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Cyclosporine a attenuates hypoxic-ischemic brain injury in newborn rats

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Abstract

Cyclosporine A (CsA) is neuroprotective in ischemic brain injuries of adult animals because it blocks the permeability transition of the mitochondrial membrane. In this study, we examined the neuroprotective effect of CsA on hypoxia-ischemia (HI)-induced brain injury in newborn rats. Seven-day-old Sprague Dawley rat pups were subjected to 2 h of 8% oxygen following a unilateral carotid artery ligation. With a single dose of CsA treatment (20 mg/kg, intraperitoneal) given immediately after HI, the HI-induced decrease in brain mitochondrial membrane potential measured with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and adenosine triphosphate levels, and increase in the brain lactate level, both apoptotic and necrotic cells measured with annexin V and propidium iodide (V-PI), and infarct area measured with 2,3,5-triphenyltetrazolium chloride (TTC) were significantly attenuated at 48 h, and the reduced brain volume also significantly improved two weeks following HI. In summary, Cyclosporine A, a mitochondrial permeability transition blocker, significantly attenuated hypoxia-ischemia induced lowering of the mitochondrial membrane potential, cerebral energy status, increased apoptotic and necrotic cells and the ensuing cerebral infarction in the immature brain.

Classification terms

Section: Disease-related Neuroscience

Key words: Cyclosporine A; Hypoxia-Ischemia, Brain; Animals, Newborn

Running title: Cyclosporine A for Neonatal Hypoxic-Ischemic Brain Injury
Introduction

Despite continuous improvement in neonatal intensive care medicine and fetal monitoring, perinatal hypoxic ischemic encephalopathy still remains a major cause of neonatal mortality and permanent neurologic sequelae, such as cerebral palsy, mental retardation, learning disability, and epilepsy, in survivors (Robertson et al., 1985; Vannucci et al., 2000; Volpe et al., 2001). No clinically effective treatment currently exists for this serious disorder. Therefore, the development of a new therapeutic modality to improve the outcome of this disease is urgently needed.

Mitochondrial damage plays a key role in the pathogenesis of both apoptotic and necrotic brain cell death after hypoxia ischemia (HI) (Rehncrona et al., 1979; Hillered et al., 1984; Nakai et al., 1997). HI activates mitochondrial permeability transition (MPT) and induces swelling by the opening of mitochondrial permeability transition pores. Loss of mitochondrial membrane potential (ΔΨ) and energy failure ultimately follow. The simultaneous release of apoptogenic substances (such as cytochrome c) and apoptosis-inducing factor from the mitochondria into the cytoplasm might subsequently initiate apoptosis, or it could cause necrotic cell death before the apoptotic programs could kick in if adenosine triphosphate (ATP) depletion is rapid and profound (Lorek et al., 1994; Liu et al., 1996; Siesjö et al., 1999; Crompton et al., 2002; Friberg et al., 2002; MacGregor et al., 2003; Wu et al., 2006). Overall, MPT appears to be the key mechanism underlying both apoptotic and necrotic cell death after HI.

Recently, the neuroprotective effects of exogenously administered cyclosporin A (CsA) have been reported both in vitro (Gogvadze et al., 1993; Seaton et al., 1998) and in vivo in adult animals with
ischemic brain injury (Li et al., 2000; Shiga et al., 1992; Matsumoto et al., 1999; Matsumoto et al., 2002; Yu et al., 2004; Akdemir et al., 2005; Borlongan et al., 2005). CsA’s neuroprotective effects are mediated by the potent and specific blocking of MPT. The ability of CsA to block MPT is not related to its inhibition of calcineurin to produce immunosuppression (Waldmeier et al., 2002) but instead is mediated through the inhibition of the matrix-specific cyclophilin D (Matsumoto et al., 1999; Matsumoto et al., 2002; Domanska-Janik et al., 2004; Yu et al., 2004; Akdemir et al., 2005; Borlongan et al., 2005). CsA is already in clinical use as an immunosuppressive agent. Therefore, any favorable experimental results can be readily translated into clinical practice. However, results obtained in adults cannot be extrapolated to neonatal medicine due to the dramatic differences in maturational stages and pathophysiology of perinatal and adult brains. Only three studies have been published until now on the effects of CsA in immature brain injury. While some beneficial effects were reported in two of these studies (Nakai et al., 2004; Leger et al., 2010), no protective effect of CsA was shown in the other study (Puka-Sundvall et al., 2001). The role of CsA in immature brain injury is still controversial, and further studies are necessary to clarify its role.

Our study was done to determine the neuroprotective effects of CsA on cerebral injury following HI in the developing brain. We tested whether CsA could attenuate the loss of $\Delta \Psi$ and the ensuing energy failure by blocking MPT, thereby inhibiting apoptosis and reducing cerebral infarction in a newborn rat pup model of cerebral HI. The $\Delta \Psi$ was measured with 5,5’6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). We evaluated cerebral energy status by measuring brain ATP and lactate levels. Apoptotic and necrotic cell death was identified by flow cytometry with a combination of
fluoresceinated annexin V and propidium iodide (V-PI). The extent of cerebral infarction was also evaluated at 48 h with 2,3,5,-triphenyl tetrazolium chloride (TTC), and at two weeks after HI with hematoxylin/eosin staining to determine whether a neuroprotective effect, if any, of CsA was transient or sustained.

Results

Weight gain

Weight gain, which is indicative of general well being in newborns, was significantly restricted in a hypoxia-ischemia control group (HC) compared to a normoxia with sham operating group (NC), starting at 3 days after HI (ten-day-old, P10). The retarded weight gain observed in HC significantly improved in a hypoxia-ischemia with CsA treatment group (HA) from 10 days after HI (Fig. 1).

Flow cytometry

Fig. 2A shows representative flow cytometric analyses of ∆Ψ using JC-1. In HC, increased cell percentage of JC-1 fluorescence shifted from upper red to lower green, indicating a collapse of ∆Ψ, was significantly increased in HC compared to NC. And this abnormality observed in HC was significantly attenuated in HA (Fig. 2B).
Representative flow cytograms of annexin V-PI 48 h after HI in each experimental group are presented in Fig. 3. In HC, the percentage of damaged (Q1, annexin V+/PI+), necrotic (Q2, annexin V+/PI+) and apoptotic (Q4, annexin V+/PI+) cells was significantly increased, and the live (Q3, annexin V-/PI-) cells significantly decreased compared to NC; these abnormalities observed in the HC group significantly improved in the HA group (Table 1).

**Brain ATP and lactate levels**

In the HC group, brain ATP levels were significantly reduced and lactate levels were significantly elevated at 48 h after HI compared to NC; these values were measured as an index of the cerebral energy status, and the abnormalities observed in HC were significantly improved in the HA group (Fig. 4).

**TTC infarct area**

Representative photomicrographs of the TTC infarct area (A) and the percentage of the infarct area defined by the loss of a normal TTC-staining pattern calculated as a percentage of the intact left hemispheric area (B) 48 h after HI in each experimental group are presented in Fig. 5. In HC, the TTC stain-negative infarct area was significantly increased compared to NC, and this increased infarct area observed in HC was significantly improved in HA (Fig. 5B).

**Measurement of brain volume**
In the HC group, there was a significant decrease in percentage of intact ipsilateral hemispheric volume compared to the NC group at two weeks after HI. The HA group showed a marked protective effect and significantly increased percentage of ipsilateral hemispheric volume compared to the HC group two weeks after HI (Fig. 6A, 6B).

**Discussion**

In the present study, CsA significantly attenuated the HI-induced retardation of body weight gain and also attenuated the increased apoptosis and necrosis, as well as the cerebral infarction in the newborn rat. Nakai et al. (2004) and Leger et al. (2010) also have reported protective effects of CsA against ischemic brain injury in fetal and newborn rat brains, respectively. CsA is already in clinical use for immune suppression even in neonates and infants (Motta et al., 2003). Taken together, these findings support the potential use of CsA as a novel therapeutic agent in perinatal hypoxic ischemic encephalopathy where any effective treatment has not been established yet.

In adult animal models, CsA blocks MPT by binding to cyclophilin-D (Rehncrona et al., 1979; Nakai et al., 1997) and prevents both in vitro and in vivo ischemic brain injury by stabilizing the mitochondrial membrane (Gogvadze et al., 1993; Seaton et al., 1998; Li et al., 2000; Matsumoto et al., 2002; Yu et al., 2004; Akdemir et al., 2005; Borlongan et al., 2005). In immature rats, secondary cerebral energy failure is
associated with mitochondrial dysfunction and swelling after HI (Lorek et al., 1994). Nakai et al. (2004) reported that CsA administration improves the recovery of fetal brain energy metabolism, inhibits mitochondrial swelling after transient in utero ischemia and subsequently reduces ischemic brain damage in the immature rat. In the present study, HI-induced reduction of ∆Ψ, as evidenced by the shift of JC-1 fluorescence from red to green as well as decreased brain ATP and increased lactate level, was significantly improved with CsA treatment in the newborn rat pups. Taken together, our findings support the assumption that CsA is neuroprotective by blocking MPT, stabilizing the mitochondrial membrane and preventing the reduction of ∆Ψ and the ensuing secondary energy failure following HI in the immature rat brain. These findings are in contrast to the results of Puka-Sundvall et al. (2001).

In the present study, we used flow cytometry with annexin V to detect exposure of phosphatidylserine outside the plasma membrane, which is the earliest and most characteristic indication of apoptosis (Vermes et al. 2004; Honda et al. 2000). PI was used to detect disruption of the plasma membrane and alterations in permeability observed in necrotic cells. Our data of significantly increased damaged, apoptotic and necrotic cells after HI, and significant attenuation of these abnormalities with CsA treatment observed in the present study implicated that because mitochondrial damage plays a key role both in apoptotic and necrotic cell death, the mitochondria could be good therapeutic targets following HI in the immature brain.

The optimal dose of CsA after HI in the immature brain has not yet been established. In the study of Puka-Sundvall et al. (2001), no protective effects were observed with 10-50 mg/kg of CsA; 10 mg/kg attenuated mild but not severe ischemic immature brain injury in the study of Leger et al. (2010). In an
adult ischemia model, Matsumoto et al. (2002) reported no neuroprotective effect when CsA was administered intraperitoneally at dosages less than 10 mg/kg, a significantly reduced infarct size at a dosage of 20 mg/kg, and increased mortality rates at dosages more than 30 mg/kg. Based on these results, we used 20 mg/kg of CsA in this study for maximal neuroprotection and minimal cytotoxic effects. Further studies will be necessary to clarify the optimum dosage.

In the present study, a single dose of CsA given immediately after HI significantly attenuated the cerebral infarction two weeks after HI, which indicated that the neuroprotective effects of CsA following HI are not transient but sustained. MPT has been known to be the key mechanism underlying both acute necrotic and delayed apoptotic cell death after HI (Robertson, 2004). Necrosis occurs rapidly resulting in acute cell death, whereas apoptotic cell death following HI is a relatively slow and multi-step process, and the entire process, from the initial trigger to the destruction of the cell, can take hours or even days (Park et al., 2006a; Kim et al., 2008). Hence, as significant attenuation of MPT with a single dose of CsA given immediately after HI could prevent not only the acute necrotic but also the delayed apoptotic cell death, the neuroprotective effects of CsA could be sustained up to two weeks after HI as observed in the present study. Overall, these findings suggest that MPT seems to the ‘critical switch’ determining cell survival or death after HI, and could be a good therapeutic target for neuroprotection (Mazzeo et al., 2009).

In summary, CsA had neuroprotective effects by blocking MPT, attenuating the lowering of $\Delta\Psi$, preventing secondary energy failure, inhibiting both apoptotic and necrotic cell death, and reducing the ensuing cerebral infarction in a newborn rat pup model of cerebral HI. Our data suggest that CsA is a
potentially novel therapeutic agent in perinatal hypoxic ischemic encephalopathy, and further clinical studies are warranted.

**Experimental procedures**

*Induction of cerebral hypoxia ischemia*

The experimental protocols described herein were reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute in Seoul, Korea. This study also followed the institutional and National Institutes of Health Guidelines for laboratory animal care. Fifty-seven, seven-day-old (P7) Sprague-Dawley rat pups (Daihan Biolink Company, Seoul, Korea) were used for the experiment. We excluded 9 rat pups that died from hypoxic ischemic injury to the brain and randomly divided the remaining 48 pups into the following three experimental groups: (1) a normoxia with sham operation group (NC, n=12), (2) a hypoxia-ischemia control group (HC, n=16) and (3) a hypoxia-ischemia with CsA treatment group (HA, n=20). Cerebral hypoxia-ischemia was induced by the modified Vannucci method as described in previous reports (Rice et al. 1981; Park et al., 2006b; Kim et al., 2008). The rat pups were anesthetized in a small jar containing cotton soaked with methoxyflurane, and deep anesthesia was maintained during the surgical procedure by placing a small plastic tube containing cotton soaked with methoxyflurane over the nose. The neck was incised along the midline, and the right common
carotid artery was permanently ligated with 4-0 silk. The total time for surgery in each animal never exceeded three minutes. After approximately two hours of postoperative stabilization, the animals were exposed to a 120-min period of hypoxia (8% O\textsubscript{2}, 92% N\textsubscript{2}) by placing them in an airtight chamber partially submerged in a 37\degree C water bath to maintain a constant thermal environment. Pups in the NC group received a sham operation without carotid artery ligation and hypoxic stress. Animals in the HA group received an intraperitoneal injection of CsA (Sigma, St Louis, MO, US) at a dosage of 20 mg/kg of body weight in 50 µL of normal saline immediately after the hypoxic insult (HI, Matsumoto et al., 2002), and an equal volume of normal saline was given in the NC and HC groups. The rat pups were then returned to their dams and twenty-four rat pups were sacrificed at 48 h after HI for measurement of brain ATP and lactate levels, flow cytometry with annexin V-PI and JC-1, and TTC infarction area determination (n=6 in NC, 8 in HC and 10 in HA). The remaining rat pups (n=6 in NC, 8 in HC and 10 in HA) were weighed daily for two weeks after HI until sacrifice for brain volume measurements.

**Preparation of brain specimens**

Under deep pentobarbital anesthesia (60 mg/kg, intraperitoneal), the skull was removed in situ, and the anterior portion of the underlying brain was cut and removed rapidly with a scalpel, frozen in liquid nitrogen and stored at -80\degree C for measurements of brain ATP and lactate levels. The rest of the fresh whole brain tissue obtained at 48 h after HI was placed in 1-2 mL of phosphate buffered saline (PBS) and transected in the coronal plane at the level of the mid-dorsal hippocampus (Vannucci et al., 2004). The
middle and posterior portions of the cerebral hemisphere were processed for TTC and flow cytometry with annexin V/PI and JC-1, respectively.

**Flow cytometry**

Mitochondrial membrane potential (ΔΨ) was estimated using fluorescent probe JC-1 (Molecular Probes, Eugene, OR, US) (Zuliani et al., 2003). The dissociated cortical cell suspensions were adjusted to a density of 1 × 106 cells/mL and stained for 20 minutes with 2.0 µg/mL of JC-1 at 37°C. JC-1 was excited with a 488-nm argon laser, and JC-1 green and orange fluorescences were recorded on FL1 (530 ± 15 nm band pass filter) and FL2 (575 ± 13 nm band pass filter) channels.

To evaluate the numbers of apoptotic and necrotic cells, the posterior portion of the ipsilateral cerebral cortex was dissociated into a single cell, and flow cytometry was done with a combination of PI (Sigma, St. Louis, MO, US) and annexin fluorescein isothiocyanate (V-FITC) (Pharmingen, San Diego, CA, US). Flow cytometric analysis was performed by a Particle Analyzing System (PAS, Partec, Münster, Germany) equipped with an argon ion laser tuned at a wavelength of 488 nm. Green annexin V-FITC fluorescence was measured at 530 ± 15 nm, and red PI fluorescence was measured at 600 nm (Park et al., 2006a; Park et al., 2006b).

**Measurement of ATP and lactate in the cerebral cortex**

Measurements of brain ATP and lactate levels are described in detail in our previous studies (Chang et
Briefly, brain ATP and lactate concentrations were measured using an ATP bioluminescence assay kit (Roche Molecular Biochemicals, Mannheim, Germany) and a lactate dehydrogenase (LDH) kit (Sigma Chemical Company, St. Louis, MO, US), respectively.

**TTC infarct area determination**

Morphometric analysis of infarct areas was performed by TTC (Sigma, St. Louis, MO, US) (Park et al., 2006). This method provides an overall measure of cell injury presented by depleted nicotinamide adenine dinucleotide phosphate (NADPH) and therefore the inability to reduce TTC to its colored form. Coronal brain sections one millimeter in thickness were incubated in PBS containing 2% TTC at 37℃ for 20 min in the dark, washed twice with PBS, and fixed with 4% paraformaldehyde for 30 min at room temperature. A cross-sectional area of the TTC-stained region for each brain slice was determined using an Optimas 6.51 Image Analysis System (Media Cybernetics Inc., Silver Springs, MD, US). The relative infarct areas are expressed as a percentage of contra-lateral hemispheric areas.

**Brain volume measurement**

After transcardiac perfusion with 0.1 M of PBS, the brains were carefully removed two weeks after HI and blocked with paraffin after overnight fixation with 4% paraformaldehyde. The 5-µm thick serial sections were made at an interval of 100 µm and stained with hematoxylin-eosin for volumetric analysis.

All volumetric qualifications were performed with an Olympus BX 40 photomicroscope (Olympus
Optical Co., Ltd., Tokyo, Japan) equipped with a high-resolution CCD camera, a motorized XYZ axis computer-controlled stage, and the Stereoinvestigator software package (ver. 6.52, Micro Bright Field, VT, US). When calculating volume, the cross-sectional areas of the region of interest (ROI) in each section were traced on the computer screen at low power with a 1.5 ×/4 × lens, and the volume of the ROI was calculated using Stereoinvestigator software according to Cavalieri's principle (Regeur et al., 1989).

Using this sampling strategy, approximately eight histology sections per brain in the affected and control hemispheres in each animal were evaluated for hemispheric measurements. For anatomical evaluation of the extent of cerebral injury, the ratio of the ipsilateral remaining cerebral hemisphere volume to the volume of the corresponding contralateral cerebral hemisphere was expressed as a percentage. An examiner blind to the treatment group quantified the results (Kim et al., 2008).

**Statistical analysis**

All data are expressed as mean ± standard deviation. The nonparametric Kruskal-Wallis test was used for the comparison of normally distributed continuous variables between the three groups, and the Mann-Whitney test was done for comparison between the two groups. A $p$-value <0.05 was considered to be statistically significant.
Acknowledgements

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References


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Research Highlights

Cyclosporine A is a mitochondrial permeability transition blocker.

Cyclosporine A attenuates hypoxic ischemic brain injury in the immature brain.
Table 1. Regional Cell Percentage of Ipsilateral Cerebral Hemisphere at 48 Hours after Hypoxia-ischemia

<table>
<thead>
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<th>NC (n=6)</th>
<th>HC (n=8)</th>
<th>HA (n=10)</th>
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<tbody>
<tr>
<td>Q1 (annexin V'/PI') (%)</td>
<td>0.2±0.2</td>
<td>1.9±0.8 *</td>
<td>0.5±0.4†</td>
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<tr>
<td>Q2 (annexin V'/PI') (%)</td>
<td>0.7±0.0</td>
<td>9.3±4.5*</td>
<td>2.4±2.0*†</td>
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<tr>
<td>Q3 (annexin V'/PI') (%)</td>
<td>89.4±0.0</td>
<td>66.3±9.9*</td>
<td>81.2±6.2*†</td>
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<tr>
<td>Q4 (annexin V'/PI') (%)</td>
<td>9.7±0.0</td>
<td>22.2±5.1*</td>
<td>15.6±3.8*†</td>
</tr>
</tbody>
</table>

NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p < 0.05 compared to NC, †p < 0.05 compared to HC.

Figure Legends

Fig. 1. Weight gain, which is indicative of general well-being in newborns, in the rat pups of each experimental group. Significantly restricted weight gain was observed in the HC group compared to the NC group starting at 3 days after hypoxia ischemia (HI), and this retarded growth observed in the HC group was significantly improved in HA rats from 10 days after HI. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p<0.05 compared to NC, †p<0.05 compared to HC.

Fig. 2. Representative flow cytometric analyses of mitochondrial membrane potential (ΔΨ) using JC-1 (A), and the percentage of low ΔΨ (B) 48 h after hypoxia ischemia (HI) in each experimental group. The shift of JC-1 fluorescence from upper red (FL2) to lower green (FL1) indicates a collapse of ΔΨ. Bars separate populations with high and low ΔΨ. Note the significantly increased subset of cells with low ΔΨ in HC brains compared to NC brains and the significant attenuation of these abnormalities in HA. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p<0.05 compared to NC, †p<0.05 compared to HC.
Fig. 3. Representative flow cytogram of annexin V binding (abscissa) versus propidium iodide (PI) uptake (ordinate) in the ipsilateral cerebral hemisphere of newborn rat brain cells in each experimental group 48 h after hypoxia ischemia (HI). Note increased percentage of damaged (Q1, annexin V+/PI−), necrotic (Q2, annexin V+/PI+) and apoptotic (Q4, annexin V+/PI−) cells, decreased live (Q3, annexin V−/PI−) cells in HC compared to NC, and attenuation of these abnormalities in HA. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group.

Fig. 4. Brain ATP and lactate levels at 48 h after hypoxia ischemia (HI) in the newborn rats. Note significantly decreased ATP and increased lactate levels in HC rats compared to NC rats, and significant attenuation of these abnormalities in HA. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p<0.05 compared to NC, †p<0.05 compared to HC.

Fig. 5. Representative photomicrographs of 2,3,5-triphenyl tetrazolium chloride (TTC) infarct area (A) and percentage of the infarct area defined by the loss of a normal TTC staining pattern calculated as a percentage of the intact left hemispheric area (B) at 48 h after hypoxia ischemia (HI) in each experimental group. Note significantly increased infarct area in HC compared to NC, and significant attenuation of these infarct areas in HA. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p<0.05 compared to NC, †p<0.05 compared to HC.

Fig. 6. Representative photomicrographs of hematoxylin/eosin staining at the anterior commissure (A) and percentage of the contralateral and ipsilateral preserved hemispheric brain volume (B) of each experimental group two weeks after hypoxia ischemia (HI). The volume of the ipsilateral remaining hemisphere was significantly lower in HC rats compared to NC rats, and this reduced volume observed in
the HC group was significantly improved in the HA group. NC, normoxia control group; HC, HI control group; HA, HI with Cyclosporine A treatment group. All data are mean ± standard deviation. *p<0.05 compared to NC, †p<0.05 compared to HC.

* p<0.05, compared with the control group.
† p < 0.05, compared with the HI group.
Fig. 1.
Fig. 2.

A.

NC  
HC  
HA

B.
Fig. 3.
**Fig. 4.**

![Bar graph showing concentrations of ATP and Lactate in different groups: NC (n=6), HC (n=5), and HA (n=10).](image-url)
Fig. 5.

A.

B.
Fig. 6.

A.

B.

Ratio of preserved hemispheric volume (topostral endotherm, %)

<table>
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<th>Group</th>
<th>n</th>
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<tr>
<td>NC</td>
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<tr>
<td>HC</td>
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<td>HA</td>
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