MS, Johnston MV. Prolonged suppression of brain nitric oxide synthase activity by 7-nitroindazole protects against

- cerebral hypoxic-ischemic injury in neonatal rat. *Brain Dev* 2001;23:349-54.
- 3) Kumral A, Baskin H, Gokmen N, Yilmaz O, Genc K, Genc S, et al. Selective inhibition of nitric oxide in hypoxic-ischemic brain model in newborn rats: is it an explanation for the protective role of erythropoietin? *Biol Neonate* 2004;85:51-4.
  - 4) Niwa M, Inao S, Takayasu M, Kawai T, Kajita Y, Nihashi T, et al. Time course of expression of three nitric oxide synthase isoforms after transient middle cerebral artery occlusion in rats. *Neurol Med Chir (Tokyo)* 2001;41:63-72; discussion 72-3.
  - 5) Zhang ZG, Chopp M, Zaloga C, Pollock JS, Förstermann U. Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. *Stroke* 1993;24:2016-21; discussion 2021-2.
  - 6) Samdani AF, Dawson TM, Dawson VL. Nitric oxide synthase in models of focal ischemia. *Stroke* 1997;28:1283-8.
  - 7) Ishida A, Ishiwa S, Trescher WH, Nakajima W, Lange MS, Blue ME, et al. Delayed increase in neuronal nitric oxide synthase immunoreactivity in thalamus and other brain regions after hypoxic-ischemic injury in neonatal rats. *Exp Neurol* 2001;168:323-33.
  - 8) Garcia-Bonilla L, Moore JM, Racchumi G, Zhou P, Butler JM, Iadecola C, et al. Inducible nitric oxide synthase in neutrophils and endothelium contributes to ischemic brain injury in mice. *J Immunol* 2014;193:2531-7.
  - 9) Voituron N, Jeton F, Cholley Y, Hasnaoui-Saadani RE, Marchant D, Quidu P, et al. Catalyzing role of erythropoietin on the nitric oxide central pathway during the ventilatory responses to hypoxia. *Physiol Rep* 2014;2:e00223.
  - 10) Calapai G, Marciano MC, Corica F, Allegra A, Parisi A, Frisina N, et al. Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *Eur J Pharmacol* 2000;401:349-56.
  - 11) Lombardero M, Kovacs K, Scheithauer BW. Erythropoietin: a hormone with multiple functions. *Pathobiology* 2011;78:41-53.
  - 12) Rangarajan V, Juul SE. Erythropoietin: emerging role of erythropoietin in neonatal neuroprotection. *Pediatr Neurol* 2014;51:481-8.
  - 13) Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, et al. In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci U S A* 1998;95:4635-40.
  - 14) Gonzalez FF, Larphaveesarp A, McQuillen P, Derugin N, Wendland M, Spadafora R, et al. Erythropoietin increases neurogenesis and oligodendroglialosis of subventricular zone precursor cells after neonatal stroke. *Stroke* 2013;44:753-8.
  - 15) Iwai M, Cao G, Yin W, Stetler RA, Liu J, Chen J. Erythropoietin promotes neuronal replacement through revascularization and neurogenesis after neonatal hypoxia/ischemia in rats. *Stroke* 2007;38:2795-803.
  - 16) Elmahdy H, El-Mashad AR, El-Bahrawy H, El-Gohary T, El-Barbary A, Aly H. Human recombinant erythropoietin in asphyxia neonatorum: pilot trial. *Pediatrics* 2010;125:e1135-42.
  - 17) Wu YW, Bauer LA, Ballard RA, Ferriero DM, Glidden DV, Mayock DE, et al. Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. *Pediatrics* 2012;130:683-91.
  - 18) Fan X, van Bel F, van der Kooij MA, Heijnen CJ, Groenendaal F. Hypothermia and erythropoietin for neuroprotection after neonatal brain damage. *Pediatr Res* 2013;73:18-23.
  - 19) Maiese K, Chong ZZ, Hou J, Shang YC. Erythropoietin and oxidative stress. *Curr Neurovasc Res* 2008;5:125-42.
  - 20) Brewer GJ. Isolation and culture of adult rat hippocampal neurons. *J Neurosci Methods* 1997;71:143-55.
  - 21) Genc K, Genc S, Baskin H, Semin I. Erythropoietin decreases cytotoxicity and nitric oxide formation induced by inflammatory stimuli in rat oligodendrocytes. *Physiol Res* 2006;55:33-8.
  - 22) Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
  - 23) Rice JE 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* 1981;9:131-41.
  - 24) Kumral A, Ozer E, Yilmaz O, Akhisaroglu M, Gokmen N, Duman N, et al. Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. *Biol Neonate* 2003;83:224-8.
  - 25) Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature* 2001;412:641-7.
  - 26) Ioroi T, Yonetani M, Nakamura H. Effects of hypoxia and reoxygenation on nitric oxide production and cerebral blood flow in developing rat striatum. *Pediatr Res* 1998;43:733-7.
  - 27) Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HH, et al. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. *Brain Res* 1994;654:85-95.
  - 28) Huang PL. Nitric oxide and cerebral ischemic preconditioning. *Cell Calcium* 2004;36:323-9.
  - 29) De Pascali F, Hemann C, Samons K, Chen CA, Zweier JL. Hypoxia and reoxygenation induce endothelial nitric oxide synthase uncoupling in endothelial cells through tetrahydrobiopterin depletion and S-glutathionylation. *Biochemistry* 2014;53:3679-88.
  - 30) Ferriero DM, Holtzman DM, Black SM, Sheldon RA. Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol Dis* 1996;3:64-71.
  - 31) Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab* 1995;15:378-84.
  - 32) Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* 1997;76:105-16.
  - 33) Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B, et al. Erythropoietin receptor signalling is required for normal brain development. *Development* 2002;129:505-16.
  - 34) Reitmeir R, Kilic E, Kilic U, Bacigaluppi M, ElAli A, Salani G, et al. Post-acute delivery of erythropoietin induces stroke recovery by promoting perilesional tissue remodelling and contralesional pyramidal tract plasticity. *Brain* 2011;134(Pt 1):84-99.
  - 35) Xiong Y, Mahmood A, Qu C, Kazmi H, Zhang ZG, Noguchi CT, et al. Erythropoietin improves histological and functional outcomes after

- traumatic brain injury in mice in the absence of the neural erythropoietin receptor. *J Neurotrauma* 2010;27:205-15.
- 36) Iwai M, Stetler RA, Xing J, Hu X, Gao Y, Zhang W, et al. Enhanced oligodendrogenesis and recovery of neurological function by erythropoietin after neonatal hypoxic/ischemic brain injury. *Stroke* 2010;41:1032-7.
- 37) Zhu C, Kang W, Xu F, Cheng X, Zhang Z, Jia L, et al. Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. *Pediatrics* 2009;124:e218-26.
- 38) Dawson DA. Nitric oxide and focal cerebral ischemia: multiplicity of actions and diverse outcome. *Cerebrovasc Brain Metab Rev* 1994;6:299-324.
- 39) Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci* 1997;20:132-9.