Original Article
Neuroprotective and regenerative effects of melatonin on hypoxic-ischemic brain injury in neonatal rats

Tingting Qiu, Meiyu Xu

Department of Pediatric Internal Medicine, The Affiliated Hospital of Nantong University, Nantong, Jiangsu, China

Abstract: To investigate the neuroprotective effect of melatonin against hypoxic-ischemic brain injury in neonatal rats and the mechanisms involved. Neonatal rats with hypoxic-ischemic brain injury were treated with melatonin at 0.01 mg per gram body weight for 7 days. The damaged area and volume of the rat brains were quantified by three-dimensional (3D) reconstructive technology. Apoptosis, regeneration, and repair of neurons in the rat brain were evaluated using terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL), 5-bromo-2'-deoxyuridine (BrdU), double cortin (DCX), and hexaribonucleotide-binding protein-3 (NeuN) assays. Seven days after hypoxic-ischemic brain injury, the damaged area and volume of the injury (16.24 ± 0.33 mm^2 and 47.8 ± 0.30 mm^3, respectively) were significantly greater than those in melatonin-treated rats (9.38 ± 0.23 mm^2 and 26.3 ± 0.28 mm^3, respectively). The TUNEL assay showed that the proportion of apoptotic neurons in the hypoxia-ischemia rats (12.2%) was significantly greater than that in melatonin-treated rats (4.3%). The percentages of DCX$^+$/BrdU$^+$ and NeuN$^+$/BrdU$^+$ neurons in melatonin-treated rats (15.3% and 11.2%, respectively) were significantly higher than those in untreated rats (5.2% and 0.2%). In conclusion, melatonin may not only reduce hypoxia-ischemia-induced brain injury but also promote nerve regeneration in neonatal rats.

Keywords: Hypoxic-ischemic encephalopathy, melatonin, apoptosis, nerve regeneration, 3D reconstruction

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is common in the neonatal period [1]. Various factors in the perinatal period may cause complete or partial hypoxia and a pause or decrease in cerebral blood flow, which always leads to brain damage [2]. Severe brain injury often causes the death of the newborn, whereas mild or moderate brain damage results in cerebral palsy, spasm, mental retardation, ataxia, etc. [2]. The morbidity of HIE is about 0.1-8%; 10-20% of affected infants die during the neonatal period, and 25-30% of the survivors will have varying degrees of neurological sequelae [3].

The development of HIE has been divided into anoxic stage and subsequent recovery stage [4]. This means that newborns with HIE experience two waves of nerve cell damage during the pathological process. The first is an irreversible process of nerve cell necrosis that occurs immediately after ischemia and anoxia, and the second is a reversible stage reflecting apoptosis of nerve cells that results in secondary brain injury [4]. The ischemia-reperfusion injury during the recovery stage is closely related to brain prognosis and has therefore been emphasized in clinical treatments [5]. The pathogenesis of cerebral ischemia-reperfusion injury is very complex. Acute energy failure, excitatory amino acid toxicity, free radical damage, and apoptosis all may lead to degeneration, death, and loss of neurons in the damaged regions [6-8].

After HIE occurs, the therapy ideally aims to inhibit apoptosis and reduce the loss of neurons [9]. On the other hand, because the newborn brain is still developing, it has a strong plasticity, so we also need to promote nerve regeneration, with the hope of there by improving the prognosis of HIE [9]. Early research indicated that the majority of nerve regeneration after ischemic-hypoxic brain damage entailed astrocyte hyperplasia [10]. Hyperplastic astrocytes can form a glial scar, which can hinder the regeneration of neurons and nerve fibers and aggravate damage to the nervous system [10].

With the advancement of nerve regeneration research, in 1992, Richards et al. and Reynolds et al. isolated neural stem cells from the striatum and hippocampus of adult rats for the first time [11, 12]. Their studies advanced our understanding of nerve regeneration after brain injury. The subventricular zone (SVZ) and subgranular zone (SGZ) are recognized as areas of continual nerve regeneration [13]. However, the neural precursor cells that proliferate in the SVZ and SGZ migrate to the olfactory bulb and dentate gyrus granular layer [14]. Thus, the precursor neurons surrounding the injured area are insufficient for neuronal regeneration [14]. Therefore, to promote the recovery of neurological function after HIE, we must enhance the proliferation of precursor cells to promote nerve regeneration at the site of injury.

Melatonin, a small molecule indole amine secreted from the pineal gland of mammals, can act not only as a kind of powerful scavenger to eliminate excess oxyradicals and hydroxyl oxyradicals within the body directly, but can also inhibit the activity of nitric oxide synthase through the activation of glutathione peroxidase, thus playing the role of an indirect antioxidant [15, 16]. Many studies have shown that this highly lipid soluble nerve endocrine hormone can not only pass through the blood-brain barrier to enter the brain but can also penetrate the hypothalamus barrier, reaching the central nervous system, where it can protect against various neurodegenerative diseases and brain injury [17]. In a rat middle cerebral artery occlusion (MCAO) model, exogenous melatonin has been shown to reduce the cerebral infarction area and enhance the neuronal anti-lipid peroxidation reaction, thus playing a role in brain protection [18]. Recent studies have shown that melatonin receptors are expressed in multiple areas during various developmental stages of the rat embryonic central nervous system [19, 20]. Melatonin receptors were also detected in mouse neural stem cells, glial cells, and neurons, indicating that melatonin may also be involved in the process of neural developmental [19, 20]. The effect of melatonin on HIE has rarely been studied. Thus, this study aimed to evaluate the protective and nerve regenerative effects of melatonin against hypoxic-ischemic brain injury in a neonatal rat HIE model.

Materials and methods

Ethics issues

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Committee on the Ethics of Animal Experiments of Nantong University.

Animal experiments

Healthy newborn Sprague-Dawley (SD) rats were raised in the laboratory animal center of Nantong University. To establish a cerebral ischemic-hypoxic neonatal rat model, the right common carotid artery of 7-day-old SD rats (n = 12) was ligatured for 30 min by an operation under ether inhalation anesthesia. The sham-operated rats experienced all surgical procedures except carotid artery ligation (n = 6). Seven days later, the cerebral ischemic-hypoxic rats were divided into two groups; one was treated with 0.01 mg per gram body weight melatonin (Sigma, St. Louis, MO, dissolved in 5% ethanol) by intraperitoneal injection (n = 6), and the other group, treated with only 5% ethanol (n = 6), was regarded as cerebral ischemic-hypoxic rats without treatment. The brains were sampled one week later to perform the following experiments. To label the proliferative neurons, all rats were intraperitoneally injected with 0.05 mg per gram body weight BrdU (Sigma, St. Louis, MO) for 5 days after surgery.

Frozen section preparation

Rat brains were sampled and fixed in 4% paraformaldehyde for 12 h at 4°C, and then sequentially treated with 20% and 30% glucose solution. Brain samples were spliced coronally at 30-μm thickness in succession.

Three-dimensional (3D) reconstruction

Coronal brain samples were photographed in succession using a Canon camera (Canon, Japan) at 2272 × 1704 pixels with a fixed focus and aperture. All images underwent batch processing including luma curve, automatic contrast control, 8-bit gray image transformation, picture inversion, and size adjustment with Photoshop 7.0 (Adobe Systems. Inc., San Jose, CA) using an HP Z600 Workstation (Hewlett-
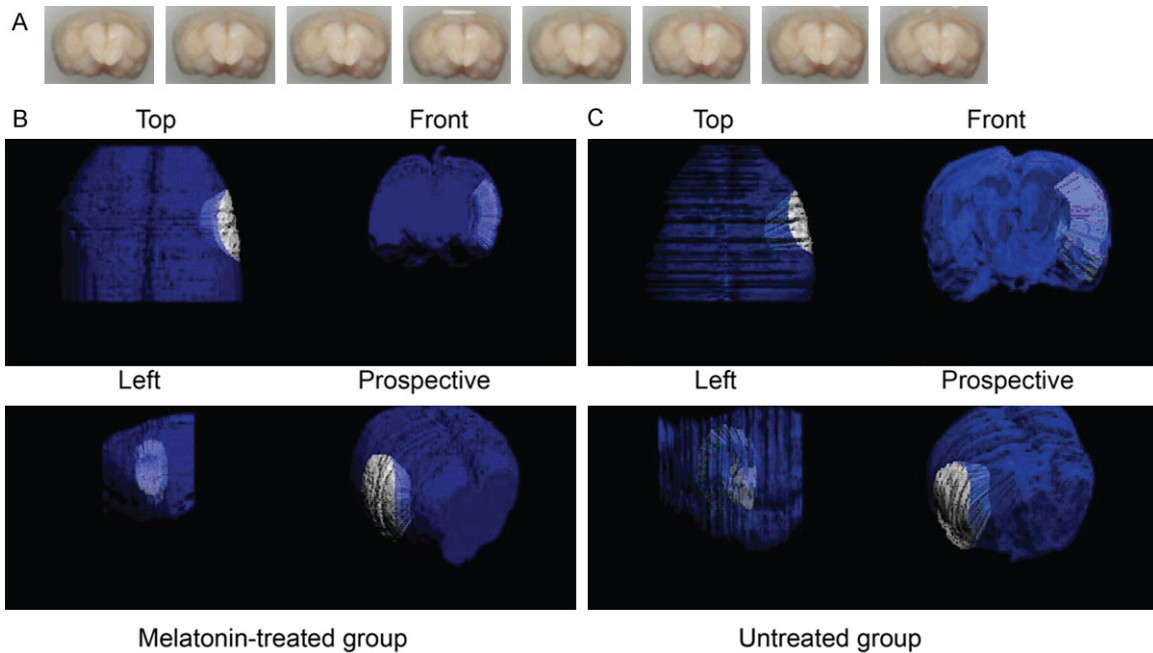
Figure 1. 3D reconstruction and hyalinized brain structures. A. Each rat cerebrum was sliced into 230 sections. B. Cerebral damage in the melatonin-treated rats was visualized by 3D reconstruction. C. The cerebral damage in untreated rats was visualized by 3D reconstruction.

Packard Co., Palo Alto, CA). A total of 230 images of 230 sections for each brain were input into 3D-DOCTOR 4.0 software (Able Software Corp, Lexington, MA). The 3D reconstruction was achieved by the following steps. All 230 images for each brain were input into a new stack frame and sorted automatically by auto alignment. Then, the three-dimensional axes, X, Y, and Z, were calibrated at 3.5, 3.5, and 30 μm, respectively. For edge extraction, white and blue were selected to represent the injury area and normal area, respectively. Finally, the 3D reconstruction was achieved by fast complex surface rendering.

Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay

To evaluate ischemia-anoxia-induced apoptosis, brain tissues were fixed on slides using 4% paraformaldehyde for 1 h. After the brain tissues were washed with 0.01 mol/L phosphate-buffered saline (PBS), they were treated with 0.1% Triton X-100 in PBS for 2 min. Then, 10 μl TUNEL (Roche, Shanghai, China) solution and 50 μL biotin-labeled solution were added to the brain tissues, followed by a 60 min incubation at 37°C. The TUNEL results were checked by an FV10i laser scanning microscope (Olympus, Japan).

Immunofluorescence assay

To study the injury and recovery of the brain in ischemic-anoxic rats, 5-bromo-2'-deoxyuridine (BrdU) was used as a neuron proliferation marker, double cortin (DCX) was used as a marker for precursor neurons and immature neurons, hexaribonucleotide-binding protein 3 (NeuN) was used to label neurons, and Hoechst was used to label nuclei.

For all of the following solutions, except where special instructions are indicated, the solvent was 0.01 mol/L PBS. From each rat brain, 10 sections were selected for the immunofluorescence assay; slides were blocked with 10% skim milk at room temperature for 30 min, and then mouse anti-BrdU, goat anti-DCX, and rabbit anti-NeuN (all purchased from Biovisualab, Shanghai, China; the primary antibodies were in 1:100 dilution) were added to the tissues on the slides and incubated at 4°C overnight. After rinsing three times in PBS, a mix of fluorescently labeled secondary antibodies including mouse anti-mouse, goat, and rabbit IgG were added to the slides and incubated at room temperature for 30 min. After washing three times in PBS, the slides were soaked in 1:1000 Hoechst (Sigma, St. Louis, MO) for 30 min at room temperature, rinsed three times in PBS,

Table 1. Total damaged volume and area comparison

Group	Total area of cerebral cortex (mm²)	Damaged area of cerebral cortex (mm²)	Total volume of cerebrum (mm³)	Total damaged volume of cerebrum (mm³)
Melatonin-treated	119.2 ± 1.1	9.4 ± 0.2*	256.4 ± 1.8	26.3 ± 0.3*
Untreated	119.6 ± 1.0	16.2 ± 0.3	259.1 ± 1.7	47.8 ± 0.3

The data are presented as the mean ± standard deviation (SD). Differences between groups were examined using t-tests. *indicates $P < 0.01$ compared with the ethanol-treated group.

and mounted using glycerol. BrdU, DCX, and NeuN were visualized using a Leica confocal microscope (Leica TCS SP8 Confocal Platform, Buffalo Grove, IL).

Statistical analyses

Continuous variables are presented as the mean ± standard deviation (SD), and categorical data are presented as a number (percentage). Differences between female and male groups were examined using t-tests or χ^2 tests according to the characteristics of the data distribution. The significance level (α) was set at 0.05 (two tailed). All statistical analyses were performed using Stata/SE 12.0 for Windows (Stata Corp LP).

Results

Melatonin decreased cerebral cortex injury

Each rat cerebrum was sliced into 230 sections (**Figure 1A**), which were then photographed and uniformly processed using Photoshop software. The processed images were then input into 3D-DOCTOR 4.0 software to restructure the 3D digital brain images and to perform measurements of the damaged area and total volume. In the cerebral ischemic-hypoxic model, obvious cerebral damage on the right side was observed, mainly distributed in the area supplied by the middle cerebral artery, including the striatum, hippocampus, and cerebral cortex (**Figure 1B** and **1C**). By contrast, in the left cerebral hemisphere, only tiny and disperse injuries were observed (**Figure 1B** and **1C**). The areas of the damaged cerebral cortex in untreated and melatonin-treated rats were 16.2 ± 0.3 mm² and 9.4 ± 0.2 mm², respectively; the area of damage to the cerebral cortex of melatonin-treated rats was significantly lower than that of the untreated rats (**Figure 1B** and **1C**; **Table 1**). The cerebral infarction volumes of the untreated and melatonin-treated rats were 47.8 ± 0.3 mm³ and 26.3 ± 0.3 mm³, respectively; the cerebral infarction volume of

the melatonin-treated rats was also significantly lower than that of untreated rats (**Figure 1B** and **1C**; **Table 1**).

Apoptosis of peripheral cortical neurons in the injury area was downregulated by melatonin after ischemic-hypoxic injury

To investigate the protective effect of melatonin on neurons in the brain injury area after ischemia and hypoxia, apoptosis of cortical cells in the ischemic hypoxic injury area was evaluated by TUNEL. As shown in **Figure 2A**, in the sham-operated group, almost no TUNEL-positive cells were seen, indicating that the sham operation had not damaged either hemisphere. The ischemic-hypoxic rats without treatment showed many TUNEL-positive cells in sections of both hemispheres. Brain sections of the ischemic-hypoxic rats treated with melatonin also showed TUNEL-positive cells in both hemispheres, but there appeared to be fewer than in the rats without treatment (**Figure 2A**). The apoptosis index (AI) of the sham-operated, untreated ischemic-hypoxic, and melatonin-treated ischemic-hypoxic rats were 0.2% ± 0.0%, 12.2% ± 1.8%, and 4.3% ± 0.7%, respectively (**Figure 2B**). The AI for the melatonin-treated rats was significantly higher than that for the sham-operated rats and significantly lower than that for the untreated ischemic-hypoxic rats, suggesting that melatonin may protect against ischemia-hypoxia-induced brain damage.

Melatonin intervention increased DCX/BrdU-NeuN/BrdU double-positive cell numbers around the area of cerebral cortex injury.

To observe whether melatonin intervention can promote the regeneration of nerves in areas corresponding to injury, proliferative neurons were labeled by BrdU, newborn neurons by DCX, and mature neurons by NeuN. As shown in **Figure 3**, untreated ischemic-hypoxic rats had a small amount of DCX-positive newborn neurons, and melatonin-treated rats showed more
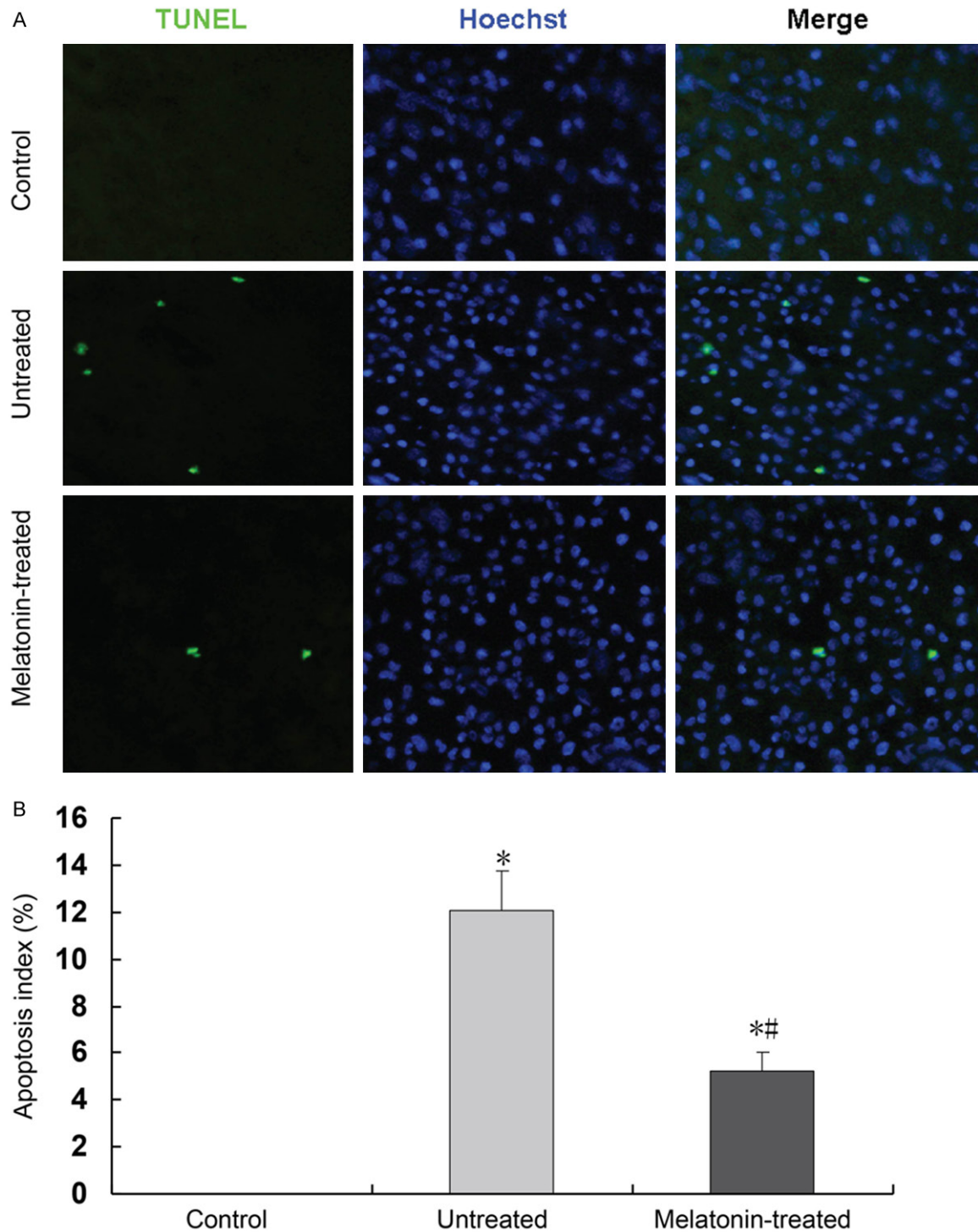
Figure 2. TUNEL assay of rat brain sections. A. Fluorescence microscope images of rat cerebral sections; nuclei of living cells were labeled with Hoechst; apoptotic cells were detected by the TUNEL assay. B. The apoptosis index (AI) of rat groups. The AI represents the percentage of TUNEL-positive neurons in the total number of neurons in the observed ranges.

DCX-positive neurons (**Figure 3A**). DCX+/BrdU+ neurons of sham-operated rats, untreated rats, and melatonin-treated ischemic-hypoxic rats comprised 0.1% ± 0.0%, 5.2% ± 0.8%, and

15.3% ± 1.5%, respectively of the total area (**Figure 3B**). The proportion of DCX+/BrdU+ neurons was significantly higher in melatonin-treated rats than in untreated rats. As shown in
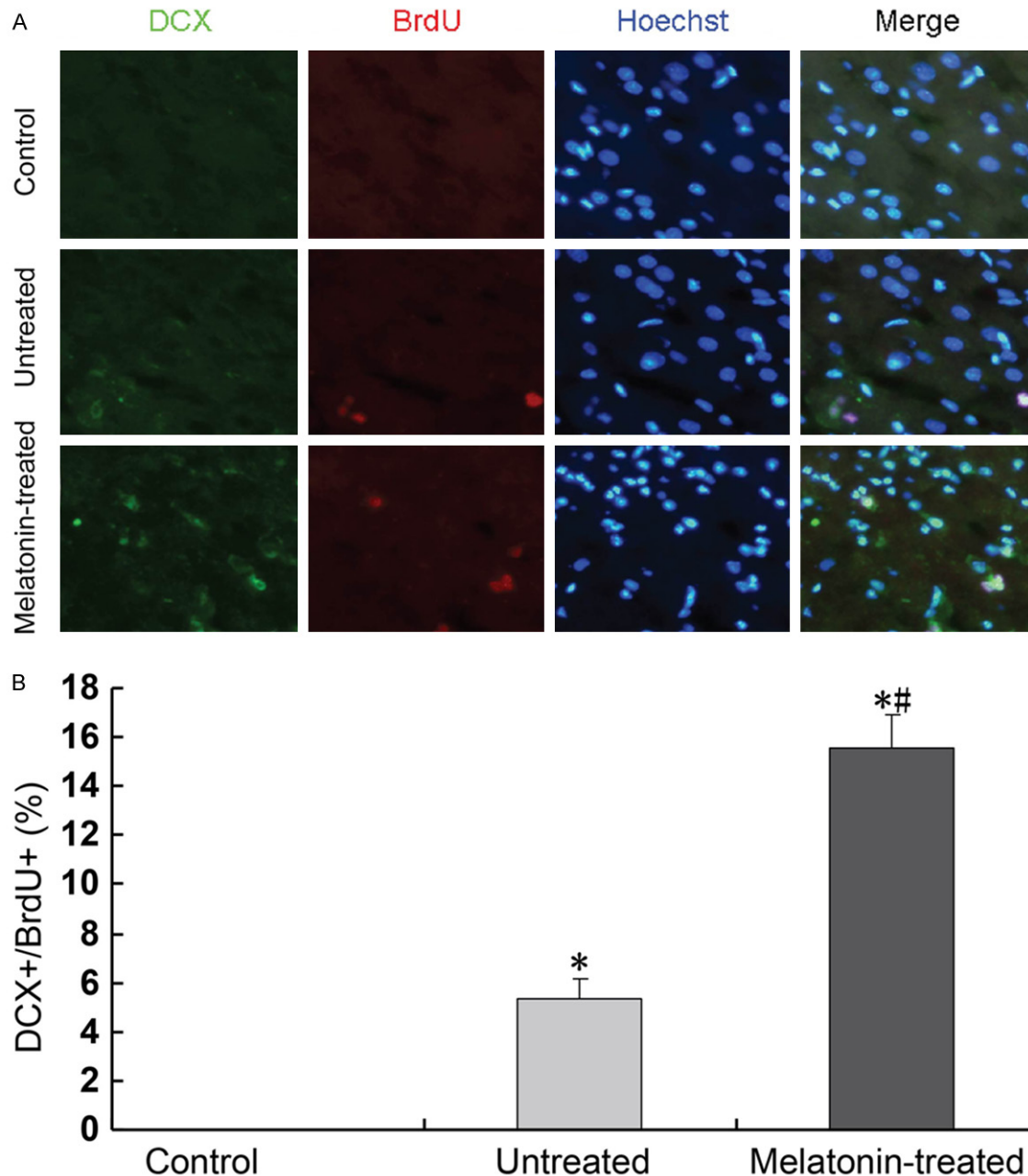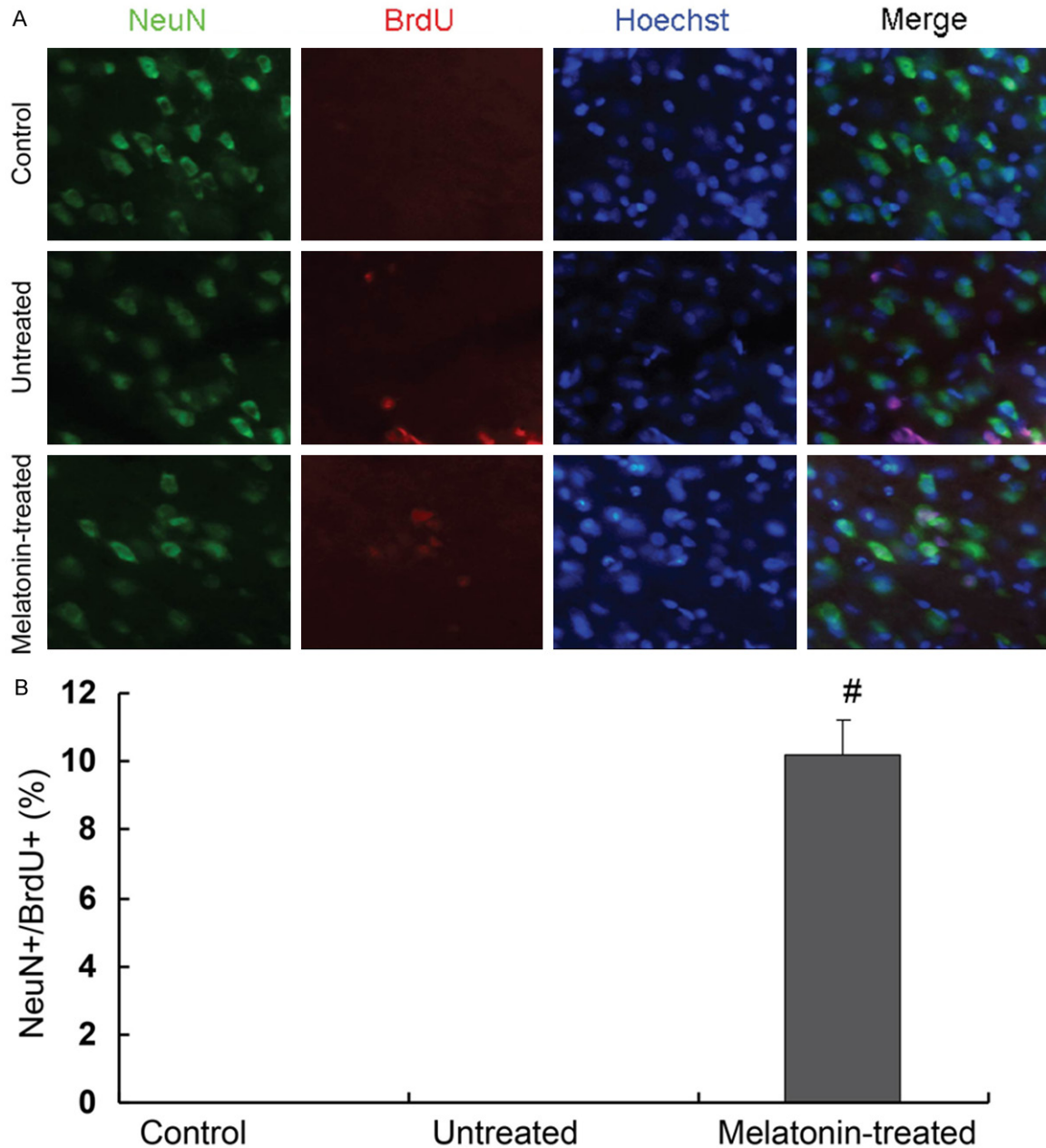
Figure 3. DCX$^+$/BrdU$^+$ neuron assay. A. Cerebral sections of sham-operated, untreated, and melatonin-treated rats were labeled with DCX, BrdU, and Hoechst; fluorescence microscope images are shown. B. DCX$^+$/BrdU$^+$ neurons were calculated and are shown as percentages of the DCX$^+$/BrdU$^+$ neurons of the total number of neurons in the observed ranges. *, significant difference compared with control rats (sham-operated); #, significant difference compared with untreated rats.

Figure 4, although the number of neurons did not differ among the sham-operated, untreated, and melatonin-treated ischemic-hypoxic rats (**Figure 4A**), the percentage of NeuN$^+$/BrdU$^+$ neurons in the above three groups were 0.1% ± 0.0%, 0.2% ± 0.1%, and 11.2% ± 1.6%, respectively (**Figure 4B**), suggesting that melatonin promoted neuron proliferation.

Discussion

The main pathological feature of secondary reperfusion injury in HIE is neuronal apoptosis [21]. A large number of free radicals are produced in the cerebral ischemia-reperfusion period. These free radicals interact with multivalent unsaturated fatty acids, causing the lipid

Figure 4. NeuN$^+$/BrdU$^+$ neuron assay. A. Cerebral sections of sham-operated, untreated, and melatonin-treated rats were labeled with NeuN, BrdU, and Hoechst; fluorescence microscope images are shown. B. NeuN$^+$/BrdU$^+$ neurons were calculated and are presented as the percentage of NeuN$^+$/BrdU$^+$ neurons in the total number of neurons in the observed ranges. #, significant difference between melatonin-treated rats and control (sham-operated) rats.

oxidation waterfall effect, and destroying important components of nerve cells [22]. Melatonin, a neuroendocrine hormone secreted by the pineal gland in mammals, has oxygen free radicals with scavenging and indirect antioxidant activities [15, 16]. In our study, melatonin treatment reduced the area and volume of hypoxia-ischemia-induced brain injury. Further data showed that the protective effect of mela-

tonin had two sources: melatonin reduced neuronal apoptosis and promoted nerve regeneration.

Apoptosis occurred mainly through two pathways: the caspase-3-dependent and caspase-3-independent pathways [23]. Fu et al. showed that melatonin inhibited caspase-3 activation and induced B-cell lymphoma 2 expression in

an in vitro hypoxia model [24]. However, Jou et al. demonstrated that melatonin decreased reactive oxygen species and reduced mitochondrial swelling caused by H_2O_2 in the cell, interrupting early apoptotic events such as exposure of the serous phosphate ester serine, calcium overload, nuclear apoptosis, etc. [25]. Thus, they suggested that the anti-apoptotic effect of melatonin was mainly due to its inhibition of mitochondrial calcium overload and depolarization of the mitochondrial membrane, which decreased membrane permeability [25]. Moreover, melatonin may play a protective role by blocking the release of cytochrome C and by activating the caspase-3 downstream motif, preventing the occurrence of nuclear enrichment [25]. The mechanism of the protective and nerve regenerative effects of melatonin against hypoxia and ischemia brain injury are not the focus of this study, and the specific regulatory mechanism of melatonin in the apoptotic pathway remains to be further elucidated.

Treatment of neonatal hypoxic-ischemic brain injury can be achieved through the blockade or reversal of the neuron apoptotic process, which would decrease cell loss. Given that the developing neonatal brain has very strong plasticity, nerve regeneration in the injured area should greatly improve the long-term prognosis. Takagi et al. found the proliferation of neural stem cells (NSCs) in the dentate gyrus of the hippocampus after transient forebrain ischemia in mice [26]. Furthermore, the BrdU-positive cells in the dentate gyrus and ventricular zone were increased at days 3, 7, and 10 after reperfusion [26]. Studies have also found that cerebral ischemia can promote the proliferation of NSCs in the hippocampal dentate gyrus, and NSCs in the SVZ and ischemic hemisphere were also increased [27]. Jin et al. also showed that, in the gerbil brain, the number of cells in the dentate gyrus increased 12-fold at 1-2 weeks after cerebral ischemia [28]. However, in the case of ischemic-hypoxic brain damage, it is difficult for these endogenously regenerated nerve cells to reach the damaged area and replace the apoptotic neurons, which is the main obstacle for self-repair and neurological function recovery [28]. In this study, DCX/BrdU and NeuN/BrdU double-positive cells were found in the brain injury region in melatonin-treated rats, suggesting that these endogenously regenerated nerve cells could reach the damaged area and facilitate nerve regeneration.

This study adopted 3D reconstruction technology to evaluate hypoxia-ischemia-induced brain injury using a rat model, as the 3D reconstruction technology is superior to traditional approaches in the assessment of brain injury [29, 30]. The 3D reconstruction data as well as cytohistologic data provide solid evidence of the protective and nerve regenerative effects of melatonin against hypoxic-ischemic brain injury in neonatal rats.

This study has several limitations. We did not perform a dose-effect analysis for melatonin; rather, we used a fixed dose (0.01 mg melatonin per gram body weight). The optimal time and time course for melatonin administration were also not explored. Finally, treatment was started 7 days after injury, and the treatment duration lasted one week. We are currently optimizing the melatonin treatment and will report our findings in subsequent reports.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Meiyu Xu, Department of Pediatric Internal Medicine, The Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong 226000, Jiangsu Province, China. Tel: +86-513-85052222; Fax: +86-513-85052222; E-mail: xumeiyu2015@126.com

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