NEURAL REGENERATION RESEARCH
Volume 7, Issue 13, May 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(13):993-999.

Melatonin combined with exercise cannot alleviate cerebral injury in a rat model of focal cerebral ischemia/reperfusion injury

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Abstract

Previous studies have demonstrated that melatonin combined with exercise can alleviate secondary damage after spinal cord injury in rats. Therefore, it is hypothesized that melatonin combined with exercise can also alleviate ischemic brain damage. In this study, adult rats were subjected to right middle cerebral artery occlusion after receiving 10 mg/kg melatonin or vehicle subcutaneously twice daily for 14 days. Forced exercise using an animal treadmill was performed at 20 m/min for 30 minutes per day for 6 days prior to middle cerebral artery occlusion. After middle cerebral artery occlusion, each rat received melatonin combined with exercise, melatonin or exercise alone equally for 7 days until sacrifice. Interestingly, rats receiving melatonin combined with exercise exhibited more severe neurological deficits than those receiving melatonin or exercise alone. Hypoxia-inducible factor 1α mRNA in the brain tissue was upregulated in rats receiving melatonin combined with exercise. Similarly, microtubule associated protein-2 mRNA expression was significantly upregulated in rats receiving melatonin alone. Chondroitin sulfate proteoglycan 4 (NG2) mRNA expression was significantly decreased in rats receiving melatonin combined with exercise as well as in rats receiving exercise alone. Furthermore, neural cell loss in the primary motor cortex was significantly reduced in rats receiving melatonin or exercise alone, but the change was not observed in rats receiving melatonin combined with exercise. These findings suggest that excessive intervention with melatonin, exercise or their combination may lead to negative effects on ischemia/reperfusion-induced brain damage.

Key Words

focal cerebral ischemia/reperfusion; melatonin; exercise; neurological function; brain tissue loss; microtubule associated protein-2; chondroitin sulfate proteoglycan 4; NG2; hypoxia-inducible factor 1 alpha; neural regeneration

Abbreviations

HIF-1 alpha, hypoxia-induced factor 1 alpha; Veh, vehicle; MCAO, middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride; OPCs, oligodendrocyte progenitor cells

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Received: 2012-01-11
Accepted: 2012-03-30
(NY20110923004/H)

Lee S, Shin J, Lee M, Hong Y, Lee SK, Lee Y, Lkhagvasuren T, Kim DW, Yang YA, Chang KT, Hong Y. Melatonin combined with exercise cannot alleviate cerebral injury in a rat model of focal cerebral ischemia/reperfusion injury. Neural Regen Res. 2012;7(13):993-999.

www.crter.cn
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doi:10.3969/j.issn.1673-5374.2012.13.005

INTRODUCTION

Brain cells have a relatively high oxygen and glucose consumption and depend almost exclusively on oxidative phosphorylation for energy production[1]. Acute ischemic stroke results from the sudden decrease or loss of blood supply to an area of the brain, resulting in a coinciding loss of neurological function[2]. This immediately leads to dysfunction of energy-dependent ion transport pumps and depolarization of neuronal and glial cells[3-4]. Consequently, raised intracellular Ca^{2+} ions induce the activation of hypoxia-induced factor 1 alpha (HIF-1α) expression[5] and generate Ca^{2+}-dependent enzymes, including nitric oxide synthase, as well as free radicals, especially reactive oxygen species. Also, transcription factors, such as HIF-1, are expressed in the brain after focal cerebral ischemia in response to changes in oxygen concentration[6-7]. HIF-1 regulates the expression of a broad range of genes facilitating cellular adaptation to low O_2 conditions. Systemic hypoxia for durations of 1, 3, or 6 hours rapidly increases the nuclear content of HIF-1α, the subunit that mainly determines HIF-1 activation in the rodent brain[8]. Increased HIF-1α expression by inhibiting prolyl 4-hydroxylase provides neuroprotection during permanent focal ischemia[8-11]. Therefore, hypoxia-damaged neural cells recover oxygen homeostasis. Diminished cerebral oxygenation affects recruitment of motor units, and supplemental O_2 enhances cerebral oxygenation[12]. At the same time, cerebral oxygen homeostasis sensors, such as HIF-1, are transcribed to turn on the expression of vascular endothelial factor, erythropoietin, and glucose transporter 1/3[7-11, 13].

Behavioral experience enhances brain function after damage, including traumatic brain injury or ischemic or hemorrhagic stroke[14]. Pre- and post-injury exercise, such as wheel running, influences brain and spinal cord neural circuitry[15-16]. A previous study suggested that 30 days of training is sufficient to induce angiogenesis within the motor cortex[17]. However, despite the lack of exercise-induced quantifiable increases in synaptogenesis in the motor cortex, exercise training does upregulate neurotropic factors that promote neuronal survival and differentiation[18]. Moreover, moderate motor activity, such as the animal running on an unobstructed walkway for several minutes a day and voluntary exercise on a running wheel, elevates expression of brain-derived neurotropic factor in the motor cortex[19].

Melatonin, the major secretory product of the pineal gland, interacts with the neuroendocrine axis and is associated with circadian rhythms. Melatonin acts as a free radical scavenger, antioxidant, and an anti-apoptotic agent[20]. Exogenous melatonin is beneficial for increasing oxygen demand under physiological and pathophysiological conditions. Previously, we have reported the synergistic effect of melatonin plus exercise in a spinal cord injury animal model[21]. In this study, we hypothesized that melatonin combined with exercise is beneficial to cerebral ischemia/reperfusion.

RESULTS

Quantitative analysis of experimental animals

Thirty-six Sprague-Dawley rats were randomly assigned to five groups: middle cerebral artery occlusion (MCAO) (MCAO + saline, *n* = 8), exercise (MCAO + exercise, *n* = 8), melatonin (MCAO + melatonin, *n* = 8) and exercise + melatonin (MCAO + exercise combined with melatonin, *n* = 8), and control groups (non-MCAO, *n* = 4). All 36 rats were included in the final analysis.

Melatonin combined with exercise resulted in neurological dysfunction

At 1, 3 and 7 days after MCAO surgery, the neurological severity score was measured (Figure 1). The neurological function of rats subjected to melatonin combined with exercise was the worst 1 day after MCAO. The neurological function of rats in the exercise group recovered significantly compared to the melatonin + exercise group ($P < 0.05$) at 7 days after MCAO. There were no significant differences among the MCAO, melatonin, and melatonin + exercise groups ($P > 0.05$).

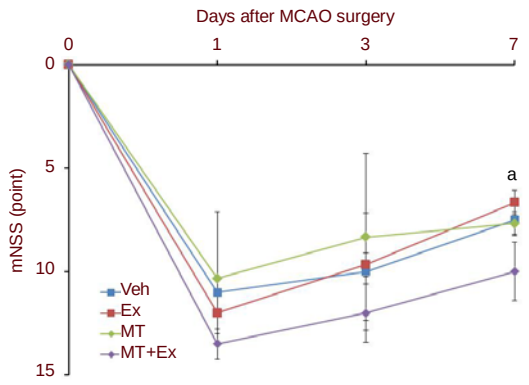

Figure 1 Melatonin combined with exercise resulted in the most severe brain damage after middle cerebral artery occlusion (MCAO) surgery. Veh: Vehicle; Ex: exercise; MT: melatonin administration. $^aP < 0.05$: Ex *vs.* MT + Ex (one-way analysis of variance followed by Tukey's *post hoc* test). Data are shown as mean ± SD.

Melatonin and exercise enhanced oxygen homeostasis

HIF-1α mRNA expression in the injured brain tissues significantly increased in the exercise + melatonin group compared to the non-MCAO group ($P < 0.01$). In the

non-MCAO groups, melatonin combined with exercise not only showed the highest HIF-1α mRNA expression among the interventional groups, but also a significant increment in the region ipsilateral to the lesion in the melatonin or exercise alone group compared to vehicle (Veh) at 7 days ($P < 0.01$) after starting therapeutic intervention (Figure 2). In the brain tissues contralateral to the MCAO lesion, HIF-1α expression was significantly increased in the melatonin or exercise alone group compared to MCAO-Veh at 7 days after MCAO ($P < 0.01$) after begining therapeutic intervention. In particular, MCAO-melatonin combined with exercise yielded the highest HIF-1α mRNA expression among the interventional groups (Figure 2).

Melatonin and exercise upregulated dendritic stability of the neurons

Reverse transcription PCR (RT-PCR) detection showed that in the brain tissues ipsilateral to the MCAO lesion, mRNA levels of MAP2, which is a specific marker of the dendritic stability of neurons, significantly increased in the MCAO-melatonin, -exercise, and -melatonin combined with exercise groups compared to non-MCAO-Veh. MCAO groups showed significantly increased MAP2 mRNA expression compared to non-MCAO-Veh ($P < 0.01$). MAP2 mRNA expression was highest in the MCAO-melatonin group. Interestingly, MCAO-melatonin combined with exercise significantly reduced MAP2 mRNA expression compared to MCAO-Veh and non-MCAO-Veh ($P < 0.01$) (Figure 3A). Figure 3B shows that in the brain tissues ipsilateral to the MCAO lesion, significantly increased chondroitin sulfate proteoglycan 4 (NG2) mRNA expression was observed in non-MCAO-melatonin and -melatonin combined with exercise groups compared to non-MCAO-Veh group ($P < 0.01$). MCAO-Veh yielded the highest NG2 mRNA expression after MCAO surgery ($P < 0.05$). NG2 mRNA expression was significantly decreased in the MCAO-melatonin combined with exercise group versus the MCAO-Veh group ($P < 0.01$) and each of the non-MCAO interventional groups ($P < 0.05$) (Figure 3B). Interestingly, in the contralateral hemispheres, NG2 mRNA expression was significantly increased in the MCAO-melatonin combined with exercise group versus the MCAO-Veh group ($P < 0.01$).

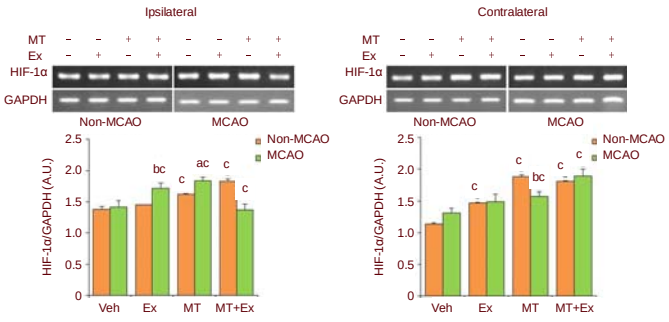

Figure 2 Pre-intervention with melatonin and/or exercise enhanced the transcription of hypoxia-inducible factor (HIF)-1α mRNA expression after focal cerebral ischemia. Brains were collected at 7 days after middle cerebral artery occlusion (MCAO) surgery. Melatonin (10 mg/kg) was administered subcutaneously for 14 days before MCAO.

Veh: Vehicle; Ex: exercise; MT: melatonin administration. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. non-MCAO-Veh, $^{c}P < 0.01$, *vs*. MCAO-Veh (one-way analysis of variance and Tukey's *post hoc* test). Data are expressed as mean ± SD.

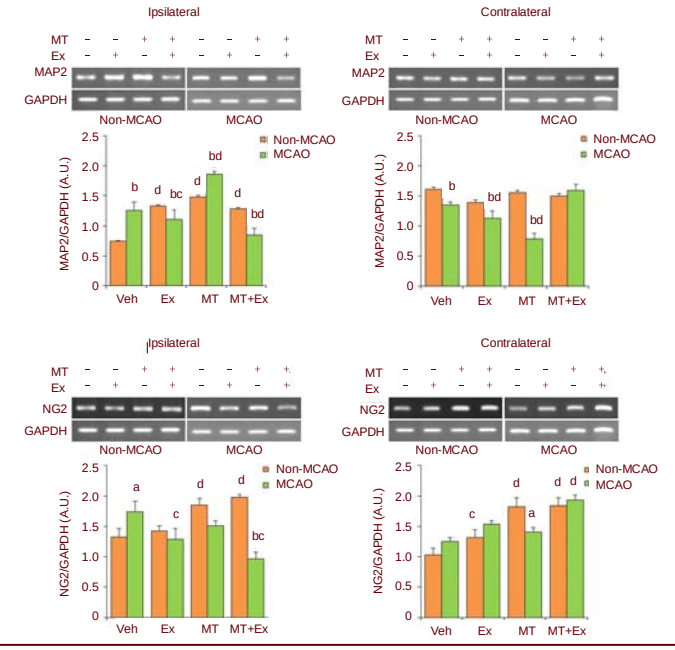

Figure 3 Pre-intervention with melatonin and/or exercise enhanced microtubule-associated protein 2 (MAP2; A), and chondroitin sulfate proteoglycan (NG2; B) mRNA levels after focal cerebral ischemia.

Brains were collected at 7 days after middle cerebral artery occlusion (MCAO) surgery. Melatonin (10 mg/kg) was administered subcutaneously for 14 days before MCAO surgery.

Veh: Vehicle; Ex: exercise; MT: melatonin administration. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. non-MCAO-Veh group; $^{c}P < 0.05$, $^{d}P < 0.01$, *vs*. MCAO-Veh (one-way analysis of variance and Tukey's *post hoc* test). Data are expressed as mean ± SD.

Melatonin and exercise reduced the loss of brain tissue

As shown in Figure 4A, triphenyltetrazolium chloride (TTC)-stained brain tissues showed necrotic and apoptotic cell death after MCAO surgery. MCAO-Veh brains showed an approximately 78% infarct area on the right hemisphere. The infarct lesion was significantly alleviated in the MCAO groups with pre-intervention with melatonin or exercise alone ($P < 0.01$). In particular, pre-intervention with melatonin resulted in red TTC-stained primary motor cortex in the ispilateral region (arrow) (Figure 4A). Interestingly, MCAO-melatonin combined with exercise showed a loss of cerebral cortex in the ipsilateral region (Figure 4A). The MCAO-Veh group had significantly reduced neuronal cells at 7 days after MCAO surgery. Not only was neuronal cell loss significantly rescued by melatonin and/or exercise at 7 days after treatment, but also microglia appeared in the infarct core zone (Figure 4B).

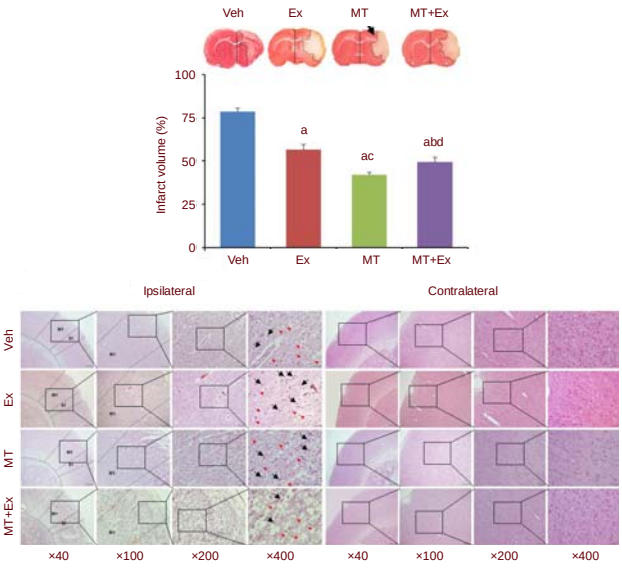

Figure 4 Loss of brain tissue was detected in rats undergoing pre-intervention with melatonin and/or exercise. Black arrows point to neuronal cells and red arrowheads indicate microglia.

(A) 2% triphenyltetrazolium chloride-stained brain tissues, and (B) hematoxylin-eosin stained brain tissues from Bregma 1.20 to −1.20 mm.

M1: Primary motor cortex; S1: sensorimotor cortex; Veh: vehicle; Ex: exercise; MT: melatonin administration; MT + Ex: melatonin combined with exercise.

$^{a}P < 0.01$, *vs*. MCAO-Veh group, $^{b}P < 0.05$, $^{c}P < 0.01$, *vs*. MCAO-Ex group, $^{d}P < 0.01$, *vs*. MCAO-MT group (one-way analysis of variance and Tukey's post hoc test). Data are expressed as mean ± SD.

DISCUSSION

HIF-1α plays a critical role in repairing neural cell damage after ischemic stroke[7]. Therefore, we expected that the overexpression of HIF-1α mRNA might induce the recovery of structural reorganization during the acute stage after focal cerebral ischemia. Thus, we applied exercise or melatonin for 14 days prior to MCAO surgery. This would enhance HIF-1α mRNA expression in non-MCAO groups. However, on the one hand, either melatonin or exercise could produce a negative effect on focal cerebral ischemia; on the other hand, melatonin or exercise could improve the neurological function and morphological recovery after focal cerebral ischemia. We also found increased HIF-1α mRNA expression in the region ipsilateral to the lesion after focal cerebral ischemia. Melatonin acts as a free radical scavenger and antioxidant and, thus, as an anti-apoptotic agent[21]. The antioxidant action of melatonin plays an important role in its known effects on protection during ischemia-reperfusion injury. However, melatonin combined with exercise did not change HIF-1α mRNA expression in MCAO groups. Similarly, the MCAO-melatonin combined with exercise group had delayed recovery of neurological function, as compared to the melatonin or exercise groups. Interestingly, the MCAO-melatonin combined with exercise group had the most severe neurological function deficits at 1 day after MCAO surgery. Nevertheless, recovery of neurological function might be achieved by a contralateral lesion brain event. Following a contralateral lesion, HIF-1α was significantly upregulated in the MCAO-melatonin and MCAO-melatonin combined with exercise groups compared to the MCAO-vehicle group. Consequently, excessive intervention might not have a beneficial effect against free radical-mediated secondary damage during the acute phase after ischemic stroke, unlike what studies have shown in spinal cord injury animal models. MAP2 is an early and sensitive marker of ischemic damage after permanent and transient focal cerebral ischemia[22].

Therefore, we compared the non-MCAO groups and the MCAO groups after each intervention and at each time point. First, all interventional groups had significantly increased MAP2 mRNA expression in the ipsilateral hemisphere, as compared to vehicle. The MCAO-exercise condition had significantly reduced MAP2 mRNA expression, as compared to MCAO-vehicle. The MCAO-vehicle condition showed a two-fold increase as compared to the non-MCAO-vehicle condition. MAP2 mRNA expression was enhanced by exogenous melatonin, indicating that exogenous melatonin might maintain the plasma melatonin concentrations after being pre-administered for 14 days. As a result, a high melatonin concentration might help restore neuronal integrity after focal cerebral ischemia. Mclver *et al* [23] reported that oligodendrocyte progenitor cells (OPCs)

are responsible for remyelination after focal cerebral ischemia, and BrdU positive cells co-localized with OPCs. Oligodendrocytes express Ca^{2+}-permeable glutamate receptors and have a low resistance to oxidative stress, which make them potentially vulnerable to injury[24]. We examined the changes in OPC marker expression. The MCAO-vehicle group had significantly increased NG2 expression, as compared to the MCAO-melatonin and/or MCAO-exercise groups. These data indicate that OPCs are characterized by high expression of non-NMDA receptors, and that they were not affected by glutamate-based ischemic brain injury. Nevertheless, melatonin or exercise did not affect a change in NG2 mRNA expression. In contrast, the contralateral region revealed an interesting result: opposite expression was observed between the ipsilateral and contralateral hemispheres. The MCAO-melatonin combined with exercise group had significantly increased NG2 mRNA expression compared to the MCAO-vehicle group.

Our results confirmed the post-ischemic upregulation of HIF-1α mRNA after focal cerebral ischemia. HIF-1α expression immediately turns on the transcription of target genes for oxygen homeostasis. Also, in a previous study, we concluded that brain-derived neurotropic factor expression affects neuronal cell survival and recovery of neurological function in an animal model of spinal cord injury[21]. In the present study, we found that the MCAO-melatonin combined with exercise group had delayed recovery of neurological function, as compared to the other interventional groups.

Furthermore, HIF-1α mRNA expression was not altered by melatonin combined with exercise in the MCAO condition. However, MAP2 and NG2 mRNA expression in the MCAO-melatonin combined with exercise condition was less than in the MCAO-vehicle condition. Notably, the ipsilateral region does not show altered gene expression following therapeutic interventions, although the contralateral region had significantly enhanced HIF-1α, MAP2, and NG2 expression.

The emphasis of studies concerning stroke is on the development of therapeutic candidates that might prevent neuronal cell death and improve recovery. Previously, we confirmed that melatonin combined with exercise could improve the function of hindlimbs and the structure of the spinal cord after spinal cord injury[21, 25-26]. However, the MCAO-melatonin combined with exercise condition might have a negative effect on brain regions ipsilateral to the lesion. Nevertheless, melatonin or exercise alone might repair the neurological symptoms and infarct lesions during acute stage focal cerebral ischemia. We suggest that neurological function might enhance HIF-1α mRNA expression in the contralateral region after MCAO. Moreover, we confirmed that MAP2 and NG2 mRNA expression was activated in glutamate-based ischemic brain injury and was remarkably enhanced in the contralateral non-injured hemisphere after treatment with melatonin combined with exercise.

In conclusion, melatonin or exercise alone in MCAO animal models can be used as therapeutic candidates. However, excessive intervention might lead to a negative effect on acute ischemic stroke. It suggests that the recovery of neurological function might potentially occur through compensatory functions of the contralateral non-injured region after acute ischemic stroke.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed at the Laboratory of Biological Clock & Aging in Inje University, Korea, from April 2010 to December 2010.

Materials

A total of 36 healthy male Sprague-Dawley rats, aged 8 weeks old, weighing 240–260 g, provided by Hyochang Science Co., Ltd., Daegu, Korea, were housed under controlled environmental conditions (23 °C), with an established photoperiod of 12 hour light/dark (lights on: 7:00 a.m.).

Methods

Establishment of MCAO

The focal cerebral ischemia/reperfusion model was induced by intraluminal occlusion of the middle cerebral artery according to a previously described method[27]. Briefly, the junction of the right common carotid artery was exposed under a surgical microscope. A 4-0 Nylon thread blunted by heating over flame was inserted from external into the internal carotid artery until the tip occluded the origin of the middle cerebral artery. After closure of the operative sites, the animals were temporarily transferred to a cage with a heating lamp. The thread was gently removed after 60 minutes of MCAO.

Neurological function evaluation

The modified neurological severity score (mNSS)[28] was tested in all animals before MCAO and at 1, 3, and 7 days after MCAO by an investigator who was blinded to the experimental groups. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). mNSS is a composite of motor, sensory, reflex, and balance tests. In the scoring of injury

severity, a score of 1 is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe the injury.

Therapeutic intervention

Melatonin was dissolved in ethanol with saline (vehicle). Rats were injected twice daily for 14 days (10 mg/kg melatonin or vehicle, subcutaneously) at 7:00 and 19:00 before MCAO was induced. Exercise was performed at 20 m/min for 30 minutes per day for a total of 6 days over 14 days[19]. The rats were placed on a moving belt facing away from an electrified grid and were trained to run in the direction opposite to the movement of the belt. This allowed them to move forward in order to avoid tail shocks (intensity, 1.0 mA; Stimulus Controller Model D48E, DRI Co., Taiwan, China). After transient stroke was induced, equal amounts of melatonin and exercise were provided during the week.

Triphenyltetrazolium chloride and hematoxylin-eosin staining for detection of infarct volume and pathological changes in brain tissue

Three fresh rat brains from each group were immediately eliminated from the skull. These brains were cut into 2 mm-thick coronal slices using a rodent brain matrix (RBM-4000C, ASI instruments, Warren, MI, USA). These brain slices were immersed for 15 minutes in 2% TTC solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C. The stained slices were transferred into 4% paraformaldehyde solution overnight and then were photographed from Bregma 1.20 mm to −1.20 mm. Rats were euthanized at 7 days using a zoletil/xylazine cocktail and perfused transcardially with cold phosphate-buffered saline followed by 4% paraformaldehyde; the brain was postfixed for 12 hours and placed in cryoprotectant (30% sucrose). The brain tissue was cut into 10-μm coronal sections on a cryostat (Microm HM525, MICROM International GmbH, Walldorf, Germany) (Bregma 1.20 mm to −1.20 mm) and was stained with hematoxylin-eosin (HE) to evaluate the loss of brain tissue. Each specimen was analyzed with a microscope digital camera (Olympus DDP70, Olympus, Tokyo, Japan) connected to a computer. Quantitative analyses were performed using Image J software (NIH, Bethesda, MD, USA).

RT-PCR for detection of HIF-1α, MAP2, NG2 mRNA expression

The brain tissues were homogenized with 1 mL Tri-reagent (Sigma-Aldrich) to extract the total RNA samples. The RNA was reverse transcribed with oligo d(T) 12−18 using reverse transcriptase #18064-014 (Invitrogen, Carlsbad, CA, USA), and this reaction mixture served as a template for the PCR. A reaction mixture (50 μL) for PCR was comprised of 2.0 μL cDNA synthesis mixture, 40 nM dNTPs, 10 pM sense and antisense primer, and 1.25 U GoTaq® DNA polymerase (Promega, Madison, WI, USA). PCR was performed following the Px2 Thermal cycler HBPX2220 protocol (Thermo Electron Corp., Waltham, MA, USA). Primer sequences are shown in Table 1.

Table 1 Oligonucleotide primers used for RT-PCR

Gene	GenBank accession No.	Primer sequence (5′ to 3′)	Product size (bp)
HIF-1α	NM_024359	F: TCA AGT CAG CAA CGT GGA AG R: TAT CGA GGC TGT GTC GAC TG	198
MAP2	NM_013066	F: CAA AGA GAA GGT GGC AAA GC R: GTG GGC AAG GGA TTT CTA CA	200
NG2	NM_010277	F: ATA CAC TGG CCT TCC ACC AG R: TCA AGC TGC AGC ATC CAT AC	110
GAPDH	BC094037	F: GTA TGA CTC CAC TCA CGG CAA A R: GGT CTC GCC TCC TGG AAG ATG	100

HIF-1α: Hypoxia-inducible factor-1α; MAP2: microtubule-associated protein 2; NG2: chondroitin sulfate proteoglycan 4; GAPDH: glyceraldehyde phosphate dehydrogenase; F: forward; R: reverse.

Statistical analysis

Measurement data are expressed as mean ± SD. Statistical analysis was performed with one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons (for melatonin and exercise) using SPSS 18.0 software (SPSS Inc., IBM, Chicago, IL, USA). A probability value of $P < 0.05$ was considered statistically significant. Investigators who were blinded to the treatment groups conducted the MCAO, observed animal behaviors and analyzed infarct size.

Acknowledgments: We would like to express to our thanks to former members of the Laboratory of Biological Clock & Aging for their help during the course of these studies.

Funding: The study was funded by the KRIBB Research Initiative Program, No. KGM0321112 to Y. Hong; BioGreen 21 Program, No. 20110301-061-542-03-00 to Y. Hong, Rural Development Administration, Republic of Korea.

Author contributions: Seunghoon Lee, Kyu-Tae Chang and Yonggeun Hong were responsible for experimental design, data collection, data analysis, and manuscript writing. Jinhee Shin, Minkyung Lee, Yunkyung Hong, Sang-Kil Lee, Youngjeon Lee and Tserentogtokh Lkhagvasuren performed the experiments. Dong-Wook Kim and Young-Ae Yang performed data analysis

and critical comments on the whole process of this study. Yonggeun Hong was in charge of research funding.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Ethics Committee for Animal Care and Use at Inje University (Approval No. 2010-21), which is certified by the Korean Association of Accreditation of Laboratory Animal Care.

REFERENCES

[1] Brouns R, De Deyn PP. The complexity of neurobiological processes in acute ischemic stroke. Clin Neurol Neurosurg. 2009;111(6):483-495.
[2] Donnan GA, Fisher M, Macleod M, et al. Stroke. Lancet. 2008;371(9624):1612-1623.
[3] Martin RL, Lloyd HG, Cowan AI. The early events of oxygen and glucose deprivation: Setting the scene for neuronal death? Trends Neurosci. 1994;17(6):251-257.
[4] Katsura K, Kristian T, Siesjo BK. Energy metabolism, ion homeostasis, and cell damage in the brain. Biochem Soc Trans. 1994;22(4):991-996.
[5] Batti L, Taylor CT, O`connor JJ. Hydroxylase inhibition reduces synaptic transmission and protects against a glutamate-induced ischemia in the CA1 region of the rat hippocampus. Neuroscience. 2010;167(4):1014-1024.
[6] Semenza GL. HIF-1: Mediator of physiological and pathological responses to hypoxia. J Appl Physiol. 2000; 88:1474-1480.
[7] Shi H. Hypoxia inducible factor 1 as a therapeutic target in ischemic stroke. Curr Med Chem. 2009;16(34): 4593-4600.
[8] Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med. 2003;54:17-28.
[9] Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721-732.
[10] Sharp FR, Bernaudin M. HIF1 and oxygen sensing in the brain. Nat Rev Neurosci. 2004;5(6):437-448.
[11] Ziello JF, Jovin IS, Haung Y. Hypoxia-inducible factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. Yale J Biol Med. 2007;80(2):51-60.
[12] Secher NH, Seifert T, Van Lieshout JJ. Cerebral blood flow and metabolism during exercise: Implications for fatigue. J Appl Physiol. 2008;104(1):306-314.
[13] Bernaudin M, Nedelec AS, Divoux D, et al. Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. J Cereb Blood Flow Metab. 2002;22(4):393-403.
[14] Will B, Galani R, Kelche C, et al. Recovery from brain injury in animals: Relative efficacy of environmental enrichment, physical exercise or formal training (1990-2002). Prog Neurobiol. 2004;72(3):167-182.
[15] Vaynman S, Gomez-Pinilla F. License to run: Exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabil Neural Repair. 2005;19(4):283-295.
[16] Ding Q, Vayman S, Akhavan M, et al. Insulin-like growth factor 1 interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. Neuroscience. 2006; 140(3):823-833.
[17] Kleim JA, Cooper NR, VandenBerg PM. Exercise induces angiogenesis but does not alter movement representations within rat motor cortex. Brain Res. 2002;934(1):1-6.
[18] Adkins DL, Boychuk J, Remple MS, et al. Motor training induces experience-specific patterns of plasticity across motor cortex and spinal cord. J Appl Physiol. 2006;101(6): 1776-1782.
[19] Klintsova AY, Dickson E, Yoshida R, et al. Altered expression of BDNF and its high-affinity receptor TrkB in response to complex motor learning and moderate exercise. Brain Res. 2004;1028(1):92-104.
[20] Cuzzocrea S, Costantino G, Gitto E, et al. Protective effects of melatonin in ischemic brain injury. J Pineal Res. 2000;29(4):217-227.
[21] Park K, Lee Y, Park S, et al. Synergistic effect of melatonin on exercise-induced neuronal reconstruction and functional recovery in a spinal cord injury animal model. J Pineal Res. 2010;48:270-281.
[22] Zhang L, Chopp M, Zhang RL, et al. Erythropoietin amplifies stroke-induced oligodendrogenesis in the rat. PLoS One. 2010;5(6):e11016.
[23] Mclver SR, Muccigrosso M, Gonzales ER, et al. Oligodendrocyte degeneration and recovery after focal cerebral ischemia. Neuroscience. 2010;169(3): 1364-1375.
[24] Wong R. NMDA receptors expressed in oligodendrocyte. Bioessays. 2006;28(5):460-464.
[25] Hong Y, Palaksha KJ, Park K, et al. Melatonin plus exercise-based neurorehabilitative therapy for spinal cord injury. J Pineal Res. 2010;49:201-209.
[26] Park S, Lee S, Park K, et al. Beneficial effects of endogenous and exogenous melatonin on neural reconstruction and functional recovery in an animal model of spinal cord injury. J Pineal Res. 2012;52:107-119.
[27] Longa EZ, Weinstein PR, Carlson S, Cummins R, et al. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989;20:84-91.
[28] Chen J, Sanberg PR, Li Y, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke. 2001;32(11): 2682-2688.

(Edited by Cheng Q, Meaney S, Wang D, Duan HB/Song LP)