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Neuroprotective effects of resveratrol via anti-apoptosis on hypoxic-ischemic brain injury in neonatal rats

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= Abstract =

Purpose : Resveratrol, extracted from red wine and grapes, has an anti-cancer effect, an antiinflammatory effect, and an antioxidative effect mainly in heart disease and also has neuroprotective effects in the adult animal model. No studies for neuroprotective effects during the neonatal periods have been reported. Therefore, we studied the neuroprotective effect of resveratrol on hypoxic-ischemic brain damage in neonatal rats via anti-apoptosis.

Methods : Embryonic cortical neuronal cell culture of rat brain was performed using pregnant Sprague-Dawley (SD) rats at 18 days of gestation (E18) for the *in vitro* approach. We injured the cells with hypoxia and administered resveratrol (1, 10, and 30 μg/mL) to the cells at 30 minutes before hypoxic insults. In addition, unilateral carotid artery ligation with hypoxia was induced in 7-day-old neonatal rats for the *in vivo* approach. We injected resveratrol (30 mg/kg) intraperitoneally into animal models. Real-time PCR and Western blotting were performed to identify the neuroprotective effects of resveratrol through anti-apoptosis.

Results : In the *in vitro* approach of hypoxia, the expression of Bax, caspase-3, and the ratio of Bax/Bcl-2, indicators of the level of apoptosis, were significantly increased in the hypoxia group compared to the normoxia group. In the case of the resveratrol-treated group, expression was significantly decreased compared to the hypoxia group. And the results in the *in vivo* approach were the same as in the *in vitro* approach.

Conclusion : The present study demonstrates that resveratrol plays neuroprotective role in hypoxic-ischemic brain damage during neonatal periods through the mechanism of anti-apoptosis. **(Korean J Pediatr 2008;51:1102-1111)**

Key Words : Resveratrol, Hypoxic-ischemic brain injury, Neuroprotective, Newborn, Apoptosis

Introduction

Cerebral hypoxic-ischemic (HI) brain injury during the perinatal period is the single most important cause of acute mortality and chronic disability in newborns, with an incidence of 1/4000 live births. About 20-50% of infants suffering from a HI insult will expire during the newborn period, and of the ones that survive 25% will experience some form of long-term consequence such as encephalopathy, motor or mental deficit, learning disability, and/or epilepsy[1]. These can include attention deficit disorders and minimal brain disorder syndromes, and may form the basis for psychiatric and neurodegenerative diseases later in life[2].

HI injury triggers biochemical events such as energy failure, membrane depolarization, brain edema, increased neurotransmitter release, increased intracellular Ca^{2+} concentration, increased production of oxygen-free radicals, increased lipid peroxidation, and decreased cerebral blood flow, leading to brain dysfunction and neuronal death. A number of pharmacological substances have been shown to protect the brain against HI injury. Among these are drugs with anti-platelet, vasodilation, or antioxidant effects. Vasodilators may improve brain perfusion and anti-platelet agents can prevent thrombosis formation. Several lines of evidence suggest roles

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for nitrogen and oxygen free radicals in the pathogenesis of brain HI injury, including evidence that a variety of free radical scavengers and antioxidants are capable of ameliorating HI injury[3]. However, the mechanisms involved in these neuroprotective effects are yet to be fully established. In spite of increased understanding of the mechanisms of cell death that underly neonatal HI brain injury, there is no clinically efficacious treatment available. Therefore, the development of new therapeutic modalities to improve the prognosis for this condition is vitally important[4, 5].

Resveratrol is a powerful antioxidant that is produced by some plants as a defense against environmental stresses. Perhaps the most notable source of resveratrol is the grapevine, which produces large amounts of resveratrol in the skins of grapes to protect against fungal diseases and sun damage[4]. It may be the beneficial agent responsible for the cardioprotective role of red wine well known worldwide as the "French paradox"[6]. Numerous studies have attempted to identify compounds responsible for red wine's apparent health benefits, and the one that has gained by far the largest amount of interest is resveratrol.

Recently, resveratrol has been found to protect kidney, brain, and heart cells from HI injury[5, 7, 8] and a recent report suggests that resveratrol inhibits the mitochondrial steps of the apoptotic process in rat brain that occur after hypoxia-reoxygenation[9].

The aim of the present study was to determine whether resveratrol can reduce cerebral injury subsequent to HI in the developing brain through an anti-apoptotic mechanism, using both *in vitro* (cell culture model of hypoxia) and *in vivo* (animal model of perinatal HI brain injury) approaches.

Materials and Methods

1. Embryonic cortical neuronal cell culture

Embryonic cortical neuronal cell culture of rat brain was performed based on the Brewer method[10]. Pregnant Sprague-Dawley (SD) rats at 18 days of gestation (E18) were anesthetized with ether for 5 minutes at room temperature and the uteruses were removed. The brains of fetal pups were dissected and cortical tissues were digested for 5 minutes at 37℃ in Hanks' Balanced Salt Solution (HBSS) (GibcoBRL, NY, USA) containing 0.25% trypsin. They were rinsed five times with 5 mL of HBSS containing 1 mM sodium pyruvate and 10 mM HEPES buffer (pH 7.4). The cells were removed in 1 mL Hanks' solution and dispersed by pipetting 6-7 times with a small-bore Pasteur pipette. After counting, cells were inoculated into Neurobasal plating medium (GibcoBRL, NY, USA) containing B27 supplement (GibcoBRL, NY, USA) (100 mL Neurobasal, 2 mL B27 supplement, 0.25 mM 2-mercaptoethanol), and cells were plated at a density of about 2×10^6 cells/mm^2 in 10 cm dishes. They were cultured in a CO_2 incubator and the culture medium was replaced every three days with feeding Neurobasal medium (100 mL Neurobasal medium, 2 mL B27 supplement, 0.25 mL glutamax Ⅰ). The cultured cells were divided into five groups, the normoxia group (n=3), the hypoxia group (n=4) and resveratrol-treated (1, 10, 30 μg/mL) groups (each group; n=3). The normoxia cells were cultured in 5% CO2 incubators and the hypoxia cells were cultured in 1% O2 incubators (94% N2, 5% CO_2) for 18 hours. The resveratrol (Sigma, MO, USA) were diluted at 20% ethanol and was administered at 30 minutes before hypoxic insult.

2. Animal protocols

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 HI brain injury was based on a modification of the Levine preparation described by Rice et al[11, 12]. Unilateral carotid artery ligation was induced in 7-day-old SD rat pups (n=18) under ether anesthesia. The neck was incised in the midline, and the left common carotid artery was permanently ligated with 5-0 silk. Total time of surgery never exceeded 5 minutes in any animal. Following surgery, pups were returned to their mothers for recovery and feeding for 1 hour. The pups were then exposed to a 2.5 hours period of hypoxia (8% oxygen, 92% nitrogen) by placing them in an airtight chamber partially submerged in a temperature controlled water bath to maintain the ambient temperature inside the chamber at a constant 37℃. The pups received an intraperitoneal injection of 30 mg/kg of resveratrol in the right lower quadrant both 30 minutes before and 30 minutes after placement in the hypoxic chamber. We supposed that it takes more than 30 minutes to reach the drug to the brain. Afterward, the pups were returned to their dams, sacrificed at 6 hours and 7 days after HI under ketamine, and whole brain tissue was harvested for analysis.

The animals were divided into four groups. In group 1 (normoxia group, n=4), the normal control animals were not exposed to HI insult and in group 2 (hypoxia group, n=4), the

animals were exposed to HI insult. In group 3 (hypoxia + resveratrol-treated group (before), n=5), the animals were injected with resveratrol at 30 minutes before HI. In group 4 (hypoxia + resveratrol-treated group (after), n=5), the animals were injected with resveratrol at 30 minutes after HI.

3. Hematoxylin and eosin (H/E) staining

Histologic studies were performed 7 days after HI 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, 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.

Each slides were taken a photograph and were measured brain area with a densitometer (Multi-gauze software, Fuji Photofilm).

4. RT-PCR and real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, total tissue (or cell material) 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 diethylpyrocarbonate (DEPC)-treated distilled water. The amount and purity of extracted RNA was determined with a spectrophotometer (Beckman, Fullerton, CA, USA). The RNA was then stored at -70℃ pending further processing.

For reverse transcription, 1 μg total RNA was reverse transcribed for 1 hour at 37℃ in a reaction mixture containing 20 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTP, (TaKaRa, Shiga, Japan), 0.5 ng Oligo-(dT) 15 primer (Promega, Madison, WI, USA), 1× RT buffer and 200 U M-MLV reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was then heated at 95℃ for 5 minutes to stop the reaction. The cDNA was stored at -20℃ pending further processing.

Real-time PCR was performed in 48-well PCR plates (Mini Opticon^{TM} Real-Time PCR System, Bio-rad Laboratories, Hercules, CA, USA) using the Finnzymes DyNAmo SYBR green qPCR kit (Finnzymes, Beverly, MA, USA). Amplification conditions are shown in Table 1 and were identical for all apoptotic and oxidant mRNAs assayed: 95℃ for 15 minutes, followed by 40 cycles of 95℃ for 45 seconds, annealing temperature for 45 seconds, and 72℃ for 45 seconds. Real-time PCR data were analyzed with LightCycler software (Bio-rad Laboratories).

5. Western blot analysis

Samples of cortical neuronal cells or 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)). Then, lysates were centrifuged and debris was removed. The protein concentrations of the supernatants were measured using the Bio-rad protein assay (Bio-rad Laboratories), with bovine serum albumin used for standards. After normalizing the concentrations, equal amounts of sample were added to the appropriate amounts of 2x sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl [pH6.8] , 200 mM DTT, 20% glycerol, 4% SDS, and 0.2% bromophenol blue). Samples were boiled for 5 minutes and loaded (40 μg) onto a 12% SDS-polyacrylamide gel.

Electrophoresis was performed and proteins were electrotransferred to polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at a constant voltage of 10 V for 30 minutes. 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 dry milk for 1 hour at room temperature. Proteins were incubated with specific primary antibodies against Bcl-2 (Santa Cruz Biotechnology, Santa

Table 1. Primer Pairs and Annealing Temperatures for Real-time PCR

Primer Name	Primer Sequence (5′-3′)	Annealing Temperature
Bcl-2	F:TTGACGCTCTCCACACACATG R:GGTGGAGGAACTCTTCAGGGA	57℃
Bax	F:TGCTGATGGCAACTTCAACT R:ATGATGGTTCTGATCAGCTCG	55℃
caspase-3	F:AATTCAAGGGACGGGTCATG R:GCTTGTGCGCGTACAGTTTC`	56℃
β-actin	F:TTGCTGATCCACATCTGCTG R:GACAGGATGCAGAAGGAGAT	53℃

Cruz, CA, USA), Bax, caspase-3 (Cell Signaling Technology, Beverly, MA, USA) at 1:1000 dilutions in TBS-T at 4℃ overnight. After four washes in a TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoreactivity was visualized through detection using enhanced chemiluminescence (ECL)-plus western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA). The bands in the exposed films were analyzed with a densitometer (Multi-gauze software, Fuji Photofilm).

6. 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 were expressed as the mean±SD, and $P<0.05$ was accepted as statistically significant.

Results

1. Morphologic recovery from neuronal cell death following hypoxic insult after treatment with resveratrol

The growth of embryonic cortical neuronal cells was observed with phase contrast microscopy (×400). On the third day after plating, most cells had scattered and attached to the plates and developed processes (Fig. 1A). Under hypoxic conditions, viability of the cultured cells decreased progressively by 18 hours after hypoxia (Fig. 1B), with half the neuronal cells having died and the number of suspended cells increased significantly in the culture medium. Morphologically, the damaged cells recovered after treatment with resveratrol on HI injury (Fig. 1C).

2. The expressions of Bcl-2, Bax and caspase-3 mRNAs as measured by real-time PCR from cultured cortical neuronal cells in 18-day-old rat embryos

The expression of Bcl-2 mRNA in the hypoxia group was significantly decreased than in the normoxia group, but recovered with resveratrol, decreasing gradually with increasing concentrations of resveratrol (1, 10 and 30 μg/mL). In contrast, the expressions of both Bax and caspase-3 mRNAs were significantly increased in the hypoxia group than in the normoxia group, again decreasing gradually with increasing concentrations of resveratrol (1, 10 and 30 μg/mL). The ratio of Bax/Bcl-2 expression was greater in hypoxia than in normoxia, decreasing after administration of resveratrol (1, 10 and 30 μg/mL) but without correlation to its concentration (Fig. 2).

3. The expressions of Bcl-2, Bax and caspase-3 in cultured cortical neuronal cells from 18-day-old rat embryos, as indicated by western blotting

The expression of caspase-3 in hypoxia was significantly increased than in normoxia and it was significantly decreased with administration of resveratrol (1 μg/mL). The expression of caspase-3 were decreased with administration of the other concentrations (10 μg/mL and 30 μg/mL), but

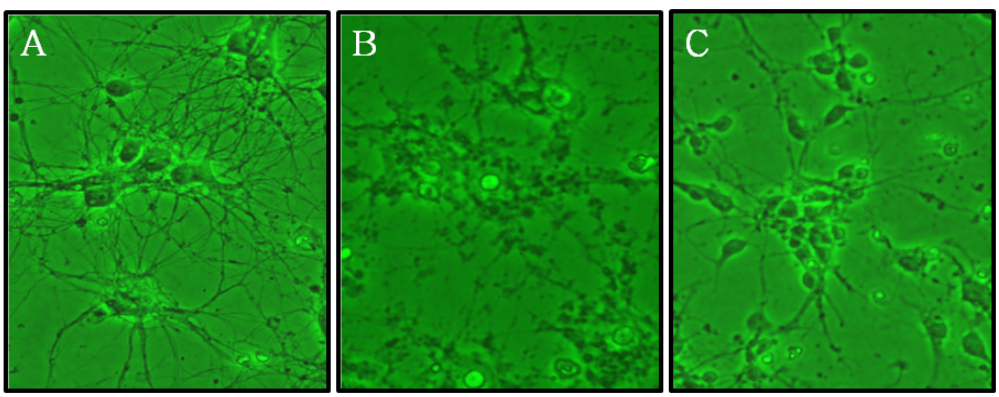

Fig. 1. High magnification (×400) photomicrographs of cultured embryonic cortical neuronal cells from 18-day-old rats (*in vitro*); the normoxia group (A), hypoxia group (B), and resveratrol-treated group (1 μg/mL) before hypoxia (C). Photomicrographs of the resveratrol-treated group (10 and 30 ug/mL) are not shown. A) The nuclear membranes are distinct, and the dendrocyte is distinct. B) The pictures shows cellular swelling, the nuclear shape is indistinct, and the dendrocyte is breaking. C) The cellular shape is generally regular.

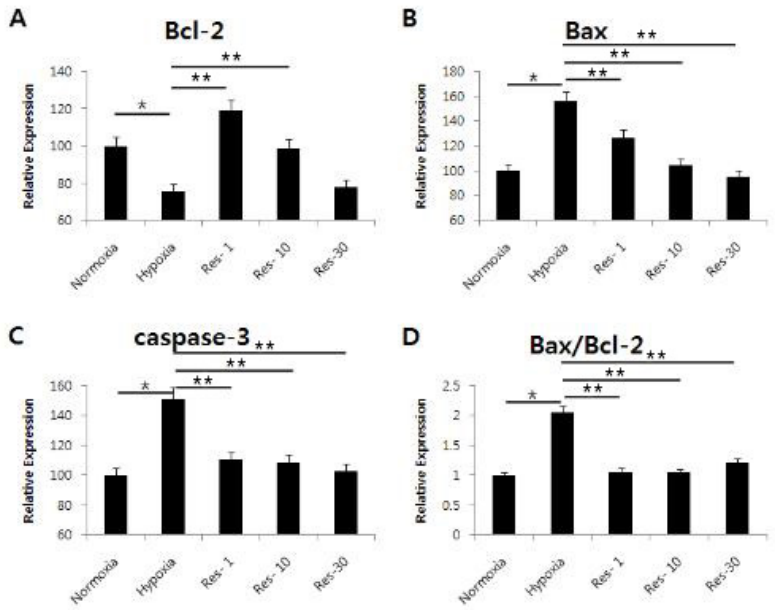

Fig. 2. Real-time PCR for Bcl-2 (A), Bax (B), and caspase-3 (C) from cultured cortical neuronal cells from 18-day-old rat embryos (*in vitro*). The ratio of Bax/Bcl-2 expression (D) is also shown. Resveratrol was administered at 1, 10, and 30 ìg/mL. Data are presented as the ratios of band intensities for normoxia, hypoxia, and resveratrol-treated groups compared to those in the normoxia group. $^{*}P<0.05$ compared with normoxia. $^{**}P<0.05$ compared with hypoxia.

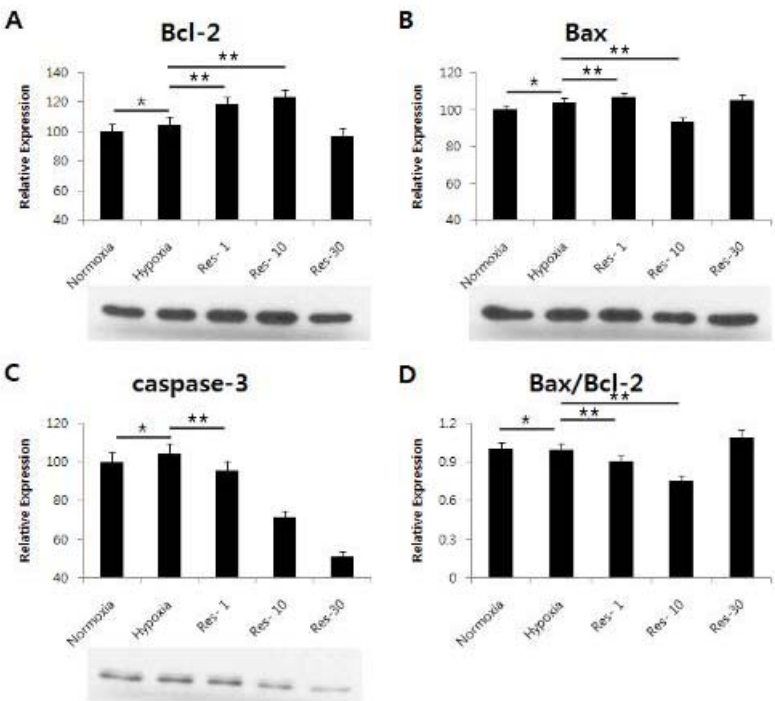

Fig. 3. Western blotting of Bcl-2 (A), Bax (B), and caspase-3 (C) from cultured cortical neuronal cells from 18-day-old rat embryos (*in vitro*). The ratio of Bax/Bcl-2 expression (D) is also shown. Resveratrol was administered at 1, 10, and 30 ìg/mL. Data are presented as the ratios of band intensities for normoxia, hypoxia, and resveratrol-treated groups compared to those in the normoxia group. $^{*}P<0.05$ compared with normoxia. $^{**}P<0.05$ compared with hypoxia.

these values were lower than it in the normoxia group. we considered two results as insignificant (Fig. 3). The expressions of Bcl-2 and Bax and the ratio of Bax/Bcl-2 yielded irregular patterns.

4. H&E staining in the resveratrol-treated animal model for perinatal HI brain injury

H&E stain of 7-day-old rat brain after HI insult revealed that brain area was decreased with hypoxia (B) compared to normoxia (A), and that rat brain was more preserved in the resveratrol-treated group before an HI insult (C) than in the resveratrol-treated group after an HI insult (D) (Fig. 4).

5. The expressions of Bcl-2, Bax and caspase-3 mRNAs as measured by real-time PCR in neonatal HI brain injury of rats

The expression of Bcl-2 mRNA in the hypoxia group was significantly decreased than the normoxia group. The expression of Bcl-2 mRNA in the resveratrol-treated group before HI brain injury was significantly increased compared with the hypoxia group. The expression of Bcl-2 mRNA in the hypoxia group was significantly decreased than the normoxia group. On the contrary the expression of Bax and caspase-3 mRNAs and the ratio of Bax/Bcl-2 expression in the hypoxia group were significantly increased than the nomoxia group. In the both resveratrol-treated groups (before and after HI brain injury), the expressions of Bax and caspase-3 mRNAs and the ratio of Bax/Bcl-2 expression were significantly decreased than the hypoxia group. but these alteration is more distinct in the resveratrol-treated group before HI brain injury. (Fig. 5).

6. The expressions of Bcl-2, Bax and caspase-3 as assayed by Western blotting for neonatal HI brain injury of rats

The expression of Bcl-2 in the hypoxia group was significantly decreased than the normoxia group. The expression of Bcl-2 in the resveratrol-treated group before HI brain injury was significantly increased compared with the hypoxia group. The expression of Bcl-2 in the hypoxia group was significantly decreased than the normoxia group. On the contrary the expression of Bax and caspase-3 and the ratio of Bax/Bcl-2 expression in the hypoxia group were significantly increased than the nomoxia group. In the both resveratrol-treated groups (before and after HI brain injury), the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 expression were significantly decreased than the hypoxia group, but these alteration is more distinct in the resveratrol-treated group before HI brain injury (Fig. 6).

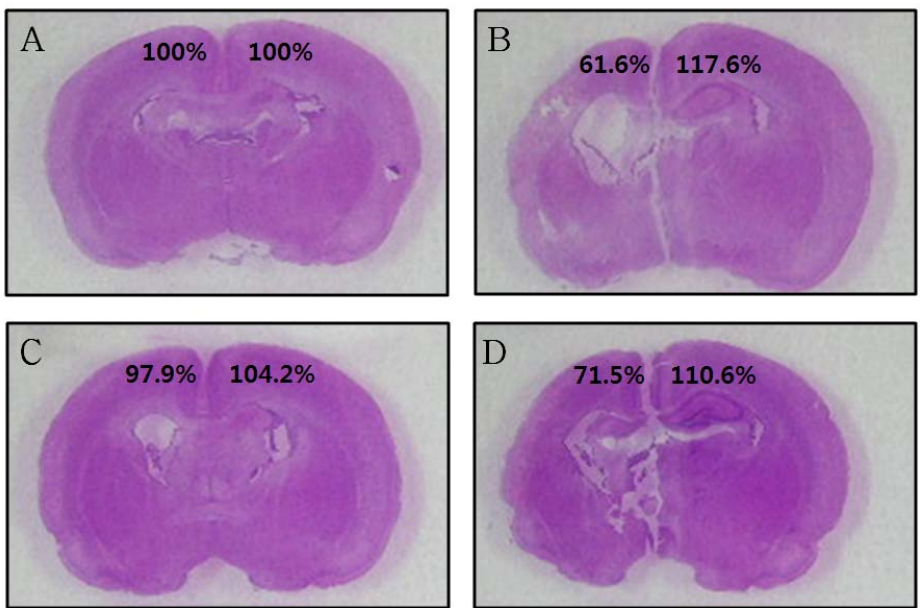

Fig. 4. Hematoxylin and eosin (H&E) staining in coronal sections of 7-day-old rat brains (*in vivo*); control group in normoxia (A), hypoxia only (B), resveratrol-treated group before HI brain injury (C), and resveratrol-treated group after HI brain injury (D). The volumes of damaged brain (left) vs. normal brain (right) are indicated as percentages of the original brain volume.

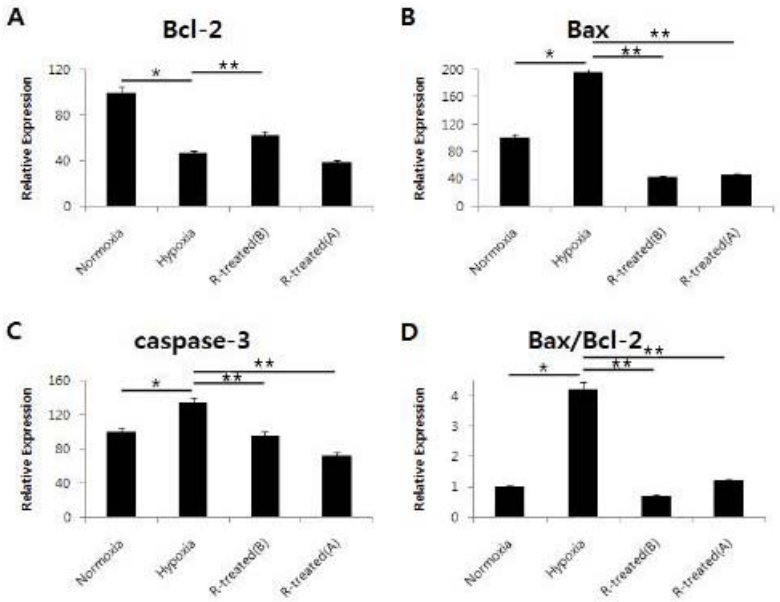

Fig. 5. Real-time PCR for Bcl-2 (A), Bax (B), and caspase-3 (C) at 6 h after HI injury (*in vivo*). The ratio of Bax/Bcl-2 expression (D) is also shown. Resveratrol was administered at 30 mg/kg. R-treated(B), resveratrol-treated group before HI insult; R-treated(A), resveratrol-treated group after HI insult. $*P<0.05$ compared with normoxia. $**P<0.05$ compared with hypoxia.

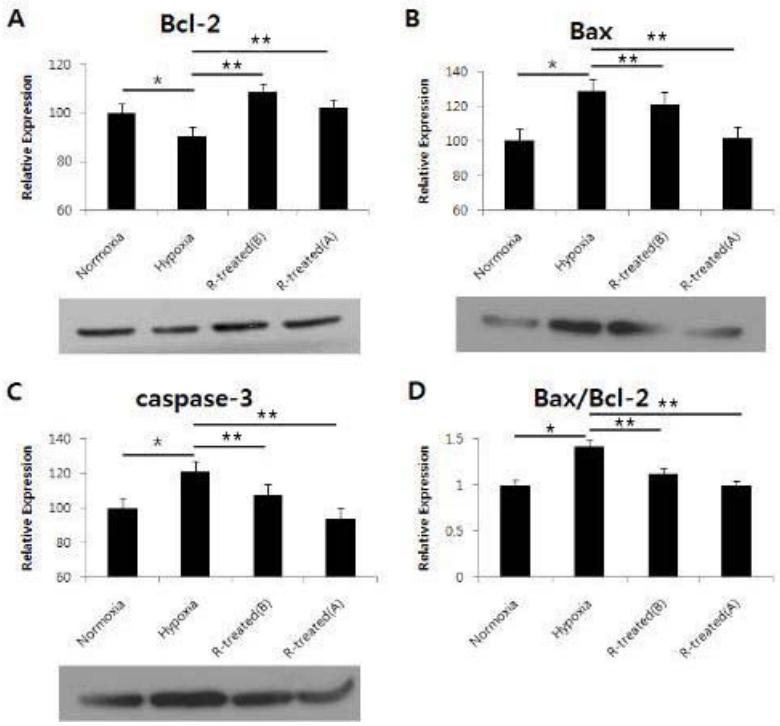

Fig. 6. Western blotting of Bcl-2 (A), Bax (B), and caspase-3 (C) at 6 h after HI injury (*in vivo*). The ratio of Bax/Bcl-2 expression (D) is also shown. Resveratrol was administered at 30 mg/kg. R-treated(B), resveratrol-treated group before HI insult; R- treated(A), resveratrol-treated group after HI insult. $*P<0.05$ compared with normoxia. $**P<0.05$ compared with hypoxia.

Discussion

Perinatal HI brain injury is an important cause of neonatal mortality and permanent neurologic sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy in survivors[13, 14]. Because of the attendant high mortality and chronic sequelae, it is very important to prevent or minimize the long-term consequences of perinatal HI injury[14]. The mechanisms of neuronal injury after an HI 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[15-17].

Cell death plays a central role in the homeostasis of an organ and in disease processes. One form of cell death, wherein the cell undergoes an ordered disassembly followed by death and phagocytosis, is known as programmed cell death or apoptosis[18]. The other form of cell death is necrosis, which result from gross and overt cellular injury causing depletion of cellular energy[19].

The family of caspases are key regulators of the apoptotic signaling pathway[20]. Generally, apoptotic caspases are categorized as initiator (caspase-8, -9) or executioner caspases (caspase-3, -6, -7)[21]. In the apoptotic process, inactive pro-caspases that exist under normal conditions as latent zymogens are cleaved into their active forms by other activated caspases. Activated executioner caspase-3 can cleave additional downstream substrates involved in apoptotic changes[22]. Caspases have been the most common target for intervention in the apoptotic cascade[23]. The Bcl family also is associated with apoptosis via activation of caspase pathway. Pro-apoptotic proteins such as Bax, Bid, and Bak promote cell death, whereas the anti-apoptotic proteins Bcl-2 and Bcl-xL may enhance cell survival[24].

Apoptosis resulting from HI is morphologically different from developmental apoptosis, and many hybrid necrotic-apoptotic phenotypes are seen[25]. From a biochemical perspective, by contrast, HI involves apoptotic processes. Indeed, key apoptotic factors such as caspase-3[26], Bcl-2[27] and Bax[28] are upregulated in pathological situations and are postulated to play prominent roles. From the results of many studies including those described above, it is evident that Bcl-2 is anti-apoptotic in nature, whereas Bax and caspase-3 are pro-apoptotic. Caspase-3, a widely studied caspase, plays an effector role in brain cell death during HI insult. It has also been demonstrated that hypoxia increases expression of Bax and that translocation of cytosolic Bax into mitochondria is an important trigger for the release of mitochondrial cytochrome c into the cytosol[29, 30]. In contrast, Bcl-2 has been demonstrated to prevent cytochrome c release and to mediate anti-apoptotic effects[31, 32].

Apoptosis is thus governed by the familiy of Bcl-2 proteins, with positive and negative regulatory members acting at serial steps along a programmed pathway[33]. Previous observations also indicate that Bcl-2 and Bax play important pathophysiological roles in the protection or acceleration of apoptosis after ischemia and/or reperfusion[34].

Resveratrol is present in grapes and red wine and is considered to be the substance responsible for the lower incidence of coronary heart disease among regular consumers of such wines[35]. In addition, several *in vitro* and *in vivo* studies have attributed this beneficial effect of resveratrol to its potent antioxidant activity[8]. Recently, a direct neuroprotective effect of resveratrol against oxidative stress has been demonstrated in PC12 cells[4].

We studied a neuroprotective effect of resveratrol using an *in vitro* cell culture model of hypoxia and an *in vivo* animal model of neonatal HI brain injury. Apoptosis was assayed by cytologic analysis through Western blotting and real-time PCR for Bcl-2, Bax and caspase-3.

The present study revealed that cells treated with resveratrol were morphologically more preserved than those in the hypoxia group. We found that the expression of the anti-apoptotic factor Bcl-2 was significantly lower in the hypoxia group than in the normoxia group. Thereafter the authors compared the hypoxia group with resveratrol-treated groups to determine the anti-apoptotic effects. The expression of Bcl-2 was significantly increased in resveratrol-treated group than hypoxia group. but there was not dose-dependent relationship. Conversely, the expression of the pro-apoptotic factors Bax and caspase-3 were significantly increased in the hypoxia group than in the normoxia group, but the expression in resveratrol-treated group were significantly increased than in hypoxia group. The ratio of Bax/Bcl-2 expression indicate the activity of apoptosis. The ratio of Bax/Bcl-2 expression was about 1 in nomoxia group, but after hypoxic insult the ratio was significantly increased. In resveratrol-treated group the ratio of Bax/Bcl-2 expression did not change. These results suggest that resveratrol might

exert a neuroprotective effect through an anti-apoptotic mechanism. But there was the limitation of this study, because these results were obvious in real-time PCR analysis but not in Western blotting analysis. we have to study additional details.

Because current neuroprotective strategies are very complicated, it is important to develop animal models to help in understanding the mechanisms leading to cell death following perinatal HI brain injury and in studying potential therapies and treatments. The most widely used animal model of perinatal HI brain injury is a modification of the Levine[11] preparation described by Rice et al[12], which utilizes a combination of ischemia produced by unilateral occlusion of the carotid artery with hypoxia achieved by the inhalation of 8% oxygen/balanced nitrogen at constant temperature (37℃) in SD rats at postnatal 7 days. This model of HI 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 that are observed when this model is employed in neonatal rats. The immature rat model has proven especially useful in numerous studies of perinatal HI 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 fetuses between the 32nd and 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, HI brain injury in 7-day-old rats can be considered as analogous to perinatal asphyxia in full-term infants[1].

The authors studied to determine the effects of resveratrol on HI insult using HI animal models by 7-day-old rats. We identified morphological preservation of brain slides in resveratrol-treated group compared with hypoxia group. In animal models anti-apoptotic factor of hypoxia group was lower and pro-apoptotic factors were significantly increased than nomoxia group. The expression of anti-apoptotic factor Bax in resveratrol-treated groups was increased than hypoxia group and the expression of pro-apoptotic factors in resveratrol-treated groups were significantly decreased than hypoxia group. These alteration was marked in resveratrol-treated group before HI insult. Theses results suggest that resveratrol might exert a neuroprotective effect more than treatment.

In conclusion, the present study demonstrates that resveratrol can regulate the expressions of Bcl-2, Bax and caspase-3 proteins in mitochondria and suppress the mitochondrial death pathway in a model of hypoxic injury in brain cells. These effects are likely to play a role in resveratrol-mediated neuroprotection and may have significant implications for the development of future therapies of HI brain injury in the perinatal period.

한 글 요 약

신생 백서의 저 산소 허혈 뇌손상에서 항세포사멸사를 통한 resveratrol의 신경보호 효과

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신진영 · 서민애 · 최은진 · 김진경
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목 적: Resveratrol은 주로 포도나무의 과실이나 잎 부위에서 추출되는 성분으로, 주로 심질환에서 암 예방 효과, 항염증 효과, 항산화 효과의 기능이 밝혀지고 있다. 최근 성인에 대한 신경보호 효과가 있는 것으로 알려졌지만. 신생아에서 연구는 아직까지 없다. 그래서 본 연구에서는 resveratrol이 신생 백서의 저 산소 허혈 뇌손상에서 신경보호 효과가 있는지를 알아보고자 실험하였다.

방 법: 재태기간 18일된 태아 백서의 대뇌피질 세포를 배양하여 1% O_2 배양기에서 저 산소 상태로 뇌세포손상을 유도하여 저 산소군, 저 산소 30분 전 resveratrol 투여군 (1, 10, 30 μg/mL)으로 나누어 정상 산소군과 비교하였다. 또한, 동물 모델에서는 생후 7일된 백서의 좌측 총 경동맥을 결찰한 후 저 산소 (8% O_2) 상태로 2.5시간 노출시켜서 저 산소 허혈 뇌 손상을 유발하였고, 뇌손상 전후 30분에 resveratrol을 체중 kg당 30 mg을 복막내로 투여하였다. 세포사멸사의 관련을 알아보기 위해 Bcl-2, Bax, caspase-3 primer를 이용한 실시간 중합효소연쇄반응과 동일 항체를 이용한 Western blotting을 시행하였다.

결 과 : 태아 백서 뇌세포 배양 실험에서 저 산소군의 경우 Bcl-2의 발현이 정상 산소군에 비해 감소하였고, Bax의 발현과 caspase-3의 발현, 그리고 Bax/Bcl-2의 비율은 증가하였다. Reaveratrol을 투여한 실험군의 경우에서는 Bcl-2 발현은 증가하였고, Bax의 발현과 caspase-3의 발현, Bax/Bcl-2의 비율은 저 산소군에 비하여 감소하는 결과를 보였다. 또한 이는 저 산소 허혈 뇌손상 동물 모델에서도 같은 결과를 보였다.

결 론 : 본 연구에서 resveratrol은 주산기 저 산소 허혈 뇌손상에서 세포사멸사 작용의 억제를 통하여 신경보호 역할을 하는 것을 알 수 있었다.

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