Stimulation of functional recovery via the mechanisms of neurorepair by S-nitrosoglutathione and motor exercise in a rat model of transient cerebral ischemia and reperfusion

Harutoshi Sakakima[a,b,1], Mushfiquddin Khan[a,1], Tajinder S. Dhammu[a], Anandakumar Shunmugavel[a], Yoshihiro Yoshida[b], Inderjit Singh[a] and Avtar K. Singh[c,d,*]

[a]*Department of Pediatrics, Medical University of South Carolina, Charleston, SC, USA*
[b]*School of Health Science, Kagoshima University, Kagoshima, Japan*
[c]*Ralph H Johnson VA Medical Center, Charleston, SC, USA*
[d]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, USA*

Abstract. *Purpose*: Stroke disability stems from insufficient neurorepair mechanisms. Improvement of functions has been achieved through rehabilitation or therapeutic agents. Therefore, we combined exercise with a neurovascular protective agent, S-nitrosoglutathione (GSNO), to accelerate functional recovery.

Methods: Stroke was induced by middle cerebral artery occlusion for 60 min followed by reperfusion in adult male rats. Animals were treated with vehicle (IR group), GSNO (0.25 mg/kg, GSNO group), rotarod exercise (EX group) and GSNO plus exercise (GSNO+EX group). The groups were studied for 14 days to determine neurorepair mechanisms and functional recovery.

Results: Treated groups showed reduced infarction, decreased neuronal cell death, enhanced neurotrophic factors, and improved neurobehavioral functions. However, the GSNO+EX showed greater functional recovery ($p < 0.05$) than the GSNO or the EX group. A GSNO sub group, treated 24 hours after IR, still showed motor function recovery ($p < 0.001$). The protective effect of GSNO or exercise was blocked by the inhibition of Akt activity.

Conclusions: GSNO and exercise aid functional recovery by stimulating neurorepair mechanisms. The improvements by GSNO and exercise depend mechanistically on the Akt pathway. A combination of exercise and GSNO shows greater functional recovery. Improved recovery with GSNO, even administered 24 hours post-IR, demonstrates its clinical relevance.

Keywords: GSNO, IR, motor exercise, neurorepair, neurobehavior, rehabilitation, S-nitrosylation, stroke

[1]Harutoshi Sakakima and Mushfiquddin Khan contributed equally to this work.

*Corresponding author: Avtar K. Singh, M.D., Professor of Pathology and Laboratory Medicine, Medical University of South Carolina, 508 Children's Research Institute, 173 Ashley Ave, Charleston, SC 29425, USA. Tel.: +1 843 792 7991; Fax: +1 843 792 3653; E-mail: khanm@musc.edu.

1. Introduction

Stroke survivors suffer from long-term disability and severe morbidity due to motor and cognitive deficits. Therapeutic exercise and pharmacological agents following stroke induce neurophysiological and neuroanatomical plasticity, leading to the recovery of

function (Kim et al., 2005; Wu et al., 2008; Ploughman et al., 2009). Animal research demonstrates that exercise enhances neuroprotection, stimulates neurorepair, and aids motor function recovery following cerebral ischemia reperfusion (IR) injury (Ke et al., 2011; Matsuda et al., 2011). As with rehabilitation therapy, therapeutic drugs including S-nitrosoglutathione (GSNO) have been shown to induce the neurorepair process following brain trauma in rats (Khan et al., 2011). Studies on human stroke indicate that combining rehabilitation with therapeutic agents might be a better strategy to induce significant recovery (Gladstone et al., 2006; Cramer, 2008). Therefore, this study investigates whether GSNO in combination with motor exercise exerts a synergistic effect in stimulating the mechanisms of neurorepair, leading to accelerated and enhanced functional recovery following IR in rats.

GSNO is an endogenous compound formed by the reaction of nitric oxide (NO) with glutathione (GSH) (Singh et al., 1996). It is an efficient S-nitrosylating agent, and the mechanism of S-nitrosylation modulates redox and protein function in health and disease (Foster et al., 2009). GSNO inhibits platelet activation, reduces embolization in humans (Radomski et al., 1992; Molloy et al., 1998; Kaposzta et al., 2002), and inhibits endothelial inflammatory events (Prasad et al., 2007). It invokes its anti-inflammatory effects on post-injury events mainly through the down regulation of the expression of NF-κB, adhesion molecules, cytokines, and inducible nitric oxide synthase (NOS) (Fortenberry et al., 2001; Khan et al., 2005; Prasad et al., 2007; Khan et al., 2009). Neuroprotective effects of GSNO are mediated by reducing neuronal apoptotic cell death and inhibiting caspase-3 activity (Khan et al., 2005; Khan et al., 2009). GSNO shows its antioxidant action through the modulation of redox (Chiueh and Rauhala, 1999); it maintains the levels of GSH and reduces the levels of peroxynitrite (Khan et al., 2006). Treatment of spinal cord injury (SCI) and traumatic brain injury (TBI) by GSNO stimulates the expression of VEGF (Chou et al., 2011) and BDNF (Khan et al., 2011), respectively. BDNF is one of a family of neurotrophins that influences neuronal proliferation, survival, and differentiation as a result of binding to its phosphorylated tyrosine kinase B receptor (pTrkB) and subsequent downstream activation of several signal transduction pathways. Both BDNF and TrkB are widely distributed throughout the brain and play critical roles in neurorepair processes (Ploughman et al., 2009). Like GSNO, exercise has been associated

with decreased cell death (Matsuda et al., 2011) as well as increased expression of BDNF/TrkB (Cui et al., 2010). Several other pharmacological agents have also been investigated for neuroprotection and motor function recovery or used as adjuvant therapy to enhance the beneficial effects of rehabilitative motor training programs (Cramer, 2008; Chollet et al., 2011). However, the potential of combination therapy of GSNO and motor exercise and the mechanisms involved therein have not been investigated following IR. Furthermore, GSNO may have an advantage over other reported experimental drugs in IR because it is a non-toxic component of the human body, and is ultimately metabolized into beneficial –SNO/NO and GSH (Foster et al., 2009; Colagiovanni et al., 2011).

In this 2-week study, we investigated whether GSNO treatment of IR with or without exercise reduces brain infarctions, decreases neuronal apoptotic cell death, and improves neurobehavioral functions via the PI3K/Akt (phosphatidylinositol 3 kinase/v-akt murine thymoma viral oncogene homolog) dependent pathway. We also examined whether an administration of GSNO at 24 hours after IR leads to decreased neurodegeneration and improved neurobehavioral functions, thus indicating the clinical relevance of GSNO therapy and providing a rationale for combining it with exercise.

2. Methods

2.1. Subjects

Subjects were male Sprague-Dawley rats ($n = 121$) weighing between 250 to 290 g at the time of surgery. All animals received humane care in compliance with the Medical University of South Carolina's (MUSC) guidance and the National Research Council's criteria for humane care. Animal procedures were approved by the institutional animal care and use committee of MUSC. The animals were allowed to acclimatize for three days before the experiments began. They were randomly divided into five groups: 1) ischemia reperfusion (IR), 2) IR+exercise treatment (EX), 3) IR+GSNO treatment (GSNO), 4) IR+GSNO+exercise treatment (GSNO+EX), and 5) sham operated control without treatment (sham). The number of animals used in each experiment is indicated in the description of figures and tables. The experimental design is presented in Fig. 1.
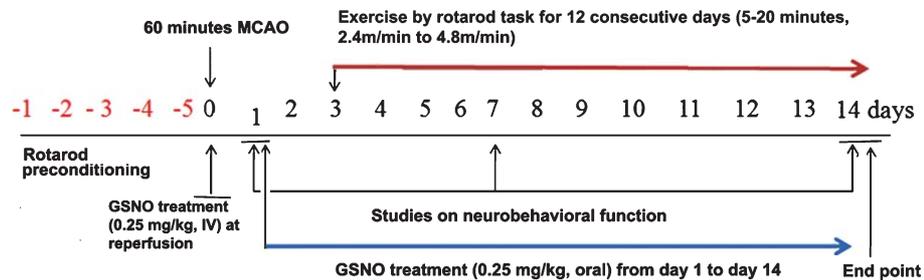
Fig. 1. Experimental protocols. Schematic showing the timeline of GSNO treatment (0.25 mg/kg body weight) begun day 0 and continued for 14 days, and motor training begun at day 3 and continued until day 14 after MCAO. Neurobehavioral assessments were performed on days 1, 7, and 14 following IR.

2.2. Middle cerebral artery occlusion (MCAO) rat model without or with exercise and/or GSNO

Rats were anesthetized by ketamine hydrochloride (80 mg/kg body weight) and xylazine (10 mg/kg body weight) administered intraperitoneally. Stroke was induced by 60 min left MCAO using an intraluminal filament as previously described (Khan et al., 2006; Matsuda et al., 2011). Regional cerebral blood flow was monitored during the occlusion and early reperfusion to ensure the obstruction of blood flow, as described (Khan et al., 2006). Reperfusion was established by withdrawal of the filament. After 7 or 14 days, animals were sacrificed with an overdose of nembutal. The brain, including the ischemic region, was analyzed for histological and immunohistochemical as well as 2,3,5-triphenyltetrazorlium chloride (TTC) studies.

In the GSNO-treated groups, GSNO (0.25 mg/kg body weight) in saline (∼250 µl) was slowly infused by jugular vein cannulation at reperfusion. From 1 day after MCAO and thereafter, GSNO was gavage fed. Physiological parameters did not alter after GSNO treatment. Details of the study on physiologic parameters in IR and GSNO-treated rats have been reported earlier (Khan et al., 2006).

2.3. Exercise training protocol

Rats were pre-trained for 5 days before the MCAO procedure. Three days after MCAO, injured rats from the EX and GSNO+EX groups were assigned to a training condition. For this training, we used a low intensity, automated 4-lane rotarod Rotamex unit (Columbus Instruments, Columbus, OH). The choice of rotating rod motor exercise was based on the report that

it improved functional outcome after stroke in rats (Ding et al., 2004). In our exercise protocol, rats were required to run at a constant speed of 2.4 m/minute for the first 2-3 days, and the speed was raised to 4.8 m/minute (20 minutes/day running duration) for the remaining days of IR. If rats fell down during the exercise session, they were placed back on the rotating rod. Animals reluctant to run were lightly touched by hand on the tail, and they started running. No animals were excluded from study based on their unwillingness to run.

2.4. Evaluation of ischemic infarct

The fresh brain coronal sections (2 mm) were immersed in a 1% solution of TTC in phosphate-buffered saline (PBS, pH 7.4) at 37°C for 10–15 min, as described previously (Khan et al., 2005). After staining, the sections were scanned to determine the ischemic infarct volume. Infarctions were measured using Scion Image software (Scion Corp., Frederick, MD). Total infarct area was multiplied by the thickness of the brain sections to obtain infarct volume as described previously (Khan et al., 2005). In order to minimize the error introduced by edema and liquefaction after infarction, an indirect method for calculating infarct volume was also used (Swanson et al., 1990; Matsuda et al., 2011). If needed, the volume was corrected. The non-infarcted area in the ipsilateral hemisphere was subtracted from that in the contralateral hemisphere, and infarct volume was calculated using the following formula: corrected percentage of infarct volume = (contralateral hemispheric volume – ipsilateral non-infarcted volume)/contralateral hemispheric volume.

2.5. Evaluation of neurological score, motor behavior, and locomotor function

Animals in each group were evaluated for neurological score and motor function test using rotarod and beam walk tasks before and at 1, 3, 7, or 14 days after reperfusion.

Neurological examination was performed by an observer masked from the identity of the groups. A neurological grading system with a 4-point scale (0–3), as described previously (Khan et al., 2005), was used: 0, no observable neurological deficit (normal); 1, failure to extend right forepaw on lifting the whole body by tail (mild); 2, circling to the contralateal side (moderate); 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe). The animals not showing paralysis at 1 h after MCAO were excluded from the study because the reduction of the blood flow may not have produced an infarction of adequate size to cause quantifiable neurological deficits in those animals.

In the motor behavior test, rats were examined before and after surgery using a beam-walking task with an elevated narrow beam (100 cm long × 2.5 cm wide). The time to traverse the beam was recorded and analyzed after three trials (60 seconds allotted time) per day. The beam walk was graded with a 6-point score (0–5), as described previously (Matsuda et al., 2011). The score 0 was given if the rat was unable to traverse the beam and could neither place the affected limbs on the horizontal surface nor maintain balance. A score of 1 was given if the rat was unable to traverse the beam or to place the affected limbs on the horizontal surface of the beam but was able to maintain balance. A score of 2 was given if the rat was unable to traverse the beam but placed the affected limbs on the horizontal surface of the beam and maintained balance. A score of 3 was given if the rat used the affected limbs in less than half of its steps along the beam. A score of 4 was given if the rat traversed the beam and used the affected limbs to aid more than 50% of its steps along the beam. A score of 5 was given if the rat traversed the beam normally with no more than two foot slips. Before surgery, the animals in all groups underwent the test to ensure that their performance score was 5.

In the motor function and balance test, rats were examined on an accelerating rotarod task by trained personnel blinded to the animal groups as described (Monville et al., 2006). Walking time on the rotarod was measured as described previously from our laboratory (Khan et al., 2009). Each rat was placed on the rotarod cylinder, and the time for which the animal remained on the drum was measured. Speed was increased from 0 rpm to 30 rpm with increments of 2 rpm every 5 seconds, and the trial ended if the animal fell down. Each animal was given three trials, and the mean latency (in seconds) of three trials was calculated for each animal.

2.6. Histology

On post-IR days 7 and 14, the animals were anesthetized with an excess dose of nembutal (150 mg/kg). Transcardial perfusion was performed with PBS followed by neutral formalin solution. Brains were removed and fixed in 10% formalin. After fixation, the tissue was processed for histology and immunohistochemistry (IHC) by following routine procedures as previously described (Chou et al., 2011). The paraffin embedded brain coronal sections (8 μM thick) were stained with hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB)-PAS, as described earlier (Pannu et al., 2007). The histological analysis was performed by an investigator who was blinded to the experimental groups.

2.7. Immunohistochemistry (IHC)

Using immunohistochemical (indirect immunoperoxidase method) analysis technique, paraffin-embedded sections (4 μM thick) from the 10% formalin-fixed brain tissues were stained for the expression of BDNF, its receptor TrkB/phospho-TrkB (pTrkB), platelet endothelial cell adhesion molecule 1 (PECAM-1), activated caspase3, Akt, phospho-Akt (pAkt) and glial fibrillary acidic protein (GFAP). The following specific antibodies were used: mouse anti-BDNF (Bio Ab Chem, Ladson, SC), rabbit anti-TrkB/pTrkB (Abcam Inc, Cambridge, MA), goat anti-PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-GFAP (Dako, carpinteria, CA), rabbit anti-Akt and anti-phosho-Akt (Cell Signaling Technology, Danvers, MA). The brain tissue sections were deparaffinized, sequentially rehydrated in graded alcohol, and then immersed in PBS (pH 7.4). Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 30 min. After rinsing three times for 10 min each with PBS, the sections were incubated for 20 min with 10% normal blocking serum in PB. They were individually incubated at 4°C overnight with the following: mouse anti-BDNF antibody (1 : 500 PBS);

rabbit anti-TrkB (1 : 200 PBS); rabbit anti-pTrkB (1 : 150 PBS); goat anti-PECAM-1 (1 : 200 PBS); rabbit anti-GFAP (1 : 1000 PBS); rabbit anti-Akt (1 : 300); rabbit anti-phosho-Akt (1 : 50). They were rinsed three times at 10 min each in PBS, and reacted with goat anti-rabbit IgG conjugated to peroxidase-labeled dextran polymer (Vector Laboratories, Burlingame, CA) for 60 min. After rinsing with PBS, the immunoreactivity was visualized with diaminobenzidine (DAB) peroxide. Counterstaining was performed with hematoxylin.

Immunostaining for rabbit anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruze, CA) was performed by immunoflorecencent methods. After reaction with the rabbit anti-caspase 3 (1 : 300) antibody at 4°C overnight followed by washing, the sections were incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (1 : 200) for 60 min. After washing, sections were counterstained with 4′,6-diamino-2-phenylindole (DAPI) for 10 min, and mounted with aqueous mounting media. Colocalization of mouse monoclonal anti-NeuN (Abcam Inc., Cambrige MA, 1 : 200) and rabbit anti-active caspase 3 immunoreactivities was performed.

Three areas in the motor cortex of the ischemic penumbra area (Fig. 3D) of each immunostained section were digitized by a 40X microscope objective with microscope and camera without visual field overlap. The ratios of BDNF, TrkB, pTrkB, PECAM-1, GFAP and active caspase 3 positive cells were quantitatively measured using Scion Image software, as described previously (Khan et al., 2011). This method of analysis was adopted since it was possible to quantify the areas of the immunostained sections.

2.8. Fluorescent TUNEL assay for detection of apoptosis

Terminal deoxynucleotidyl transferase-mediated biotinylated UTPnick and labeling (TUNEL) assay was performed in the ischemic penumbra region as shown in Fig. 3D, using a DNA fragmentation/flurorescence staining TUNEL apoptosis detection kit (Millipore Corporation, Temecla, CA), according to the manufacturer's protocol. For double labeling, brain sections from three animals in each experimental group were probed with neuronal marker NeuN or caspase 3 and visualized by fluorescence microscopy, as described previously (Khan et al., 2005). Three areas from the penumbra area

of each TUNEL stained section were digitized by a 40X microscope objective with microscope and camera without visual field overlap. TUNEL positive cells were counted in each group.

2.9. Inhibition of PI3 kinase/Akt pathway by LY294002 compound

Animals were infused with PI3 kinase inhibitor LY294002 compound (5 mg/kg in DMSO) intraperitoneally in GSNO and EX+GSNO groups to examine its effect on neuroprotection, recovery of neurobehavioral function and the expression of Akt/pAkt. The first dose of the inhibitor was administered 24 h before MCAO. The same dose was repeated from day 0 to 6 following MCAO (total 8 dose). The animals were sacrificed at day 7 after MCAO, and studies on TTC, neurological score, rotarod latency and the expression of Akt/pAkt were performed as described elsewhere for 14-day groups. The data were compared with the 7-day groups.

2.10. Statistical analysis

Data are expressed as mean ± standard error (SE). Student t tests (for comparison between two groups) or one-way ANOVA (for comparison of 3 or more groups) followed by post hoc Fisher's protected least significant differences (PLSD) tests were used for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

After acclimatization with rotarod and beam walk tasks, all the ischemic animals were subjected to MCAO (60 min), followed by reperfusion (7 or 14 days). Ischemic animals were treated with either GSNO (0.25 mg/kg) at reperfusion and daily thereafter, motor exercise (starting at day 3 after reperfusion and continuing for 12 consecutive days), or both. The dose and the route for GSNO were based on our previous studies on IR and TBI (Khan et al., 2006; Khan et al., 2009). Neurobehavioral functions were evaluated at days 1, 7, and 14, and brain infarctions were measured at day 7 or 14 after reperfusion. Although brain infarctions occur before 48–72 hours in IR animal models (Liu et al., 2009), we measured it at day 7 and 14 to show that GSNO-mediated improved functional

recovery may be due, in part, to reduced infarct volume. Histological and immunohistochemical studies were performed using fixed tissues at day 14 of reperfusion. The experimental protocols are visualized in Fig. 1.

3.1. GSNO and/or exercise reduces infarction and improves neurological score measured at day 14 after IR

GSNO significantly reduced infarct volume from $57.5 \pm 5.4\%$ (IR) to $39.5 \pm 5.8\%$ ($p < 0.05$). However, GSNO+EX reduced the volume further to $29.9 \pm 6.8\%$
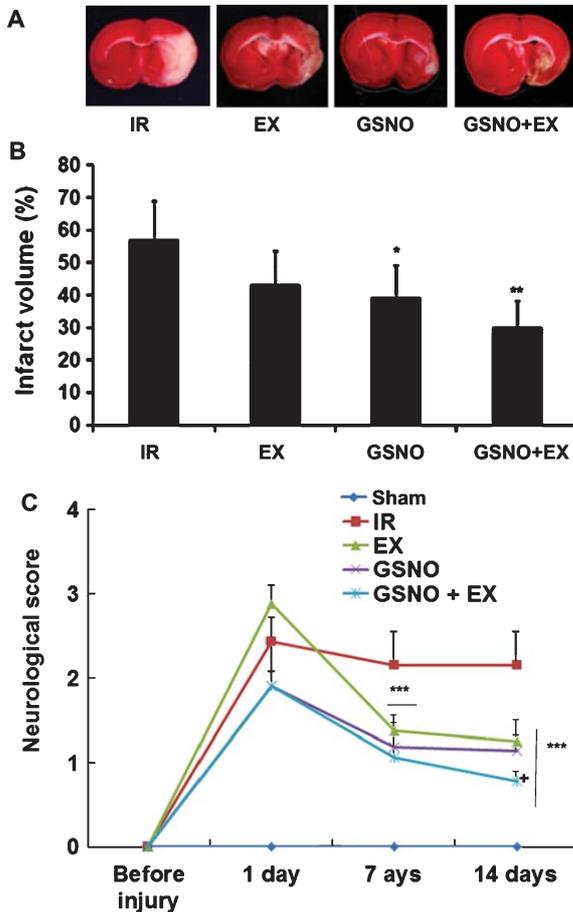

Fig. 2. Representative TTC stained brain sections (# 3 out of six consecutive sections from cranial to caudate region) (A) and infarct volume (B) at 14 days of reperfusion, and neurological score (C) at day 1, 7, 14 of reperfusion after 60 min MCAO. GSNO treatment and/or exercise training reduced brain infarctions and improved neurological score. Data are presented as mean $\pm$ SE ($n = 8$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. IR, $+p < 0.05$ vs. EX.

($p < 0.01$) (Fig. 2A, B). EX alone did not show a significant decrease in infarctions. All treated groups had significantly improved neurological scores as compared to the IR group; however, the GSNO+EX group showed greater score improvement than the EX and GSNO groups at day 14 of reperfusion (Fig. 2C).

In a subgroup, GSNO treatment initiated at 24 h for 14 days significantly improved neurological (Fig. 6C) and motor (Fig. 6D) functions. However, there was no statistically significant reduction in infarct volume in the GSNO treated group (Fig. 6A, B).

3.2. GSNO and/or exercise reduces the expression of active caspase-3 and TUNEL staining of neuronal cells determined at day 14 after IR

The expression of caspase 3 and the number of TUNEL-stained cells increased in the penumbra area (Fig. 3D) of the IR group (Fig. 3A, B). Treated groups showed significantly reduced expression of caspase 3 and a decreased number of TUNEL positive cells (Fig. 3A, B). TUNEL staining colocalized with caspase 3 (Fig. 3C upper panel) and neuronal marker NeuN (Fig. 3C lower panel), indicating apoptotic cell death of neurons.

3.3. GSNO and/or exercise increases BDNF, TrkB, pTrkB, PECAM-1, LFB and decrease GFAP and cellular infiltration determined at day 14 after IR

The expression of BDNF, TrkB, pTrkB, PECAM-1 and the levels of LFB (myelin) decreased, the expression of GFAP (reactive astrocytes) and the degree of cellular infiltration increased following IR (Fig. 4). While GSNO and/or exercise significantly increased the expression of BDNF, TrkB, pTrkB and PECAM-1 (Fig. 4A, B), the treatments significantly decreased GFAP expression. The treated groups also showed increased myelin levels (LFB staining) and reduced cellular infiltration (H&E staining) (Fig. 4C).

3.4. GSNO plus exercise accelerates the rate and improve the degree of functional recovery

Both GSNO and exercise significantly improved walking (rotarod task, Fig. 5A) and balancing/coordination (beam task, Fig. 5B) compared to the IR group at day 7 as well as 14. However, the combination of GSNO and exercise significantly accelerated
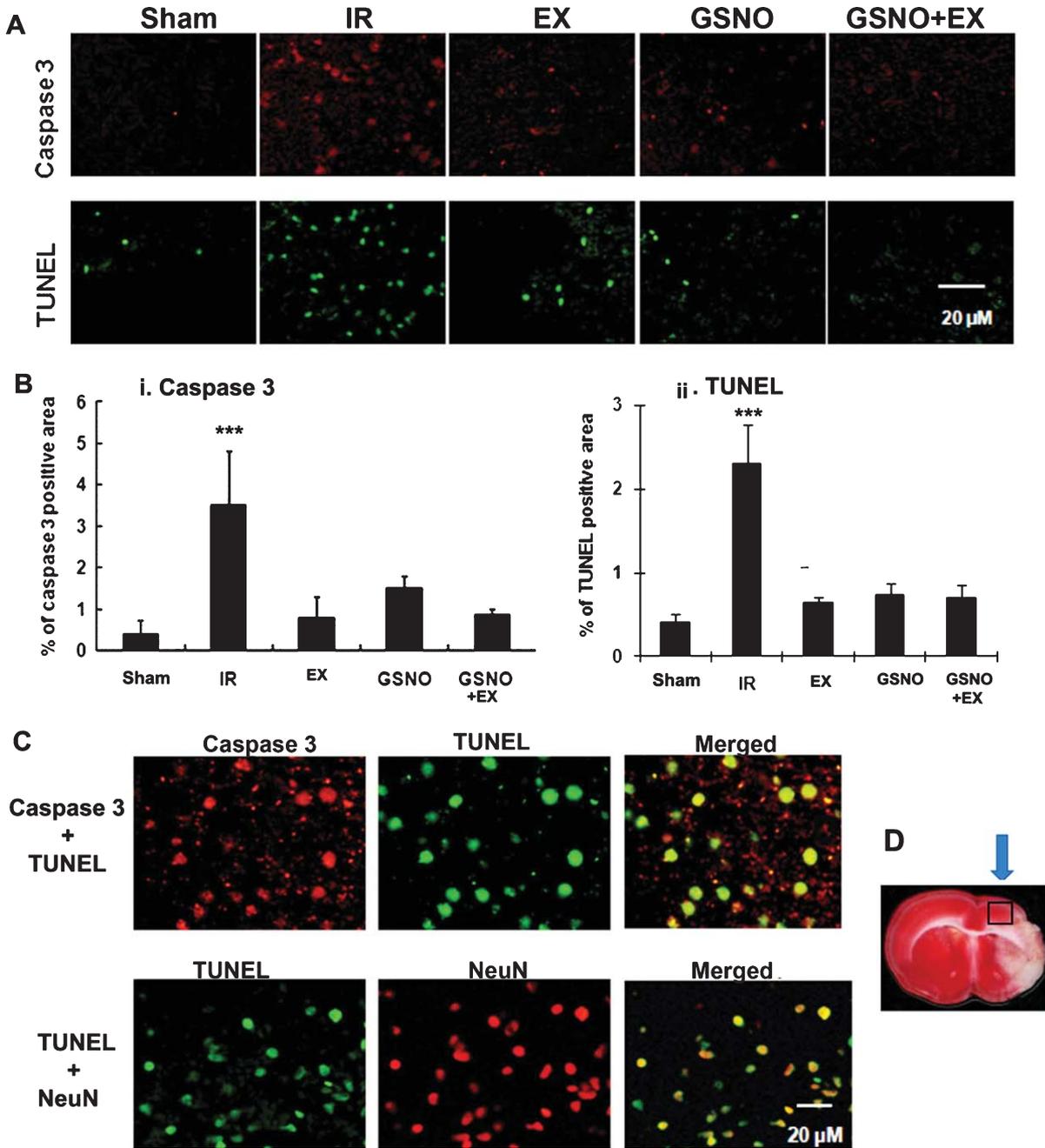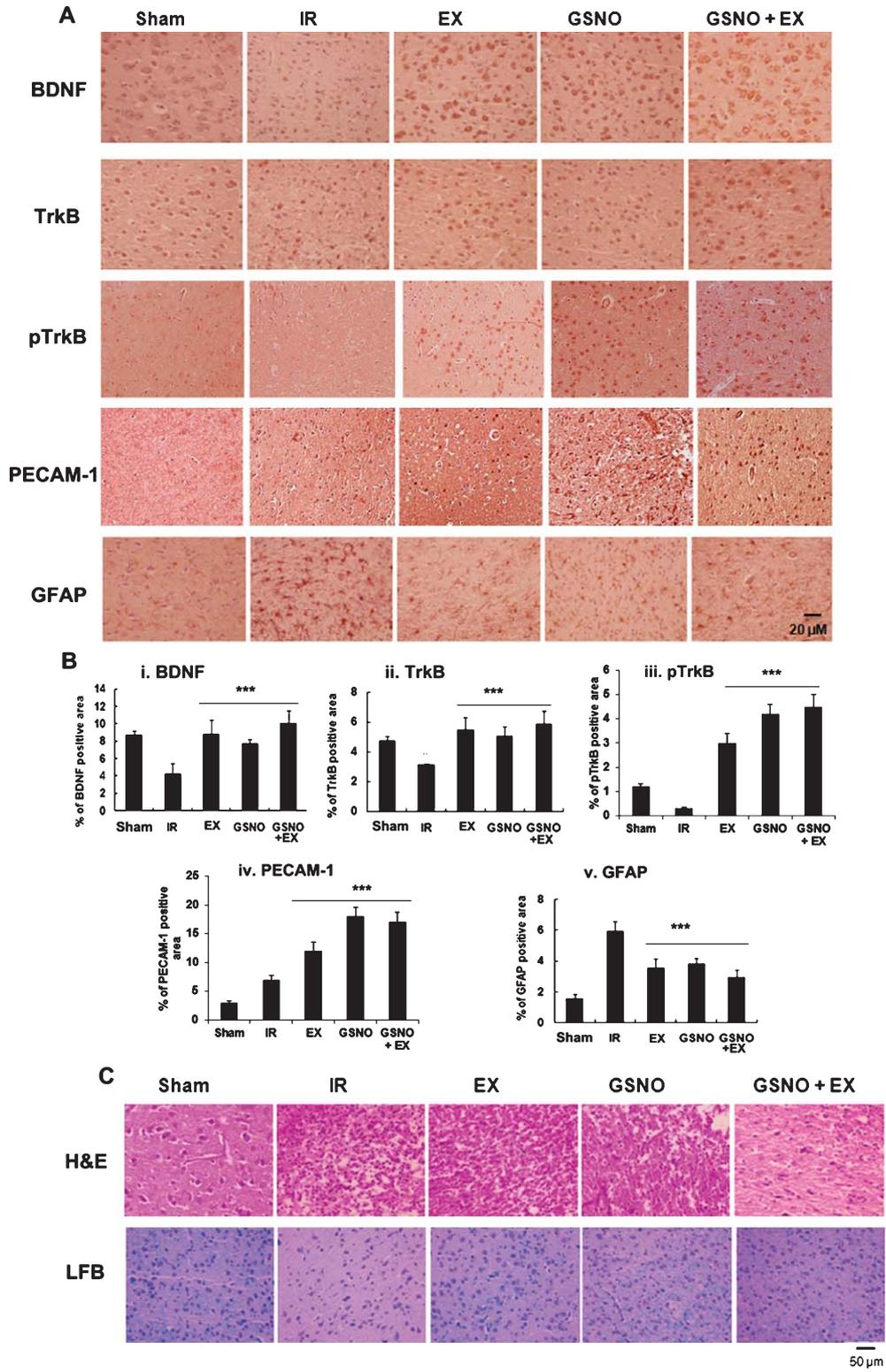
Fig. 3. Photomicrographs of immunofluorescence staining of caspase 3 (A, upper panel) and TUNEL (A, lower panel); graph counting of caspase 3 (Bi) and TUNEL positive cells (Bii); photomicrographs of colocalization of TUNEL with caspase 3 (C, upper panel) and NeuN (C, lower panel); and cortical penumbral area on representative TTC stained section (under rectangle) used for the immunostaining and histological studies (D) at 14 days after IR. Caspase 3 and TUNEL positive cells in IR groups were increased. GSNO treatment and/or exercise training reduced the number of caspase 3 and TUNEL positive neuronal cells. Data are presented as mean ± SE. ***$p < 0.001$ vs. sham, GSNO, EX and GSNO+EX, ($n = 3$).
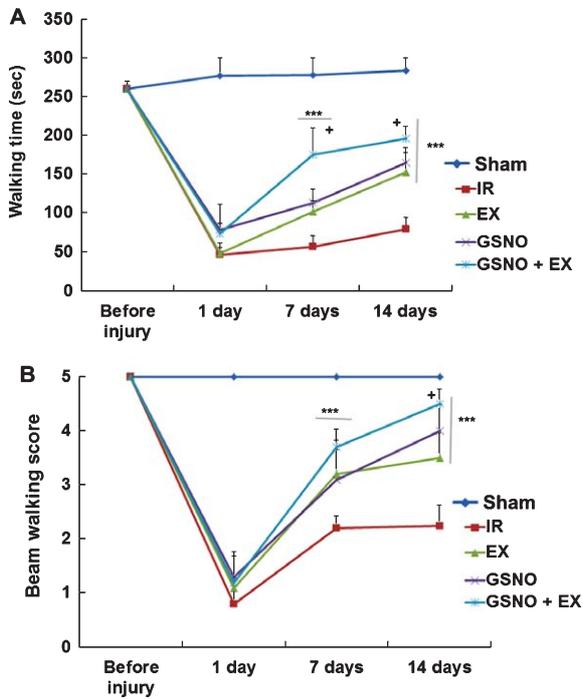
Fig. 5. Rotarod (A) and beam walk (B) tasks. Walking time (rotarod) and walking score (beam balance) significantly increased in treated groups compared with IR group at day 7 and 14. However, GSNO+EX group showed improved walking time compared with GSNO or EX groups. Data are presented as mean $\pm$ SE ($n = 8$). ***$p < 0.001$ vs. IR, +$p < 0.05$ vs. EX.

walking ability and provided a greater degree of recovery than GSNO or EX alone, as shown in Fig. 5A and Fig. 2C.

3.5. Inhibition of PI3 kinase/Akt pathway blunts the neuroprotective and neurorestorative effects of GSNO and exercise evaluated at day 7 after IR

Twenty four hours pre-MCAO treatment followed by daily treatment up to 6 days after IR with PI3 kinase inhibitor LY294002 (LY; 5 mg/kg in DMSO) blocked the therapeutic effect of GSNO as well as that of exercise. No changes in neurological score (Table 1), brain infarctions or motor function (Fig. 7)

were observed in GSNO or EX groups treated with LY compound compared with IR. Expectedly, LY treatment down regulated the expression of pAkt and not Akt (Fig. 8).

4. Discussion

In this 2-week IR study, GSNO or motor exercise provided neuroprotection, reduced neuronal cell death, maintained tissue structure, and aided functional recovery by stimulating the expression of neurorepair mediators BDNF/TrkB/pTrkB via activating Akt. Furthermore, GSNO in combination with exercise accelerated the rate and enhanced the degree of recovery.

Stroke is a multimechanistic and biphasic acute and chronic disease. While the acute phase is associated with cell death and secondary injury, the chronic phase shows insufficient neurorepair mechanisms. Clinical trials document that most monotherapies failed due to the lack of drug efficacy in the chronic phase (Lo, 2008; Moskowitz et al., 2010). Rehabilitation has long been used to improve neurological function in the chronic phase. However, its efficacy is slow and limited. An ideal therapy will ameliorate the injury in both phases and may therefore include a combination of rehabilitation and a mechanism-based therapeutic compound with proven efficacy. We opted for motor (rotarod) exercise combined with the neuroprotective and neurorepair agent GSNO. Rotarod exercise is an established motor balance and coordination training intervention (Hamm, 2001). It supports endurance and coordinated movement (Hoffman et al., 2008; Kline et al., 2008).

GSNO, an endogenous NO metabolome regulator, is present in the brain and other organs (Kluge et al., 1997). It is involved in cell signaling via S-nitrosylation of target proteins, including NF-κB, caspase-3, and endothelial NOS (Marshall and Stamler, 2001; Khan et al., 2005; Prasad et al., 2007). An exogenous administration of GSNO or enhancement of the levels of endogenous GSNO protects against cardiac ischemic injury (Konorev et al.,

Fig. 4. Photomicrographs of DAB staining of immunoreactivity of BDNF, TrkB, pTrkB, PECAM-1, GFAP (A); graphs of counts of BDNF, TrkB, pTrkB, PECAM-1, GFAP positive cells (B); and photomicrographs of H&E (C, upper panel) and LFB (C, lower panel) stainings in cortical penumbral area at 14 days after IR. GSNO treatment and/or exercise training enhanced the expression of BDNF, TrkB, pTrkB and PECAM-1; and the staining of LFB (myelin). GSNO and exercise also decreased the expression of GFAP and reduced the infiltration and tissue deformation in the cortical penumbra. Data are presented as mean $\pm$ SE. ***$p < 0.001$ vs. IR ($n = 3$).
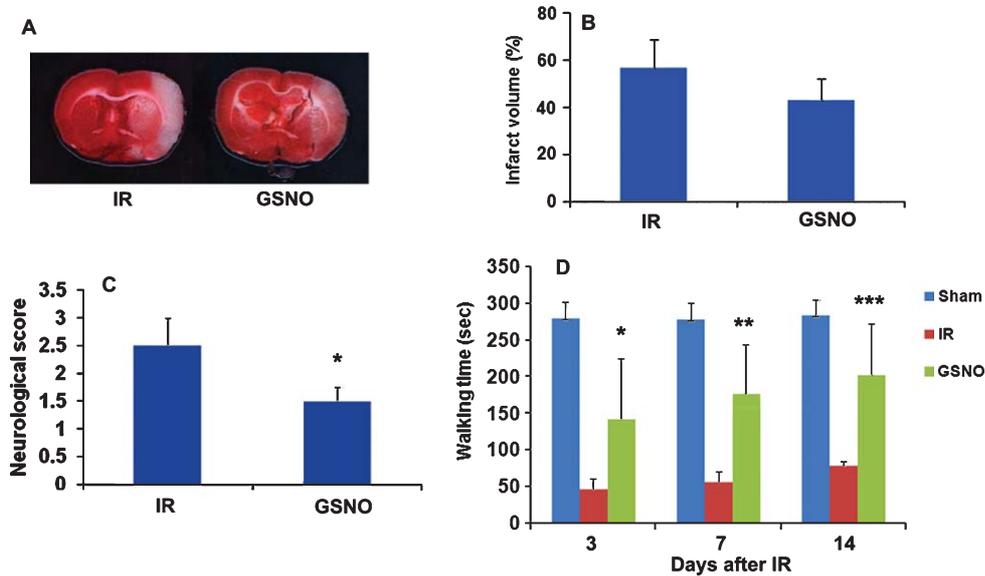
Fig. 6. Representative TTC stained brain section (# 3 out of six consecutive sections) (A), infarct volume (B), and neurological score (C) at 14 days after IR, and rotarod task (D) at day 3, 7 and 14. GSNO treatment, initiated at 24 hours after reperfusion, showed improved neurological score and motor function (rotarod latency). Data are presented as mean $\pm$ SE ($n = 7$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. IR.

Table 1
Effect of inhibition of PI3 kinase/Akt pathway on neurological score following IR

	1st day	7th day
Sham	0	0
IR	2.4 ± 0.3	2.1 ± 0.4
EX	2.9 ± 0.1	1.4 ± 0.2***
GSNO	1.9 ± 0.2	1.2 ± 0.3***
EX+LY	3 ± 0.0	2 ± 0.0
GSNO+LY	2 ± 0.0	2 ± 0.0

Neurological score at day 7 after IR. Animals were treated with LY294002 (LY) compound (5 mg/kg) 1 day before MCAO and daily thereafter for 6 days. GSNO treatment and EX training were performed as described in Methods. Neither GSNO nor exercise improved neurological score in LY-treated animals. Data are presented as mean $\pm$ SE ($n = 7$). ***$p < 0.001$ vs. IR, GSNO+LY, EX+LY.

1995; Lima et al., 2009), supporting the theory that, when channeled adequately into S-nitrosylation, GSNO shows therapeutic potential (Que et al., 2005). However, the potential of S-nitrosylation therapy and the status of GSNO are under-explored in stroke. Decreased synthesis of GSNO in a setting of reduced GSH levels, as in IR (Khan et al., 2006), combined with its degradation by inflammation-induced increased activity of GSNO reductase (Que et al., 2005), may contribute to the reduced levels of GSNO in stroke. Therefore, we anticipated that both phases of IR would

have decreased levels of GSNO and that its exogenous administration would provide neurovascular protection and stimulate the neurorepair process. The first dose of GSNO was administered early to identify the neurorepair mechanisms and to compare results with our previous studies of the anti-inflammatory and anti-ischemic effects of GSNO (Khan et al., 2005; Khan et al., 2006). Moreover, the clinical relevance of the treatment in this study is shown in the other set of experiments (Fig. 6) in which the first dose of GSNO was administered as late as 24 hours.

Previously, we documented that GSNO reduces secondary injury, protects the brain, and improves neurological function in IR (Khan et al., 2005; Khan et al., 2006) and TBI (Khan et al., 2009). We have also observed that a low dose of GSNO promotes functional recovery in a 2-week study of TBI (Khan et al., 2011). In this study, administration of low dose GSNO either at reperfusion (Fig. 2) or at 24 h after reperfusion (Fig. 6) and daily thereafter not only reduced brain injury but also improved neurological scores. Exercise alone could not significantly reduce infarct volume (Fig. 2A, B) because infarctions occur before 48–72 hours after IR and exercise was initiated 72 hour following ischemia. However, exercise improved neurobehavioral functions (Figs. 2C and 5), indicating that the reduced deficits are related with the
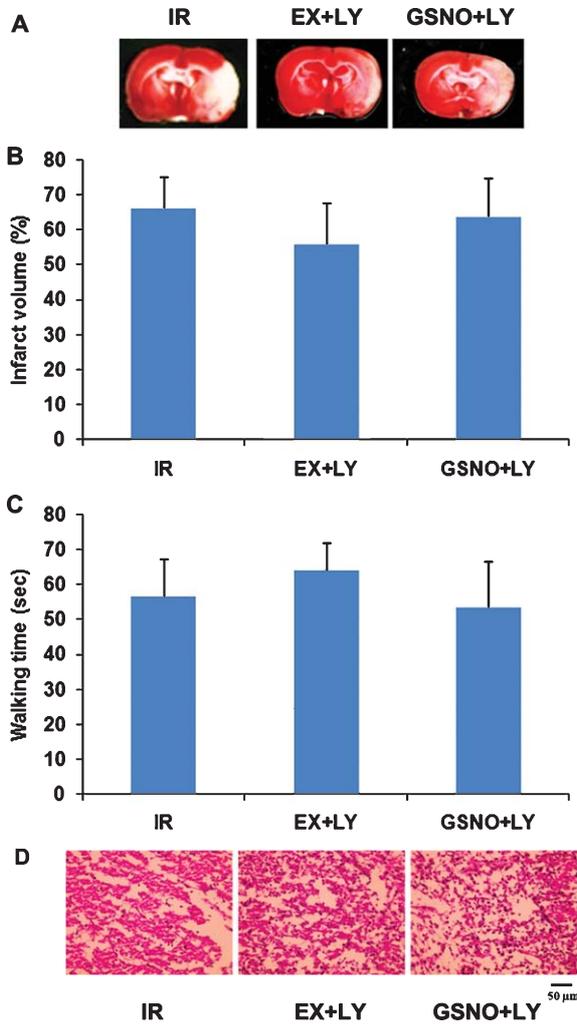
Fig. 7. Representative TTC stained brain section (# 3 out of six consecutive sections from cranial to caudate region) (A), infarct volume (B), rotarod task (C), and H&E staining (D) at day 7 after IR. One day pre-MCAO and 6 days thereafter, treatment with LY294002 compound blunted infarct reduction and improved walking in GSNO and EX groups (*n* = 7).
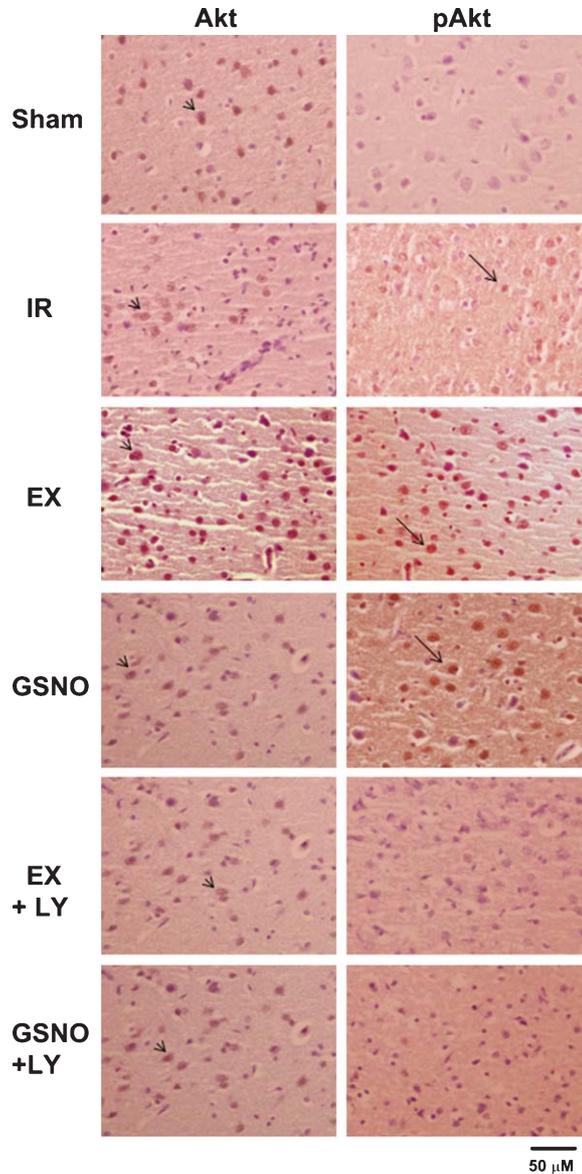

Fig. 8. Photomicrographs of DAB staining of immunoreactivity of Akt and pAkt at day 7 after IR. Expression of pAkt was remarkably reduced in IR. Both GSNO and EX enhanced the expression of pAkt. However, a single 1-day pre-MCAO and 6 days thereafter treatment with LY294002 compound reduced GSNO and EX-mediated increased expression of pAkt (*n* = 3).

exercise-mediated decreased neuronal apoptotic cell death (Fig. 3).

Similar to our acute phase IR study with GSNO (Khan et al., 2005), the treatment also reduced the expression of caspase 3 and neuronal apoptotic cell death at day 14 after IR (Fig. 3), indicating the validity of the treatment in both phases. As in TBI, GSNO in this IR study stimulated the neurorepair mediator BDNF and its receptor TrkB/pTrkB (Fig. 4A, B) and maintained the levels of myelin (Fig. 4C). Improved functional recovery via late modulation of

AMPA receptor signaling is also dependent on BDNF (Clarkson et al., 2011). The beneficial effects of rehabilitation on recovery and synaptic plasticity, but not on infarction, have been negated by treatment with antisense BDNF oligonucleotide, indicating that BDNF is required for functional recovery following stroke

(Ploughman et al., 2009). Behavioral recovery through enhanced neuroplasticity has also been observed by antagonizing a raised GABA-mediated tonic neuronal inhibition (Clarkson et al., 2010). In our study, GSNO treatment, likely via stimulation of neurotrophic factors (Fig. 4), improved motor functions as evaluated by two different tasks (rotarod and beam walk), supporting the rationale that GSNO has the ability to improve neurobehavioral functions (Fig. 5). The rotarod exercise had an effect on neuroprotection and neurorepair similar to but less than that with GSNO (Figs. 2–5).

Because the benefit of exercise in neurorepair has been linked with the activation of the PI3 kinase/Akt pathway (Chen and Russo-Neustadt, 2005), we used a selective PI3 kinase inhibitor LY (Cheng et al., 2010) to confirm this mechanism in our study. LY treatment reversed not only the effect of exercise but also the beneficial effect of GSNO, indicating that GSNO also provides neuroprotective and neurorepair action via activation of Akt (Table 1 and Fig. 7). The effect of LY was supported by reduced pAkt expression in both GSNO and exercise animals (Fig. 8). We previously reported that GSNO stimulates VEGF in a rat SCI study, suggesting that GSNO may have up regulated VEGF via activating the Akt pathway in this study. Increased expression of PECAM-1 in the GSNO group (Fig. 4) further supports the involvement of a VEGF-dependent neurorepair mechanism. Akt is known to up regulate hypoxia inducible factor 1 alpha (HIF1α), a transcription factor of VEGF, leading to the stimulation of VEGF (Cheng et al., 2010). HIF1α has also been reported to be stabilized directly by GSNO (Wellman et al., 2004). The expression of HIF1α and VEGF is reduced by the PI3 kinase inhibitor LY, supporting the mechanism that VEGF is up regulated by Akt activity (Cheng et al., 2010). Neurorepair activity by VEGF is mediated through the induction of neurotrophic factors and PECAM-1 (angiogenesis), which together promote neurogenesis in animal models of chronic brain injury (Lok et al., 2007).

Later, we combined exercise with GSNO to investigate whether the combination has a synergistic effect on improvement of the neurobehavioral functions compared with GSNO or exercise alone. Figures 2C (neurological score) and 5A (walking time on rotated rod) show that the combination therapy provides greater functional improvement than GSNO or exercise alone. These data support the potential of GSNO as an adjuvant therapy of translational value in rehabilitation following stroke. However, there are several

caveats to this study. First, a two-week time frame for a rehabilitation study is relatively short. Second, further studies are needed to investigate the GSNO-mediated signals downstream to Akt involved in the neurorepair process. Finally, the levels of endogenous GSNO and the expression of its degrading enzymes, including GSNO reductase, are yet to be determined.

In conclusion, these studies demonstrate that GSNO accelerates the recovery of neurological and motor functions and enhances the benefit of exercise by stimulating the expression of neurotrophic factor BDNF/pTrkB and activating Akt. The clinical relevance of GSNO therapy is supported by improved neurobehavioral functions even when the treatment was initiated 24 hours after IR. GSNO is an endogenous component of the human body, and its exogenous administration has no known side effects or toxicity when used for other indications (Konorev et al., 1995; Molloy et al., 1998; Aledia et al., 2002; Kaposzta et al., 2002; Snyder et al., 2002; Colagiovanni et al., 2011). These findings make GSNO an attractive candidate to be investigated in humans for neurorepair and rehabilitation following stroke.

References

Aledia, A.S., Tran, L.M., King, B.O., Serna, D.L., Eng, J., Jones, B.U., Chen, J.C. & Roum, J.H. (2002). S-nitrosoglutathione preserves platelet function during *in vitro* ventricular assist device circulation. *Asaio J*, *48*(5), 526-531.

Chen, M.J. & Russo-Neustadt, A.A. (2005). Exercise activates the phosphatidylinositol 3-kinase pathway. *Brain Res Mol Brain Res*, *135*(1-2), 181-193.

Cheng, X.W., Kuzuya, M., Kim, W., Song, H., Hu, L., Inoue, A., Nakamura, K., Di, Q., Sasaki, T., Tsuzuki, M., Shi, G.P.,

Okumura, K. & Murohara, T. (2010). Exercise training stimulates ischemia-induced neovascularization via phosphatidylinositol 3-kinase/Akt-dependent hypoxia-induced factor-1 alpha reactivation in mice of advanced age. *Circulation*, *122*(7), 707-716.

Chiueh, C.C. & Rauhala, P. (1999). The redox pathway of S-nitrosoglutathione, glutathione and nitric oxide in cell to neuron communications. *Free Radic Res*, *31*(6), 641-650.

Chollet, F., Tardy, J., Albucher, J.F., Thalamas, C., Berard, E., Lamy, C., Bejot, Y., Deltour, S., Jaillard, A., Niclot, P., Guillon, B., Moulin, T., Marque, P., Pariente, J., Arnaud, C. & Loubinoux, I. (2011). Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): A randomised placebo-controlled trial. *Lancet Neurol*, *10*(2), 123-130.

Chou, P.C., Shunmugavel, A., Sayed, H.E., Desouki, M.M., Nguyen, S.A., Khan, M., Singh, I. & Bilgen, M. (2011). Preclinical use of longitudinal MRI for screening the efficacy of s-nitrosoglutathione in treating spinal cord injury. *J Magn Reson Imaging*, *33*(6), 1301-1311.

Clarkson, A.N., Huang, B.S., Macisaac, S.E., Mody, I. & Carmichael, S.T. (2010). Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature*, *468*(7321), 305-309.

Clarkson, A.N., Overman, J.J., Zhong, S., Mueller, R., Lynch, G. & Carmichael, S.T. (2011). AMPA receptor-induced local brain-derived neurotrophic factor signaling mediates motor recovery after stroke. *J Neurosci*, *31*(10), 3766-3775.

Colagiovanni, D.B., Borkhataria, D., Looker, D., Schuler, D., Bachmann, C., Sagelsdorff, P., Honarvar, N. & Rosenthal, G.J. (2011). Preclinical 28-day inhalation toxicity assessment of s-nitrosoglutathione in beagle dogs and wistar rats. *Int J Toxicol*, *30*(5), 466-477.

Cramer, S.C. (2008). Repairing the human brain after stroke. II. Restorative therapies. *Ann Neurol*, *63*(5), 549-560.

Cui, X., Chopp, M., Zacharek, A., Roberts, C., Buller, B., Ion, M. & Chen, J. (2010). Niacin treatment of stroke increases synaptic plasticity and axon growth in rats. *Stroke*, *41*(9), 2044-2049.

Ding, Y., Li, J., Lai, Q., Rafols, J.A., Luan, X., Clark, J. & Diaz, F.G. (2004). Motor balance and coordination training enhances functional outcome in rat with transient middle cerebral artery occlusion. *Neuroscience*, *123*(3), 667-674.

Fortenberry, J.D., Owens, M.L., Chen, N.X. & Brown, L.A. (2001). S-nitrosoglutathione inhibits TNF-alpha-induced NFkappaB activation in neutrophils. *Inflamm Res*, *50*(2), 89-95.

Foster, M.W., Hess, D.T. & Stamler, J.S. (2009). Protein S-nitrosylation in health and disease: A current perspective. *Trends Mol Med*, *15*(9), 391-404.

Gladstone, D.J., Danells, C.J., Armesto, A., McIlroy, W.E., Staines, W.R., Graham, S.J., Herrmann, N., Szalai, J.P. & Black, S.E. (2006). Physiotherapy coupled with dextroamphetamine for rehabilitation after hemiparetic stroke: A randomized, double-blind, placebo-controlled trial. *Stroke*, *37*(1), 179-185.

Hamm, R.J. (2001). Neurobehavioral assessment of outcome following traumatic brain injury in rats: An evaluation of selected measures. *J Neurotrauma*, *18*(11), 1207-1216.

Hoffman, A.N., Malena, R.R., Westergom, B.P., Luthra, P., Cheng, J.P., Aslam, H.A., Zafonte, R.D. & Kline, A.E. (2008). Environmental enrichment-mediated functional improvement after experimental traumatic brain injury is contingent on task-specific neurobehavioral experience. *Neurosci Lett*, *431*(3), 226-230.

Kaposzta, Z., Clifton, A., Molloy, J., Martin, J.F. & Markus, H.S. (2002). S-nitrosoglutathione reduces asymptomatic embolization after carotid angioplasty. *Circulation*, *106*(24), 3057-3062.

Ke, Z., Yip, S.P., Li, L., Zheng, X.X. & Tong, K.Y. (2011). The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: A rat brain ischemia model. *PLoS One*, *6*(2), e16643.

Khan, M., Im, Y.B., Shunmugavel, A., Gilg, A.G., Dhindsa, R.K., Singh, A.K. & Singh, I. (2009). Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *J Neuroinflammation*, *6*, 32.

Khan, M., Jatana, M., Elango, C., Paintlia, A.S., Singh, A.K. & Singh, I. (2006). Cerebrovascular protection by various nitric oxide donors in rats after experimental stroke. *Nitric Oxide*, *15*(2), 114-124.

Khan, M., Sakakima, H., Dhammu, T.S., Shunmugavel, A., Im, Y.B., Gilg, A.G., Singh, A.K. & Singh, I. (2011). S-Nitrosoglutathione reduces oxidative injury and promotes mechanisms of neurorepair following traumatic brain injury in rats. *J Neuroinflammation*, *8*(1), 78.

Khan, M., Sekhon, B., Giri, S., Jatana, M., Gilg, A.G., Ayasolla, K., Elango, C., Singh, A.K. & Singh, I. (2005). S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. *J Cereb Blood Flow Metab*, *25*(2), 177-192.

Kim, M.W., Bang, M.S., Han, T.R., Ko, Y.J., Yoon, B.W., Kim, J.H., Kang, L.M., Lee, K.M. & Kim, M.H. (2005). Exercise increased BDNF and trkB in the contralateral hemisphere of the ischemic rat brain. *Brain Res*, *1052*(1), 16-21.

Kline, A.E., Hoffman, A.N., Cheng, J.P., Zafonte, R.D. & Massucci, J.L. (2008). Chronic administration of antipsychotics impede behavioral recovery after experimental traumatic brain injury. *Neurosci Lett*, *448*(3), 263-267.

Kluge, I., Gutteck-Amsler, U., Zollinger, M. & Do, K.Q. (1997). S-Nitrosoglutathione in Rat Cerebellum: Identification and Quantification by Liquid Chromatography-Mass Spectrometry. *J Neurochem*, *69*(6), 2599-2607.

Konorev, E.A., Tarpey, M.M., Joseph, J., Baker, J.E. & Kalyanaraman, B. (1995). S-nitrosoglutathione improves functional recovery in the isolated rat heart after cardioplegic ischemic arrest-evidence for a cardioprotective effect of nitric oxide. *J Pharmacol Exp Ther*, *274*(1), 200-206.

Lima, B., Lam, G.K., Xie, L., Diesen, D.L., Villamizar, N., Nienaber, J., Messina, E., Bowles, D., Kontos, C.D., Hare, J.M., Stamler, J.S. & Rockman, H.A. (2009). Endogenous S-nitrosothiols protect against myocardial injury. *Proc Natl Acad Sci U S A*, *106*(15), 6297-6302.

Liu, F., Schafer, D.P. & McCullough, L.D. (2009). TTC, fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. *J Neurosci Methods*, *179*(1), 1-8.

Lo, E.H. (2008). Experimental models, neurovascular mechanisms and translational issues in stroke research. *Br J Pharmacol*, *153*(Suppl 1), S396-S405.

Lok, J., Gupta, P., Guo, S., Kim, W.J., Whalen, M.J., van Leyen, K. & Lo, E.H. (2007). Cell-cell signaling in the neurovascular unit. *Neurochem Res*, *32*(12), 2032-2045.

Marshall, H.E. & Stamler, J.S. (2001). Inhibition of NF-kappa B by S-nitrosylation. *Biochemistry*, *40*(6), 1688-1693.

Matsuda, F., Sakakima, H. & Yoshida, Y. (2011). The effects of early exercise on brain damage and recovery after focal cerebral infarction in rats. *Acta Physiol (Oxf)*, *201*(2), 275-287.

Molloy, J., Martin, J.F., Baskerville, P.A., Fraser, S.C. & Markus, H.S. (1998). S-nitrosoglutathione reduces the rate of emboliza-tion in humans. *Circulation*, *98*(14), 1372-1375.

Monville, C., Torres, E.M. & Dunnett, S.B. (2006). Comparison of incremental and accelerating protocols of the rotarod test for the assessment of motor deficits in the 6-OHDA model. *J Neurosci Methods*, *158*(2), 219-223.

Moskowitz, M.A., Lo, E.H. & Iadecola, C. (2010). The science of stroke: Mechanisms in search of treatments. *Neuron*, *67*(2), 181-198.

Pannu, R., Christie, D.K., Barbosa, E., Singh, I. & Singh, A.K. (2007). Post-trauma Lipitor treatment prevents endothelial dys-function, facilitates neuroprotection, and promotes locomotor recovery following spinal cord injury. *J Neurochem*, *101*(1), 182-200.

Ploughman, M., Windle, V., MacLellan, C.L., White, N., Dore, J.J. & Corbett, D. (2009). Brain-derived neurotrophic factor con-tributes to recovery of skilled reaching after focal ischemia in rats. *Stroke*, *40*(4), 1490-1495.

Prasad, R., Giri, S., Nath, N., Singh, I. & Singh, A.K. (2007). GSNO attenuates EAE disease by S-nitrosylation-mediated

modulation of endothelial-monocyte interactions. *Glia*, *55*(1), 65-77.

Que, L.G., Liu, L., Yan, Y., Whitehead, G.S., Gavett, S.H., Schwartz, D.A. & Stamler, J.S. (2005). Protection from experimental asthma by an endogenous bronchodilator. *Science*, *308*(5728), 1618-1621.

Radomski, M.W., Rees, D.D., Dutra, A. & Moncada, S. (1992). S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*. *Br J Pharmacol*, *107*(3), 745-749.

Singh, S.P., Wishnok, J.S., Keshive, M., Deen, W.M. & Tannenbaum, S.R. (1996). The chemistry of the S-nitrosoglutathione/glutathione system. *Proc Natl Acad Sci U S A*, *93*(25), 14428-14433.

Snyder, A.H., McPherson, M.E., Hunt, J.F., Johnson, M., Stam-ler, J.S. & Gaston, B. (2002). Acute effects of aerosolized S-nitrosoglutathione in cystic fibrosis. *Am J Respir Crit Care Med*, *165*(7), 922-926.

Swanson, R.A., Morton, M.T., Tsao-Wu, G., Savalos, R.A., David-son, C. & Sharp, F.R. (1990). A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab*, *10*(2), 290-293.

Wellman, T.L., Jenkins, J., Penar, P.L., Tranmer, B., Zahr, R. & Lounsbury, K.M. (2004). Nitric oxide and reactive oxygen species exert opposing effects on the stability of hypoxia-inducible factor-1alpha (HIF-1alpha) in explants of human pial arteries. *Faseb J*, *18*(2), 379-381.

Wu, H., Lu, D., Jiang, H., Xiong, Y., Qu, C., Li, B., Mahmood, A., Zhou, D. & Chopp, M. (2008). Simvastatin-mediated upregula-tion of VEGF and BDNF, activation of the PI3K/Akt pathway, and increase of neurogenesis are associated with therapeu-tic improvement after traumatic brain injury. *J Neurotrauma*, *25*(2), 130-139.