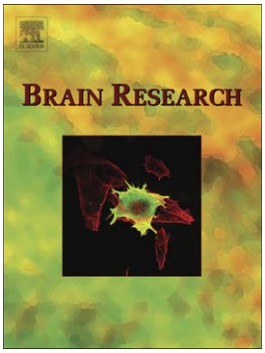

Cyclosporine a attenuates hypoxic-ischemic brain injury in newborn rats

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PII: S0006-8993(10)01829-9
DOI: doi: 10.1016/j.brainres.2010.08.047
Reference: BRES 40684

To appear in: *Brain Research*

Accepted date: 17 August 2010

Please cite this article as: Jong Hee Hwang, Jang Hoon Lee, Kyung-Hoon Lee, Eun Joo Bae, Dong Kyung Sung, Yun Sil Chang, Won Soon Park, Cyclosporine a attenuates hypoxic-ischemic brain injury in newborn rats, *Brain Research* (2010), doi: 10.1016/j.brainres.2010.08.047

[Title Page]

Complete title: **Cyclosporine A attenuates hypoxic-ischemic brain injury in newborn rats**

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Number of text pages of the manuscript (including figures and tables): 23 pages

Number of figures and tables: 6 figures

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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 ($\Delta\Psi$) 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 shame 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 $\Delta\Psi$ using JC-1. In HC, increased cell percentage of JC-1 fluorescence shifted from upper red to lower green, indicating a collapse of $\Delta\Psi$, 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 $\Delta\Psi$, 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 $\Delta\Psi$ 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_2, 92% N_2) by placing them in an airtight chamber partially submerged in a 37□ 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℃ 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 ($\Delta\Psi$) 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*

al., 1998; Park *et al.*, 1998). 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

This work was supported by a Korea Research Foundation grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-521-E00062) and the IN-SUNG Foundation of Medical Research (C-A5-841-1).

References

Akdemir, G., Ergüngör, M.F., Sezer, M., Albayrak, L., Dağlioğlu, E., Kilinç K., 2005. Therapeutic efficacy of intraventricular cyclosporine A and methylprednisolone on a global cerebral ischemia model in rats. Neurol. Res. 27, 827-834.

Borlongan, C.V., Yu, G., Matsukawa, N., Xu, L., Hess, D.C., Sanberg, P.R., Wang, Y., 2005. Acute functional effects of cyclosporine-A and methylprednisolone treatment in adult rats exposed to transient ischemic stroke. Life Sci. 76, 1503-1512.

Chang, Y.S., Park, W.S., Lee, M., Kim, K.S., Shin, S.M., Choi, J.H., 1998. Effect of hyperglycemia on brain cell membrane function and energy metabolism during hypoxia-ischemia in newborn piglets. Brain Res. 798, 271-280.

Crompton, M., Barksby, E., Johnson, N., Capano, M., 2002. Mitochondrial intermembrane junctional complexes and their involvement in cell death. Biochimie. 84, 143-152.

Domanska-Janik, K., Bużanska, L., Dłużniewska, J., Kozłowska, H., Sarnowska, A., Zabłocka, B., 2004. Neuroprotection by cyclosporin A following transient brain ischemia correlates with the inhibition of the early efflux of cytochrome C to cytoplasm. Brain Res. Mol. Brain Res. 121, 50-59.

Friberg, H., Wieloch, T., 2002. Mitochondrial permeability transition in acute neurodegeneration. Biochimie. 84, 241-250.

Gogvadze, V., Richter, C., 1993. Cyclosporine A protects mitochondria in an *in vitro* model of hypoxia/reperfusion injury. FEBS Lett. 333, 334-338.

Hillered, L., Siesjö, B.K., Arfors, K.E., 1984. Mitochondrial response to transient forebrain ischemia and recirculation in the rat. J. Cereb. Blood Flow Metab. 4, 438-446.

Honda, O., Kuroda, M., Joja, I., Asaumi, J., Takeda, Y., Akaki, S., Togami, I., Kanazawa, S., Kawasaki, S., Hiraki, Y., 2000. Assessment of secondary necrosis of Jurkat cells using a new microscopic system and double staining method with annexin V and propidium iodide. Int J Oncol. 16, 283-288.

Kim, B.R., Shim, J.W., Sung, D.K., Kim, S.S., Jeon, G.W., Kim, M.J., Chang, Y.S., Park, W.S., Choi. E.S., 2008. Granulocyte stimulating factor attenuates hypoxic-ischemic brain injury by inhibiting apoptosis in neonatal rats. Yonsei Med J. 49, 836-842.

Leger, P.L., De Paulis, D., Branco, S., Bonnin, P., Couture-Lepetit, E., Baud, O., Renolleau, S., Ovize, M., Gharib, A., Charriaut-Marlangue, C., 2010. Evaluation of cyclosporine A in a stroke model in the immature rat brain. Exp Neurol. Jun 16.

Li, P.A., Kristián, T., He, Q.P., Siesjö, B.K., 2000. Cyclosporin A enhances survival, ameliorates brain damage, and prevents secondary mitochondrial dysfunction after a 30-minute period of transient cerebral ischemia. Exp. Neurol. 165, 153-163.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147-157.

Lorek, A., Takei, Y., Cady, E.B., Wyatt, J.S., Penrice, J., Edwards, A.D., Peebles, D., Wylezinska, M., Owen-Reece, H., Kirkbride, V., Copper, C.E., Aldridge, R.F., Roth, S.C., Brown, G., Delpy, D.T., Reynolds, E.O.R., 1994. Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr. Res. 36, 699-706.

MacGregor, D.G., Avshalumov, M.V., Rice, M.E., 2003. Brain edema induced by *in vitro* ischemia: causal

factors and neuroprotection. J. Neurochem. 85, 1402-1411.

Matsumoto, S., Friberg, H., Ferrand-Drake, M., Wieloch, T., 1999. Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion. J. Cereb. Blood Flow Metab. 19, 736-741.

Matsumoto, S., Isshiki, A., Watanabe, Y., Wieloch, T., 2002. Restricted clinical efficacy of cyclosporine A on rat transient middle cerebral artery occlusion. Life Sci. 72, 591-600.

Mazzeo, A.T., Beat, A., Singh, A., Bullock, M.R., 2009. The role of mitochondrial transition pore, and its modulation, in traumatic brain injury and delayed neurodegeneration after TBI. Exp Neurol. 218;363-370.

Motta, M., Cavazza, A., Migliori, C., Chirico, G., 2003. Autoimmune haemolytic anaemia in a newborn infant. Arch. Dis. Child Fetal Neonatal Ed. 88, F341-F342.

Nakai, A., Kuroda, S., Kristián, T., Siesjö, B.K., 1997. The immunosuppressant drug FK506 ameliorates secondary mitochondrial dysfunction following transient focal cerebral ischemia in the rat. Neurobiol. Dis. 4, 288-300.

Nakai, A., Shibazaki, Y., Taniuchi, Y., Miyake, H., Oya, A., Takeshita, T., 2004. Role of mitochondrial permeability transition in fetal brain damage in rats. Pediatr. Neurol. 30, 247-253.

Park, W.S., Sung, D.K., Kang, S., Koo, S.H., Kim, Y.J., Lee, J.H., Chang, Y.S., Lee, M., 2006a. Therapeutic window for cyclohexamide treatment after hypoxic-ischemic brain injury in neonatal rats. J. Korean Med. Sci. 21, 490-494.

Park, W.S., Sung, D.K., Kang, S., Koo, S.H., Kim, Y.J., Lee, J.H., Chang, Y.S., Lee, M., 2006b. Neuroprotective effect of cyclohexamide on hypoxic-ischemic brain injury in neonatal rats. J. Korean Med. Sci. 21, 337-341.

Park, W.S., Chang, Y.S., Lee, M., 1998. Effect of induced hyperglycemia on brain cell membrane function and energy metabolism during the early phase of experimental meningitis in newborn piglets. Brain Res. 798, 195-203.

Puka-Sundvall, M., Gilland, E., Hagberg, H., 2001. Cerebral hypoxia-ischemia in immature rats: involvement of mitochondrial permeability transition? Dev. Neurosci. 23, 192-197.

Regeur, L., Pakkenberg, B., 1989. Optimizing sampling designs for volume measurements of components of human brain using a stereological metheod. J Microsc. 155; 113-121.

Rehncrona, S., Mela, L., Siesjö, B.K., 1979. Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. Stroke 10, 437-446.

Rice, J.E., 3rd, Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann. Neurol. 9, 131-141.

Robertson, C., Finer, N., 1985. Term infants with hypoxic-ischemic encephalopathy: outcome at 3.5 years. Dev. Med. Child Neurol. 27, 473-484.

Robertson, C., 2004. Mitochondrial dysfunction contributes to cell death following traumatic brain injury in adult and immature animals. J Bioenerg Biomember. 36;363-368.

Seaton, T.A., Cooper, J.M., Schapira, A.H.V., 1998. Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins. Brain Res. 809, 12-17.

Shiga, Y., Onodera, H., Matsuo, Y., Kogure, K., 1992. Cyclosporin A protects against ischemia-reperfusion injury in the brain. Brain Res. 595, 145-148.

Siesjö, B.K., Elmér, E., Janelidze, S., Keep, M., Kristián, T., Ouyang, Y.B., Uchino, H., 1999. Role and

mechanisms of secondary mitochondrial failure. Acta. Neurochir. Suppl. 73, 7-13.

Vannucci, R.C., 2000. Hypoxic-ischemic encephalopathy. Am. J. Perinatol. 17, 113-120.

Vannucci, R.C., Towfighi, J., Vanuucci SJ., 2004. Secondary energy failure after cerebral hypoxia-ischemia in the immature rat J. Cereb. Blood Flow Metab. 24, 1090-1097.

Volpe, J.J., 2001. Hypoxic-ischemic encephalopathy: clinical aspects in unit 3: hypoxic-ischemic encephalopathy, *In*: Volpe, J.J., eds. Neurology of the Newborn, 4th Ed. Philadelphia, PA, U.S.A.: W.B. Saunders, p331-396.

Waldmeier, P.C., Feldtrauer, J.J., Qian, T., Lemasters, J.J., 2002. Inhibition of the mitochondrial permeability transition by the nonimmunosuppressive cyclosporin derivative NIM811, Mol Pharmacol. 62, 22–29.

Wu, L., Shen, F., Lin, L., Zhang, X., Bruce, I.C., Xia, Q., 2006. The neuroprotection conferred by activating the mitochondrial ATP-sensitive K^+ channel is mediated by inhibiting the mitochondrial permeability transition pore. Neurosci. Lett. 402, 184-189.

Yu, G., Hess, D.C., Borlongan, C.V., 2004. Combined cyclosporine-A and methylprednisolone treatment exerts partial and transient neuroprotection against ischemic stroke. Brain Res. 1018, 32-37.

Zuliani. T., Duval, R., Jayat, C., Schnébert, S., André, P., Dumas, M.,Ratinaud, M.H., 2003. Sensitive and reliable JC-1 and TOTO-3 double staining to assess mitochondrial transmembrane potential and plasma membrane integritu: interest for cell death investigations. Cytometry A. 54; 100-108.

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

	NC (n=6)	HC (n=8)	HA (n=10)
Q1 (annexin V^-/PI^+) (%)	0.2±0.2	1.9±0.8 *	0.5±0.4†
Q2 (annexin V^+/PI^+) (%)	0.7±0.0	9.3±4.5*	2.4±2.0*†
Q3 (annexin V^-/PI^-) (%)	89.4±0.0	66.3±9.9*	81.2±6.2*†
Q4 (annexin V^+/PI^-) (%)	9.7±0.0	22.2±5.1*	15.6±3.8*†

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, $^\dagger p < 0.05$ 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, $^\dagger p<0.05$ 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, $^\dagger p<0.05$ 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 $\pm$ standard deviation. $^*p<0.05$ compared to NC, $^\dagger p<0.05$ 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 $\pm$ standard deviation. $^*p<0.05$ compared to NC, $^\dagger p<0.05$ compared to HC.

Fig. 6. Representative photomicrographs of hematoxylin/eosin staining at the anterior commisure (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, $^{\dagger}p<0.05$ compared to HC.

$^{*}p<0.05$, compared with the control group.

$^{\dagger}p < 0.05$, compared with the HI group.

Fig.1.

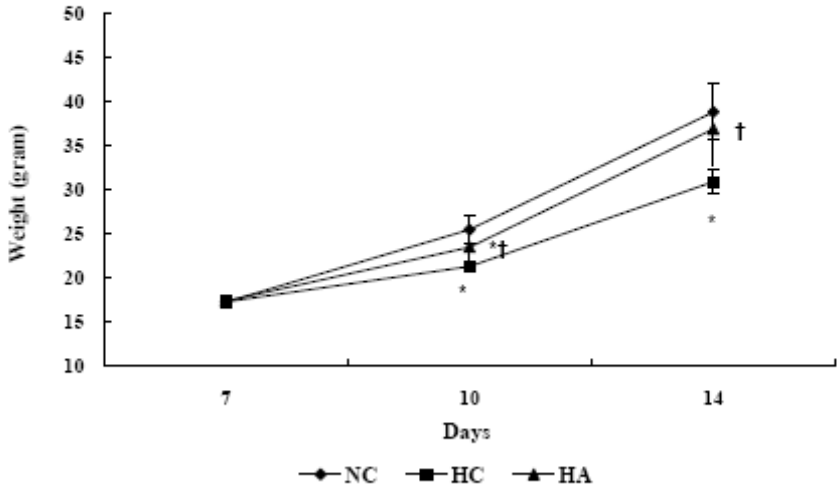
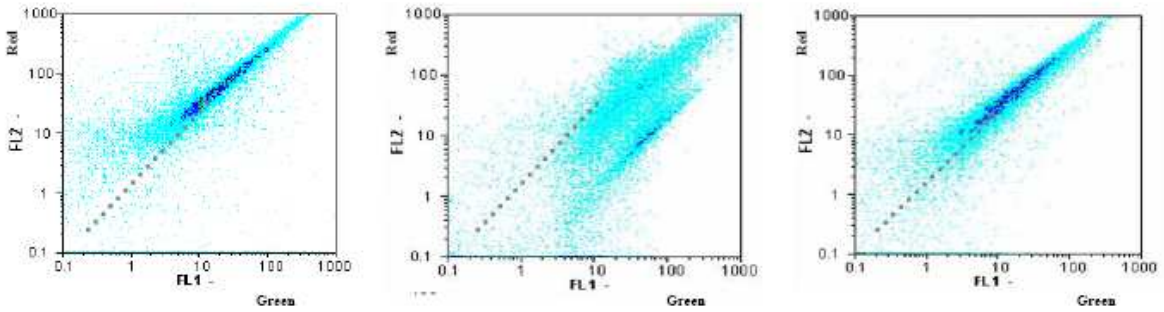


Fig.2.

A.



NC HC HA

B.

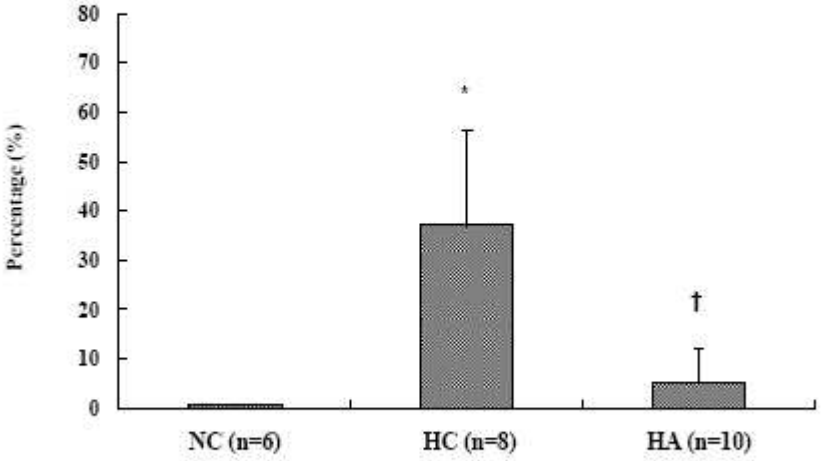
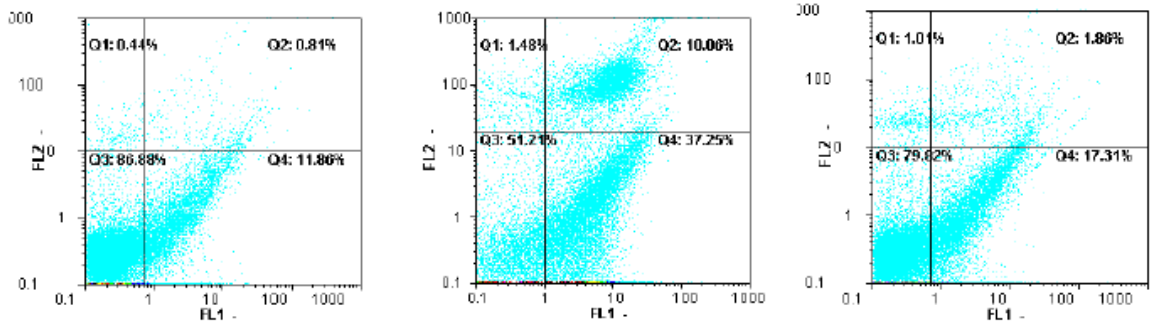


Fig.3.



NC HC HA

Fig.4.

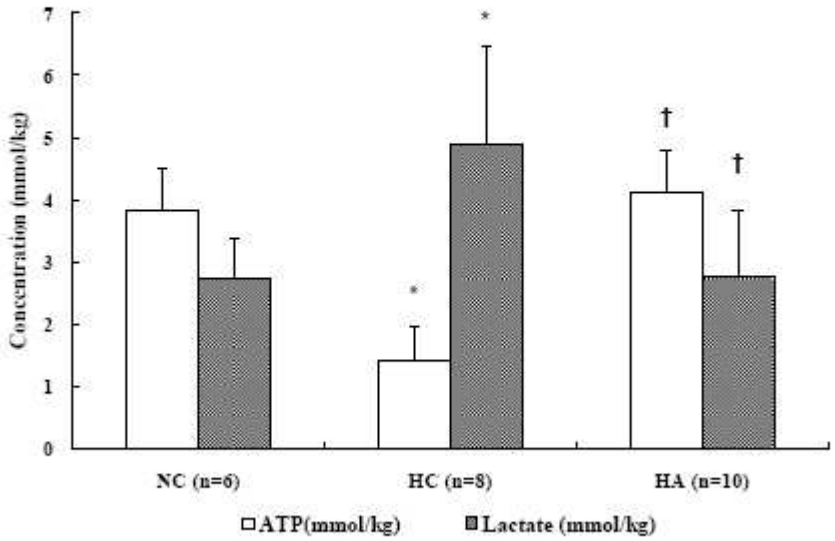


Fig.5.

A.

NC HC HA

B.

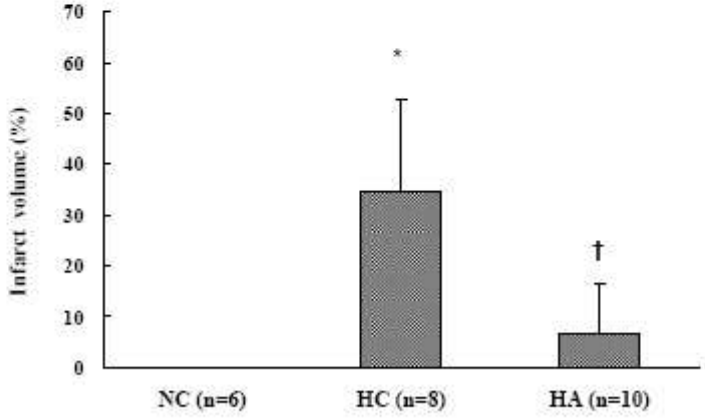


Fig.6.

A.

NC HC HA

B.