

## Neuroprotective effects of geneticin (G418) via apoptosis in perinatal hypoxic-ischemic brain injury

Mi Ju, M.D., Hyun Ju Lee, M.D., Sun Ju Lee, M.D.\*, Eo Su Seo, M.D.<sup>†</sup>  
 Hye Jin Park, M.D., Kye Yang Lee, M.D., Gyeong Hoon Lee, M.D.  
 Eun Jin Choi, M.D., Jin Kyung Kim, M.D., Jong Won Lee, Ph.D.<sup>†</sup>  
 Hai Lee Chung, M.D. and Woo Taek Kim, M.D.

Department of Pediatrics\*, Department of Ophthalmology<sup>†</sup>, School of Medicine, DongGuk University, Kyeong-Ju  
 Department of Pediatrics, Department of Biochemistry<sup>†</sup>, School of Medicine, Catholic University of Daegu, Daegu, Korea

**Purpose :** Some antibiotics were known to exert neuroprotective effects in the animal model of hypoxic-ischemic (H-I) brain injury, but the mechanism is still unclear. A recent study reported that geneticin (G418), an aminoglycoside antibiotic, increased survival of human breast cancer cells by suppressing apoptosis. We investigated the neuroprotective effects of systemically administrated geneticin via anti-apoptosis following the H-I brain injury

**Methods :** Seven-day-old Sprague-Dawley rat pups were subjected to unilateral (left) common carotid artery occlusion followed by 2.5 hours of hypoxic exposure and the cortical cell culture of rat brain was done under a hypoxic incubator. Apoptosis was measured in the injured hemispheres 7 days after H-I insult and in the injured cells from hypoxic chamber using morphologic analysis by Terminal dUTP Nick-end Labeling(TUNEL) assay and immunohistochemistry for caspase-3, and cytologic analysis by western blot and real time PCR for bax, bcl-2, and caspase-3.

**Results :** The gross appearance and hematoxylin and eosin stain revealed increased brain volume in the geneticin-treated animal model of perinatal H-I brain injury. The TUNEL assay revealed decreased apoptotic cells after administration of geneticin in the cell culture model of anoxia. Immunohistochemistry showed decreased caspase-3 expression in geneticin-treated cortical cell culture. Western blot and real-time PCR showed decreased caspase-3 expression and decreased ratio of Bax/Bcl-2 expression in geneticin-treated animal model.

**Conclusion :** Geneticin appears to exert a neuroprotective effect against perinatal H-I brain injury at least via anti-apoptosis. However, more experiments are needed in order to demonstrate the usefulness of geneticin as a preventive and rescue treatment for H-I brain injuries of neonatal brain. (Korean J Pediatr 2008;51:170-180)

**Key Words :** Hypoxic-ischemic brain injury, Anti-apoptosis, Perinatal, Geneticin, G418

### Introduction

Hypoxic-ischemic (H-I) brain injury during the perinatal period remains the single major cause of acute mortality and chronic disability in newborn infants, leading to permanent neurodevelopmental sequelae<sup>1)</sup>. The incidence of asphy-

xia at birth is estimates to be 0.2-0.4% in full-term newborn infants and approaches 60% in preterm infants. About 20-50% of infants suffering from H-I encephalopathy expire during the neonatal period, and 25-30% of the survivors exhibit permanent neurodevelopmental abnormalities such as mental retardation, learning disability, epilepsy, and cerebral palsy<sup>2, 3)</sup>.

H-I brain injury can also result in attention deficit disorders and minimal brain disorder syndromes, and may in addition form the basis for psychiatric and neurodegenerative diseases later in life<sup>4)</sup>. H-I brain injury triggers biochemical events such as energy failure, membrane depolarization,

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책임저자: 김우택, 대구가톨릭대학병원 소아과청소년과

Correspondence: Woo Taek Kim, M.D.

Tel: 053)650-4250 Fax: 053)622-4240

E-mail: woodykim@cu.ac.kr

brain edema, increased neurotransmitter release, increased intracellular  $Ca^{++}$ , increased production of oxygen-free radicals, increased lipid peroxidation, and decreased cerebral blood flow, leading to brain dysfunction and neuronal death<sup>5</sup>). neuronal death begins during the H-I insult and extends into the recovery period following resuscitation. It causes immediate neuronal loss during the insult itself (primary neuronal cell death), after which other mechanisms become operative either immediately after the injury (reactive cell death or reperfusion injury) or hours or days later (delayed neuronal cell death)<sup>6</sup>). The injured brain tissue undergoes selective neuronal necrosis or infarction and the immediate area surrounding the infarct consists of neurons with either necrosis or apoptosis.

The penumbral area appears most amenable to reversal of cellular injury through therapeutic intervention which includes prophylactic therapy during or immediately after the H-I insult and rescue therapy during delayed neuronal death. Although there are experimental therapeutic interventions that may ameliorate perinatal H-I brain injury, currently there is no clinical treatment for this disorder. The inhibition of molecular pathways leading to apoptosis may provide a novel therapeutic opportunity for treatment of perinatal H-I brain injury<sup>2, 3, 7</sup>.

Cell death from perinatal H-I brain injury may result not only the result of classic necrosis but also may involve apoptosis. While necrosis is the dominant mechanism in severe ischemic insults, the prevention of apoptosis may potentially expand the therapeutic window for the treatment of perinatal H-I brain injury. The degradation of cells by apoptosis depends on the activation of caspases, and caspase-3, a widely studied caspase, plays an important role in neuronal cell death after an H-I insult<sup>5, 8</sup>). In addition, the Bcl-2 family of proteins also involves in apoptosis. Pro-apoptotic proteins such as Bax, Bid, and Bak promote cell death, whereas anti-apoptotic proteins such as Bcl-2 and Bcl-xL may enhance cell survival<sup>9</sup>). Recently several antibiotics such as tetracycline derivatives, doxycycline and minocycline, and ceftriaxone have been shown to be neuroprotective in animal models of H-I brain injury. Especially the administration of minocycline either before or after an H-I insult substantially blocked tissue damage by preventing the formation of activated caspase-3<sup>10-14</sup>). A recent study represent that geneticin, an antibiotic of aminoglycosides, made human breast cancer cells survive by suppressing apoptosis, even more resistant to hypoxia<sup>15</sup>.

We investigated a neuroprotective effect of geneticin through anti-apoptosis using in vitro cell culture model of anoxia and in vivo animal model of perinatal H-I brain injury. Apoptosis was identified using morphologic analysis by TUNEL assay and immunohistochemistry for caspase-3, and cytologic analysis by Western blot and real-time PCR for bax, bcl-2, and caspase-3.

## Materials and Methods

### 1. Animal model

This study was performed in accordance with the approved animal use guidelines of the Catholic University of Daegu. The protocol for the newborn animal model of H-I brain injury was based on a modification of the Levine<sup>16</sup> preparation described by Rice et al.<sup>17</sup>. Sprague-Dawley rat pups at postnatal day 7 (P7, day of birth is postnatal day 1) were used to test perinatal H-I brain injury. Eighteen pups were anesthetized with ketamine and underwent permanent unilateral (left) common carotid artery ligation. Following a 1-hour recovery and feeding period, the pups underwent a 2.5-hour period of anoxia (92%  $N_2$ , 8%  $O_2$ ) in an airtight container. The container was submerged in a 37 °C water bath to maintain a constant thermal environment during this period. Following hypoxia, the pups were returned to their dam. The pups received an intraperitoneal injection of 0.1  $\mu$ g/kg of geneticin in the right lower quadrant 30 minutes both before and after placement in the hypoxic chamber. The pups were sacrificed 7 days after H-I insult and brain tissues were immediately removed. The ipsilateral (injured) hemispheres of the animal were separated and prepared, and tissue homogenates were stored at -70°C pending further processing.

### 2. Embryonic cortical neuronal cell culture

The cortical cell culture procedure for rat brain based on the Brewer<sup>18</sup> method was employed. Sprague-Dawley rats pregnant for 18 days were anesthetized with ether for 5 minutes at room temperature and the uteruses were removed. The brains of fetal pups were dissected and brain cortical tissues were digested for 5 minutes at 37°C in Hank's balanced salt solution (HBSS) (Gibco BRL, USA) containing 0.25% trypsin. They were rinsed five times with 5 mL of HBSS containing 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). The cells were removed in 1 mL Hank's solution,

pipetted 6–7 times with a small-bore Pasteur pipette, and dispersed. After cell counting, they were inoculated into Neurobasal plating media supplemented with B27 (Gibco BRL, USA) (100 mL Neurobasal, 2 mL B27 supplement, 0.25 mL glutamax I, 0.1 mL 25 mM glutamate, 0.1 mL 25 mM 2-mercaptoethanol), and cells were plated at a density of about  $2 \times 10^6$  cells/mm<sup>2</sup> in 8 well chamber slides. They were cultured in CO<sub>2</sub> chamber, and 1/5 of the total volume of the culture solutions were replaced every three days with feeding Neurobasal media (Gibco BRL, USA) (100 mL Neurobasal medium, 2 mL B27 supplement, 0.25 mL glutamax I). The cultured cells were divided two groups as the normoxia group and the anoxia group. The normoxia group was cultured in 5% CO<sub>2</sub> incubators and the anoxia group was cultured in 1% O<sub>2</sub> incubators (94% N<sub>2</sub>, 5% CO<sub>2</sub>) for 2–3 days with cell counting. After the determination was made of sufficient cellular injury, cells were divided into geneticin-treated (10 µg/mL) and control groups.

### 3. TUNEL stain

Cultured cells on 8 well chamber slides were rinsed with phosphate buffered solution (PBS) and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature. Afterward, cells were treated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 minutes on ice and rinsed twice with PBS for 2 minutes, then were mixed with labeling solution and enzyme solution from the in situ cell death detection kit (TUNEL kit, Roche, Germany) at 50 µL per sample, and incubated for 1 hour at 37°C. After rinsing three times with PBS for 2 minutes, cells were treated with DAPI (4,6-diamidino-2-phenylindole) (Sigma, USA) (target concentration 1 g/mL) for 10 minutes at room temperature and rinsed with PBS again. The cells were plated with antifading solution and observed using Zeiss Axiophot microscope attached fluorescent viewing device. The TUNEL-stained cells were visualized as green-colored cells using the F09 filter set (BP450-490, FT510, LP 520), and the DAPI-stained cells were seen as blue-colored cells using the F02 filter set (G365, FT 395, LP 420). A triple band pass filter (ex: 400/495/570, beam splitter: 410/505/585, em: 460/530/610) for simultaneous detection of DAPI-stained and TUNEL-stained cells in the same view was used to photograph the cells. The TUNEL-stained cells were detected as cells with marked condensation of chromatin compared to DAPI stain, or as cells with apoptotic bodies as seen with an optic microscope. Cell counts were

done at 600 × magnification at 10 different sites in each chamber. TUNEL-stained cells only were counted as apoptotic, and the total cells count was calculated as the total number of TUNEL-stained cells plus DAPI-stained cells.

### 4. Hematoxylin and eosin (H & E) stain

Histologic studies were performed 7 days after H-I insult. After intracardiac perfusion with saline, the brains were removed, fixed in 4% paraformaldehyde, embedded in paraffin, and prepared for light microscopy. Four µm sections were mounted on glass cover slides. Sections were deparaffinized in xylene for 30 minutes and serially treated with 100% (5 minutes), 96% (10 minutes), and 70% (10 minutes) ethanol. Slides were stained with hematoxylin, rinsed for a few seconds with water to remove excess stain, placed in 1% eosin and then rinsed briefly in water to remove the acid, and washed through the following series: 70%, 95%, and 100% ethanol at 5 minutes each. Finally, the slides were transferred to xylene for clearing, mounted, and covered with cover slips.

### 5. Immunohistochemistry

Immunohistochemistry was performed 7 days after H-I insult. After intracardiac perfusion with saline, the brains were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Eight-micrometer-thick sections were cut from the bregma and mounted on coated slides. Sections were deparaffinized, rehydrated, washed in tris buffered saline (TBS), and microwaved two times for 2 minutes in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by treatment with 3% H<sub>2</sub>O<sub>2</sub>. Sections were permeabilized with 0.3% Triton X-100 (Sigma Chemical Co. USA), blocked in 10% goat serum for 60 minutes at room temperature, and incubated with a primary antibody against caspase-3 (Cell signaling, USA) at 4°C overnight. Sections were next washed twice in TBS for 10 minutes, followed by 1-hour incubation with the biotinylated second antibody (Santa Cruz Biotechnology, USA). After two 10 minute TBS washes, the sections were incubated with the avidin-biotin complex (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) in TBS for 1 hour. The signal was visualized by incubating in 0.06% 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co.) and 0.006% H<sub>2</sub>O<sub>2</sub> in 0.1 M Tris-HCl (pH 7.6). Sections were dehydrated, counterstained with Neutral Red, mounted with Depex (Serva), and covered with cover slips.

## 6. Western Blot

Samples of brain tissue were homogenized and total protein was extracted using a protein lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 0.5% Nonidet P-40, 100 mM phenylmethylsulfonyl fluoride [PMSF], 1 mg/mL leupeptin, 1 mg/mL aprotinin, and 1 M 1,4-dithio-DL-threitol [DTT]). Equivalent aliquots of proteins were boiled in loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 20% glycerol, 4% sodium dodecyl sulfate (SDS), and 0.2% bromophenol blue) for 5 minutes. Samples contain in equal amounts of protein (40  $\mu$ g) were subjected to a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were then electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBS-T buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk for 1 hour at room temperature. Proteins were visualized following incubation with specific primary antibodies of Bcl-2 (Santa Cruz Biotechnology, USA), Bax (Cell signaling, USA) and caspase 3 (Cell signaling, USA) at 4°C overnight. After four washes in a TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated second antibodies (Santa Cruz Biotechnology, USA) for 1 hour at room temperature. Immunoreactivity was detected using enhanced chemiluminescence (ECL). The bands in the exposed films were analyzed by densitometer (Multi-gauge software).

## 7. RNA extraction and Real Time-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen corporation, Carlsbad, CA). Briefly, total tissue was homogenized in 1 ml of TRIzol reagent and total RNA was separated from DNA and proteins by extracting with chloroform and precipitating using isopropanol. The precipitate was washed twice in 75% ethanol, air-dried, and re-diluted in diethylpycocarbonate (DEPC)-treated distilled water. The amount and purity of extracted RNA was quantitated with a spectrophotometry (Backman). The RNA was then stored at -70°C pending further processing. For reverse transcription, 1  $\mu$ g total RNA was reverse transcribed for 1 hour at 37°C in a reaction mixture containing 20 U RNase inhibitor (Promega, Madison, WI), 1 mM dNTP (TaKaRa), 0.5 ng Oligo-(dT) 15 primer (Promega, Madison, WI), 1X RT buffer and 200 U M-MLV reverse transcriptase (Promega, Madison, WI). The reaction mixture was then heated at 95°C for 5

minutes to stop the reaction. The cDNA was then stored at -20°C pending further processing.

Real-time PCR was performed in 48 well PCR plates (MJ mini gradient thermal cycler) using the FINNZYMES DyNAmo SYBR green qPCR kit. Amplification conditions were the same for all apoptotic mRNAs assayed: 95°C for 15 minutes, followed by 40 cycles of 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 45 seconds. Real-time PCR data were analysed with LightCycler software (BIORAD, USA).

The primers used were as follows: caspase-3 sense 5'-AAT TCA AGG GAC GGG TCA TG-3' caspase-3 antisense 5'-GCT TGT GCG CGT ACA GTT TC-3'; Bcl-2 sense 5'-TTG ACG CTC TCC ACA CAC ATG-3' Bcl-2 antisense 5'-GGT GGA GGA ACT CTT CAG GGA-3'; Bax sense 5'-TGC TGA TGG CAA CTT CAA CT-3' Bax antisense 5'-ATG ATG GTT CTG ATC AGC TCG-3'.

## 8. Statistical analysis

Data were analyzed using the SPSS version 12 statistical analysis package. Examined data were assessed using the T-test, GLM (general lineal model), and ANOVA. In each test, the data are expressed as the mean SD and  $P=0.05$  was accepted as statistically significant.

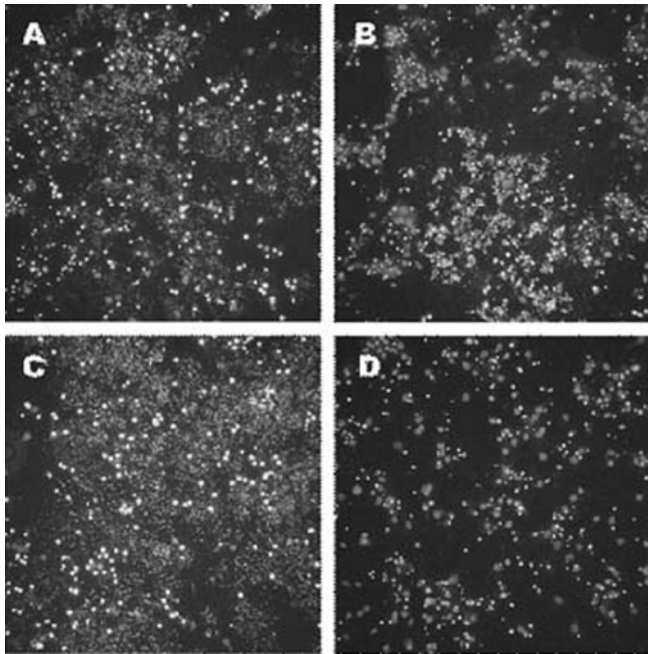
## Results

### 1. Decreased counts of TUNEL-stained apoptotic cells in genetin-treated groups of cortical cell culture from rat brain

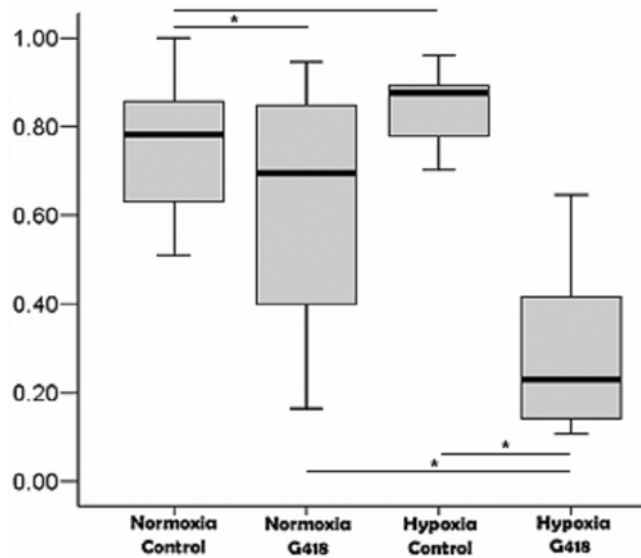
Apoptotic ratio is defined as the ratio of TUNEL-stained cells to the total cells (TUNEL-stained cells plus DAPI-stained cells). Genetin-treated groups in normoxia (B) and anoxia (D) appeared to contain fewer TUNEL-stained cells than were present in control groups in normoxia (A) and anoxia (C) (Fig. 1).

In normoxia, the mean apoptotic ratios of the control and genetin-treated groups were 0.76 (0.16) and 0.62 (0.27), respectively, which is a statistically significant difference between the two groups ( $P<0.01$ ). In anoxia, the mean apoptotic ratios of the control and genetin-treated groups were 0.84 (0.08) and 0.30 (0.20), respectively, also with a statistically significant difference between the two groups ( $P<0.01$ ). In the control group, the mean apoptotic ratio of normoxia was 0.76 (0.16) and was less than the ratio for

anoxia of 0.84 (0.08), but the difference between the two groups in this case was not statistically significant ( $P>0.05$ ). In the geneticin-treated group, the mean apoptotic



**Fig. 1.** TUNEL (Terminal dUTP Nick-end Labeling) assay control-stained with DAPI (4,6-diamidino-2-phenylindole) revealed green-colored cells with TUNEL-positive stain and blue-colored cells with DAPI stain ( $\times 600$ ). (A) for control group in normoxia, (B) for geneticin-treated group in normoxia, (C) for control group in hypoxia, (D) for geneticin-treated group in hypoxia.

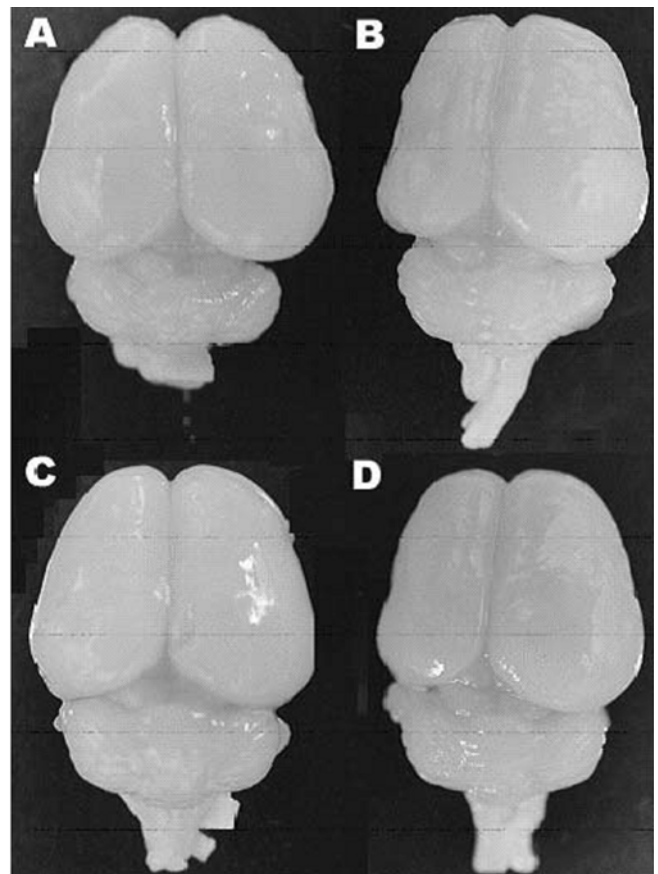


**Fig. 2.** Box analysis revealed control group in normoxia, geneticin-treated group in normoxia, control group in hypoxia, and geneticin-treated group in hypoxia with TUNEL assay.  $P<0.01$ .

ratio for normoxia was 0.62 (0.27) and was less than the ratio for anoxia of 0.30 (0.20), and the difference between the two groups was statistically significant ( $P<0.01$ ) (Fig. 2). Therefore, the TUNEL assay showed decreased ratios of TUNEL-stained apoptotic cells in geneticin-treated cultures in the in vitro cell culture model of anoxia.

## 2. Increased brain volume by gross appearance and H&E stain in the geneticin-treated animal model for perinatal HI brain injury

The gross appearance together with H&E staining of 7-day-old rat brain after H-I insult revealed that brain volume was more greatly decreased in anoxia (B) than in normoxia (A), and that rat brain was better protected in animals treated with geneticin before an HI insult (C) than in animals treated after an H-I insult (D) (Fig. 3, 4).

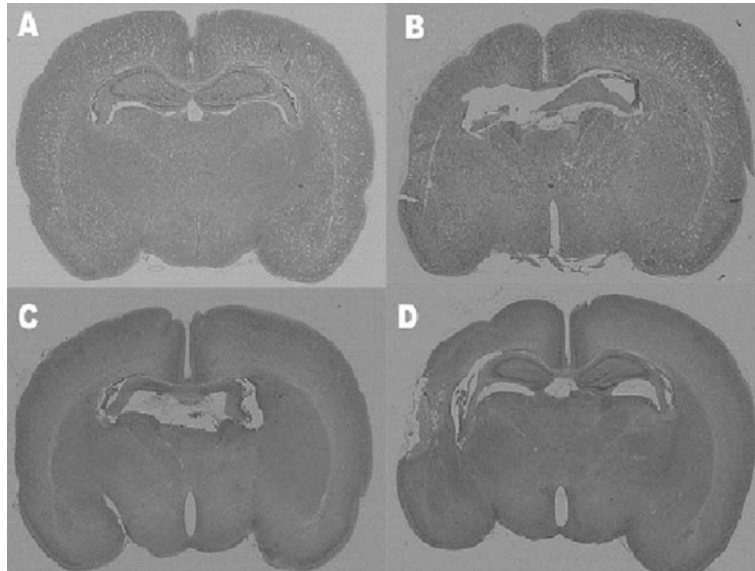


**Fig. 3.** Gross appearance of 7-day-old rat brains revealed: (A) for control group in normoxia, (B) for control group in hypoxia, (C) for geneticin-treated group in hypoxia, (D) for geneticin-treated group after hypoxia.

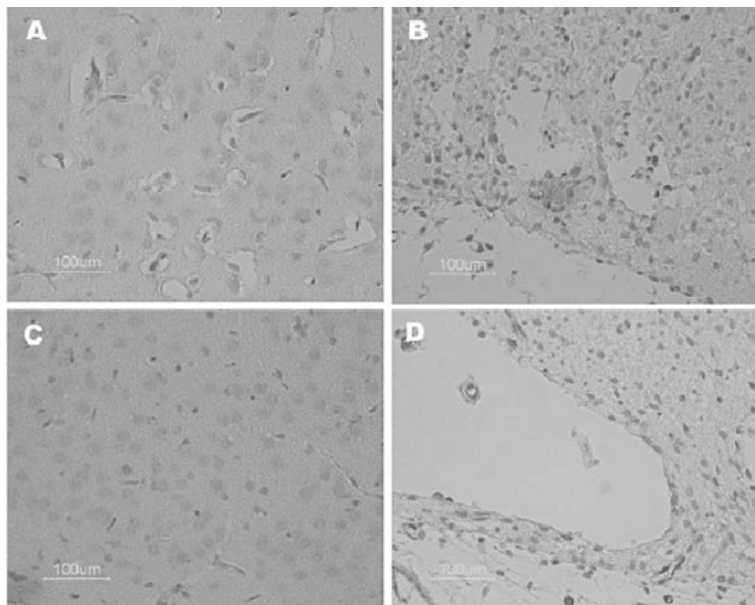
### 3. Decreased expression of Bax by immunohistochemistry in geneticin-treated cortical cells

Immunohistochemistry for caspase-3 seven days after H-I insult revealed that apoptotic cells were more greatly

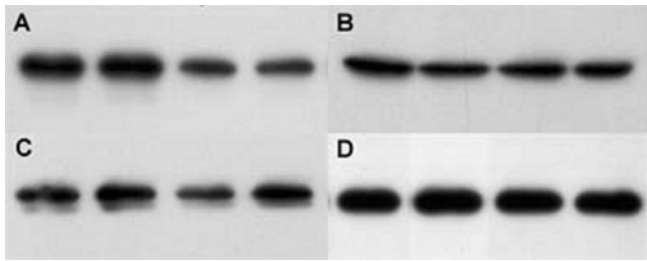
increased in anoxia (B) than in normoxia (A), and more decreased in cells treated with geneticin before an H-I insult (C) than after an H-I insult(D) (Fig. 5).



**Fig. 4.** Hematoxylin and eosin (H&E) stain in coronal sections of 7-day-old rat brains revealed; (A) for control group in normoxia, (B) for control group in hypoxia only, (C) for geneticin-treated group before hypoxia, (D) for geneticin-treated group after hypoxia.



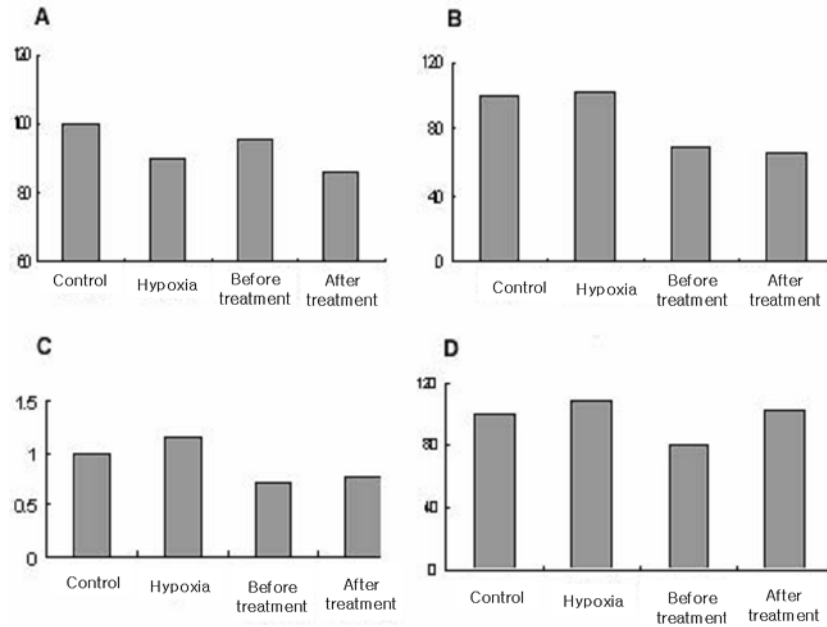
**Fig. 5.** Light microscopic view of immunoreactivity for caspase-3 expressions in rat brains revealed; (A) for control group in normoxia, (B) for control group in hypoxia, (C) for geneticin-treated group before hypoxia, (D) for geneticin-treated group after hypoxia.



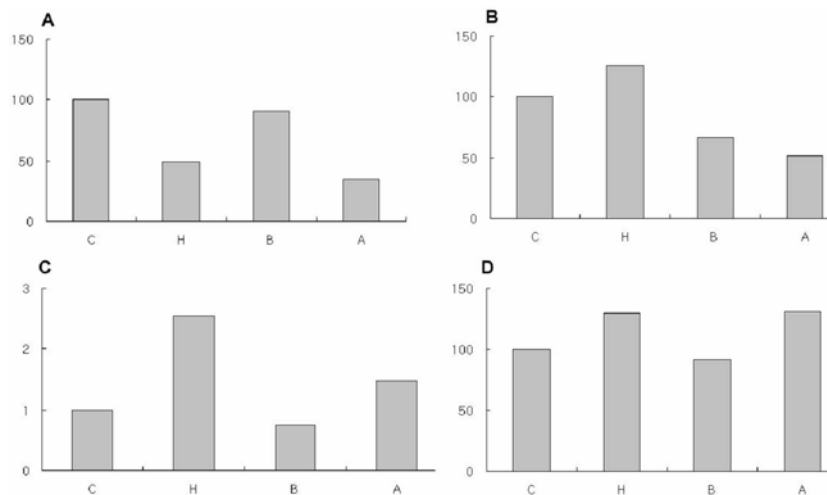
**Fig. 6.** Western blotting revealed; (A) for Bax, (B) for Bcl-2, (C) for caspase-3, (D) for actin.

#### 4. Decreased ratio of Bax/Bcl-2 expression and caspase-3 expression detected by western blotting in the geneticin-treated animal model

The ratio of Bax/Bcl-2 expression and the expression of caspase-3, which are indicators of the level of apoptosis, were more increased in anoxia than in normoxia and more decreased before administration of geneticin than after administration of geneticin (Fig 6, 7).



**Fig. 7.** Histogram of Western blotting revealed; (A) for Bcl-2, (B) for Bax, (C) for Bax/Bcl-2 ratio, (D) for caspase-3.



**Fig. 8.** Histogram of real-time PCR revealed; (A) for Bcl-2, (B) for Bax, (C) for Bax/Bcl-2 ratio, (D) for caspase-3. (C, Control; H, Hypoxia; B, before treatment; A, after treatment)

### 5. Decreased ration of Bax/Bcl-2 expression and caspase-3 expression using real-time PCR in the geneticin-treated animal model

The ratio of Bax/Bcl-2 expression and the expression of caspase-3 were more increased in anoxia than in normoxia, and more decreased before administration of geneticin than after administration of geneticin (Fig. 8).

## Discussions

The antibiotic geneticin, also known as G418 sulfate, is a water-soluble aminoglycosides. It is structurally related to gentamycin but has inhibitory activity against a much wider variety of prokaryotic and eukaryotic organisms, including protozoa and helminths. It is used most extensively in transfection experiments to select eukaryotic cells that have acquired neomycin resistance genes, although its mechanism of action is still elusive. It is not normally used as a standard antibiotic due to its toxicity. It is known to be toxic to bacteria, yeast, protozoa, helminthes, and mammalian cells<sup>19, 21</sup>. A recent study reported that geneticin increased survival of human breast cancer cells by suppressing apoptosis, and that it also made these cells more resistant to hypoxia<sup>15</sup>. Some antibiotics such as the tetracycline derivative, doxycycline and the cephalosporin antibiotic, exhibited neuroprotective effects through anti-apoptosis in an animal model of neonatal hypoxia-ischemia<sup>10-14</sup>. Therefore, we investigated the neuroprotective effect of geneticin through anti-apoptosis using an in vitro cell culture model of hypoxia and an in vivo animal model of perinatal H-I brain injury. Apoptosis was identified through morphologic analysis by TUNEL assay and by immunohistochemistry for caspase-3, and through cytologic analysis by Western blotting and real time PCR for bax, bcl-2, and caspase-3.

In the present study, the TUNEL assay revealed decreased TUNEL-staining of apoptotic cells after administration of geneticin in the in vitro cell culture model of hypoxia. Gross appearance and H & E staining of 7-day-old rat brain after H-I insult revealed greater brain volume for geneticin-treated animals than for non-treated animals in the geneticin-treated animal model of perinatal H-I brain injury. Immunohistochemistry revealed decreased expression of caspase-3 in geneticin-treated cortical cells from rat brain. Western blot analysis and real-time PCR revealed a decreased ratio of Bax/Bcl-2 expression and expression of caspase-3 in gene-

ticin-treated animals in the animal model of perinatal H-I brain injury. These results suggest that geneticin may exert neuroprotective effects against perinatal H-I brain injury including at least anti-apoptosis.

H-I encephalopathy in the perinatal period is an important cause of permanent damage to central nervous system (CNS) cells, and may result in neonatal death or be manifested later as cerebral palsy or mental deficiency. Because of high mortality and chronic sequelae, it is very important to prevent or minimize the long-term consequences of perinatal H-I injury<sup>2, 22</sup>. The mechanisms of neuronal injury after an H-I insult remain unclear, but they are thought to include energy depletion, free radical damage, cytokine-mediated inflammatory reactions, excitotoxicity, neurotransmitter alterations, accumulation of extracellular glutamate and activation of glutamate receptors, accumulation of cytosolic calcium, caspase-dependent cell death, and both necrosis and apoptosis<sup>22-24</sup>. Necrosis includes swelling of the cytoplasm and leads to organelle dissolution and rupture of the plasma membranes, allowing the cellular contents to leak out into the extracellular space. Apoptosis, by contrast, is programmed cell death, in which the cell undergoes nuclear and cytoplasmic condensation, and apoptotic bodies, are phagocytosed by neighboring cells and rapidly degraded<sup>25</sup>. Apoptosis is a delayed event that can be the result of less severe insults and is associated with the activation of caspase.

Caspases are expressed as proenzymes containing three subunits and become activated following proteolytic processing and association of their large and small subunits. Once activated, caspases cleave proteins in a relatively substrate-specific manner, which provides for the morphological changes observed in cells during apoptosis<sup>26</sup>. The activation of caspase-3 can occur via cytokine-mediated receptor activation or via an alteration of the mitochondrial membrane potential. Caspases have been the most common target for intervention in the apoptotic cascade<sup>5</sup>. The Bcl family is also associated with apoptosis via activation of caspase pathway. The Bcl-2 family of proteins, a major cell death regulator, controls directly the mitochondrial membrane permeability. This family consists of anti-apoptotic proteins (Bcl-2, Bcl-xL, and Bcl-w) and pro-apoptotic proteins including multidomain proteins such as Bax and Bak and single-domain BH3-only proteins such as Bid, Bik and Bim<sup>9</sup>. Apoptosis is assayed by several methods morphologically by electron microscopy, H & E or Nissl-staining, TUNEL staining, and DNA-laddering that detect internucle-



osomal cleavage, and cytologic analyses by FACS (fluorescence-activated cell sorter), western blot, and real time PCR of proteins involved in the apoptotic machinery<sup>8, 25, 27, 28</sup>. In the present study, we employed morphologic analyses by TUNEL assay and immunohistochemistry for caspase-3, together with cytologic analyses by western blot and real-time PCR for bax, bcl-2, and caspase-3.

Neurodevelopmental outcome following perinatal H-I brain injury depends substantially on the nature and extent of neuronal destruction and the degree of energy failure<sup>29</sup>. H-I injury may be partially prevented by intervention with neuroprotective agents. Currently known neuroprotective agents and factors include oxygen-free radical inhibitors and scavengers, monosialogangliosides, calcium channel blockers, glucocorticosteroids, phenobarbital, hyperglycemia, carbon dioxide, hypoxic preconditioning<sup>2</sup>, excitatory amino acid antagonists<sup>30</sup>, inhibitors of nitric oxide production<sup>31</sup>, growth factors<sup>23</sup>, hypothermia<sup>32</sup>, inhibitors of caspase activity<sup>8</sup>, edaravone<sup>33</sup>, ceramide<sup>34</sup>, magnesium<sup>35</sup>, several antibiotics<sup>10-14</sup>, and apoptosis inhibitors<sup>8</sup>.

As current neuroprotective strategies are very complicated, it is important to find animal models to help in understanding of the mechanisms leading to cell death after perinatal H-I brain injury and in studying the potential therapies and treatments. The most widely used animal model of perinatal H-I brain injury is a modification of the Levine<sup>16</sup> preparation described by Rice et al.<sup>17</sup> which utilizes a combination of ischemia produced by unilateral occlusion of carotid artery together with hypoxia achieved by the inhalation of 8% oxygen/balanced nitrogen at constant temperature (37°C) in Sprague-Dawley rats at postnatal 7 day. This model of H-I brain injury results in a reproducible pattern of hemispheric injury that is ipsilateral but not contralateral to the carotid ligation. There are prominent features of both apoptosis and necrosis when this model is performed in neonatal rats. The immature rat model has proved especially useful for numerous studies of perinatal H-I brain injury. The 7-day-old rat was originally chosen for study because the neurological events at postnatal day 1 are comparable to those in human fetus between the 32nd to 34th weeks of gestation, i.e., cerebral cortical neuronal layering is complete, the germinal matrix is involuting, and white matter has as yet undergone little myelination. Hence, H-I brain injury in 7-day-old rats can be considered as analogous to perinatal asphyxia in the full-term infants<sup>36</sup>.

Tetracyclines inhibited microglial activation and protected

hippocampal neurons against global brain ischemia in gerbils, even when administered after the H-I insult<sup>10</sup>. Doxycycline was used in a model of focal CNS reperfusion injury in order to determine its efficacy for preserving neurological function, and it attenuated post-ischemic decreases in *in vivo* leukocyte counts and inhibited *in vitro* leukocyte adhesion. Doxycycline has also been suggested to play a role in reducing clinical CNS reperfusion<sup>37</sup>, and has been shown to reduce cleaved caspase-3 and microglial activation in an animal model of neonatal hypoxia-ischemia<sup>14</sup>. Tetracycline derivatives and ceftriaxone, a cephalosporin antibiotic, have been reported to protect neurons against apoptotic death by ionizing radiation in primary cortical cultures<sup>12</sup>. Arvin et al.<sup>13</sup> showed that minocycline administered either immediately before or immediately after H-I insult substantially blocks tissue damage in a rodent model of neonatal H-I brain injury, and they suggested that minocycline treatment prevents both the formation of activated caspase-3, a known effector of apoptosis, and the appearance of a calpain-cleaved substrate that is a marker of excitotoxic/necrotic cell death. Even in normoxia- and hypoxia- induced cortical cell cultures of rat brain, minocycline was found to be neuroprotective via an anti-apoptotic mechanism. Geneticin is an aminoglycoside antibiotic and has also shown neuroprotective properties. Previously we found that geneticin exerts a protective effect against hypoxic injury in rat kidney (unpublished).

The present study demonstrated that geneticin treatment results in decreased numbers of apoptotic cells, greater brain volume, a decreased ratio of Bax/Bcl-2 expression, and decreased expression of caspase-3 in the context of perinatal H-I brain injury.

In conclusion, geneticin appears to exert neuroprotective effects through anti-apoptotic in both *in vitro* and *in vivo* experimental studies of perinatal H-I brain injury. However, more experiments are needed in order to demonstrate the usefulness of geneticin as a preventive and rescue treatment for H-I brain injuries of neonatal brain.

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한 글 요약

주산기 저산소성 허혈성 뇌손상에서 항고사를 통한 geneticin (G418)의 신경보호 효과

동국대학교 의과대학 소아과학교실\*, 안과학교실†  
 대구가톨릭대학교 의과대학 소아과학교실, 생화학교실‡

주 미 · 이현주 · 이선주\* · 서역수† · 박혜진 · 이계향  
 이경훈 · 최은진 · 김진경 · 이종원‡ · 정혜리 · 김우택

**목적 :** 몇몇 항생제가 저산소성 허혈성 뇌 손상에서 뇌 보호 효과를 가진 것으로 밝혀졌지만 아직까지 그 기전에 대해 잘 알려지지 않고 있다. 최근 아미노글루코사이드 계열의 항생제인 G418(geneticin)이 항고사에 의한 암세포 생존을 증가 시키는 것으로 알려졌으며 본 연구는 G418이 주산기 저산소성 허혈성 뇌손상에서 세포 고사를 억제함으로써 뇌 보호 효과를 나타내는 지를 알아보고자 하였다.

**방법 :** 임신 18일된 백서의 대뇌피질 신경세포를 배양하여 정상산소 상태군과 저산소 상태군으로 나누고, 두 군을 각각 대조군과 G418 10 µg/mL으로 처리한 군으로 나누어서 TUNEL 분석과 caspase 3에 대한 면역조직화학검사를 하였고, 생후 7일된 신생 백서의 좌측 총경동맥을 결찰 후 절단하고 저산소 상태를 유도하여 G418을 저산소 상태 유도 전 30분과 유도 후 30분에 복강 내로 0.1 µg/kg 투여하고 7일 후에 희생시켜 H&E 염색과 caspase-3에 대해 Western blot과 real-time PCR를 하였다.

**결과 :** TUNEL 분석상 정상산소 상태군과 저산소 상태군 모두에서 대조군보다 G418 투여군에서 통계학적으로 유의하게 고사세포가 감소되었다( $P < 0.01$ ). Caspase-3에 대한 면역조직화학검사에서 저산소 손상 전에 G418을 투여한 군에서 손상 후에 투여한 군보다 Caspase-3 발현이 적게 나타났다. 저산소 손상 7일 후에 얻은 신생 백서 뇌의 육안적 관찰과 H & E 염색에서 저산소 상태군의 뇌 용적이 정상산소 상태군의 경우보다 감소된 것을 알 수 있었고, 저산소 손상 전에 G418을 투여한 군에서 손상 후에 투여한 군보다 뇌 조직의 손상이 더 적은 것을 볼 수 있었다. Bax/Bcl-2, caspase-3에 대해 Western blot과 real-time PCR 한 경우에 G418 투여한 군에서 Bax/Bcl-2 비율과 caspase-3 발현이 감소되었다.

**결론 :** 주산기 저산소성 허혈성 뇌 손상에서 G418는 뇌 조직의 고사를 억제함으로써 뇌보호 효과를 나타내었다.

References

1) Vannucci RC. Hypoxic-ischemic encephalopathy. Am J Perinatol 2000;17:113-20.  
 2) Vannucci RC, Perlman JM. Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics 1997;100:1004-14.

3) Vannucci RC, Connor JR, Mauger DT, Palmer C, Smith MB, Towfighi J, et al. Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res 1999;55:158-63.  
 4) Weitzdoerfer R, Pollak A, Lubec B. Perinatal asphyxia in the rat has lifelong effects on morphology, cognitive functions, and behavior. Semin Perinatol 2004;28:249-56.  
 5) Calvert JW, Zhang JH. Pathophysiology of an hypoxic-ischemic insult during the perinatal period. Neurol Res 2005; 27:246-60.  
 6) Williams CE, Gunn A, Gluckman PD. Time course of intracellular edema and epileptiform activity following prenatal cerebral ischemia in sheep. Stroke 1991;22:516-21.  
 7) Vannucci RC. Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. Pediatr Res 1990;27:317-26.  
 8) Han BH, Xu D, Choi J, Han Y, Xanthoudakis S, Roy S, et al. Selective, reversible caspase-3 inhibitor is neuroprotective and reveals distinct pathways of cell death after neonatal hypoxic-ischemic brain injury. J Biol Chem 2002;277:30128-36.  
 9) Yin W, Cao G, Johnnides MJ, Signore AP, Luo Y, Hickey RW, et al. TAT-mediated delivery of Bcl-xL protein is neuroprotective against neonatal hypoxic-ischemic brain injury via inhibition of caspases and AIF. Neurobiol Dis 2006;21:358-71.  
 10) Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracycline Inhibit Microglial Activation and Are Neuroprotective in Global Brain Ischemia. Proc Natl Acad Sci USA 1998;22:15769-74.  
 11) Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 1999;96:13496-500.  
 12) Tikka T, Usenius T, Tenhunen M, Keinanen R, Koistinaho J. Tetracycline Derivatives and Ceftriaxone, A Cephalosporin Antibiotic, Protect Neurons Against Apoptosis Induced by Ionizing Radiation. J Neurochem 2001;78:1409-14.  
 13) Arvin KL, Han BH, Du Y, Lin SZ, Raul SM, Holtzman DM. Minocycline Markedly Protects the Neonatal Brain Against Hypoxic-Ischemia Injury. Ann Neurol 2002;52:54-61.  
 14) Jantzie LL, Cheung PY, Todd KG. Doxycycline reduces cleaved caspase-3 and microglial activation in an animal model of neonatal hypoxia-ischemia. J Cereb Blood Flow Metab 2005;25:314-24.  
 15) Lee TS, Park SH, Lee JW, Lim SH. The effect of Geneticin on the survival of a human breast cancer cells under hypoxic condition. J Korean Breast Cancer Soc 2002;5:279-83.  
 16) Levine S. Anoxic-ischemic encephalopathy in rats. Am J Pathol 1960;6:1-17.  
 17) Rice JE 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 1981;9:131-41.  
 18) Brewer GJ. Isolation and culture of adult rat hippocampal neurons. J Neurosci Meth 1997;71:143-55.  
 19) Loebenberg D, Counells M, Waitz JA. Antibiotic G-418, a New Micromonospora-Produced Aminoglycoside with Activity Against Protozoa and Helminths. Antimicrob Agents

- Chemother 1975;7:811-15.
- 20) Eustice DC, Wilhelm JM. Mechanisms of action of aminoglycoside antibiotics in eucaryotic protein synthesis. *Antimicrob Agents Chemother* 1984;26:53-60.
  - 21) Coggan JS, Kovacs I, Thompson SH. The aminoglycoside G418 suppresses muscarinic receptor-activated calcium release in stably transfected murine N1E-115 neuroblastoma cells. *Neurosci Lett* 1994;11:247-50.
  - 22) Volpe JJ. Perinatal brain injury: from pathogenesis to neuroprotection. *Ment Retard Dev Disabil Res Rev* 2001;7:56-64.
  - 23) Johnston BM, Mallard EC, Willians CE, Gluckman PD. Insulin-like growth factor-1 is a potent neuronal rescue agent after hypoxic-ischemic injury in fetal lambs. *J Clin Invest* 1996;97:300-8.
  - 24) Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ. Early Neurodegeneration after Hypoxia-Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death Is Apoptosis. *Neurobiol Dis* 2001;8:207-19.
  - 25) Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev* 1993;14:133-51.
  - 26) Banasiak KJ, Xia Y, Haddad GG. Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol* 2000; 62:215-49.
  - 27) Hill IE, MacManus JP, Rasquinha I, Tuor UI. DNA fragmentation indicative of apoptosis following unilateral cerebral hypoxia-ischemia in the neonatal rat. *Brain Res* 1995;676: 398-403.
  - 28) Nakajima W, Ishida A, Lange MS, Gabrielson KL, Wilson MA, Martin LJ, et al. Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. *J Neurosci* 2000;20:7994-8004.
  - 29) White A. Systemic review of therapy after hypoxic-ischaemic brain injury in the perinatal period. *Semin Neonatol* 2000;5:33-40.
  - 30) McDonald JW, Silverstein FS, Johnston MV. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Res Rev* 1990; 15:41-70.
  - 31) Ishida A, Trescher WH, Lange MS, Johnston MV. Prolonged suppression of brain nitric oxide synthase activity by 7-nitroindazole protects against cerebral hypoxic-ischemic injury in neonatal rat. *Brain Dev* 2001;23:349-54.
  - 32) Thoresen M, Bagenholm R, Loberg EM, Apricena F, Kjellmer I. Post-hypoxic cooling of neonatal rats provides protection against brain injury. *Arch Dis Child Fetal Neonatal Ed* 1996;74:F3-F9
  - 33) Yasuoka N, Nakajima W, Ishida A, Takada G. Neuroprotection of Edaravone on hypoxic-ischemia brain injury in neonatal rats. *Brain Res* 2004;19:129-39.
  - 34) Chen Y, Ginis I, Hallenbeck JM. The protective effect of ceramide in immature rat brain hypoxia-ischemia involves up-regulation of bcl-2 and reduction of TUNEL-positive cells. *J Cereb Blood Flow Metab* 2001;21:34-40.
  - 35) Turkyilmaz C, Turkyilmaz Z, Atalay Y, Soylemezoglu F, Celasun B. Magnesium pre-treatment reduces neuronal apoptosis in newborn rats in hypoxia-ischemia. *Brain Res* 2002;15:133-7.
  - 36) Vannucci RC, Vannucci SJ. Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* 2005;27:81-6.
  - 37) Clark WM, Calcagno FA, Gabler WL, Smith JR, Coull BM. Reduction of Central Nervous System Reperfusion Injury in Rabbits Using Doxycycline treatment. *Stroke* 1994;25:1411-5.