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ORIGINAL ARTICLE

Neuroprotective Effect of Chunghyuldan (Qing Xue Dan) on Hypoxia-Reoxygenation Induced Damage of Neuroblastoma 2a Cell Lines

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ABSTRACT **Objectives**: Chunghyuldan (CHD), a combinatorial drug that has anti-hyperlipidemic and anti-inflammatory activities, has been shown to reduce infarct volume in a focal ischemia–reperfusion rat model. To explore the molecular basis of CHD's neuroprotective effect, we examined whether CHD shows a cell-protective activity and has a regulatory effect on Bax and/or B-cell leukemia/lymphoma 2 (Bcl-2) expression in mouse neuroblastoma 2a (N2a) cells subjected to hypoxia-reoxygenation (H/R). **Methods**: In order to evaluate the effects of CHD on the cytotoxicity induced from hypoxia or H/R condition, lactate dehydrogenase (LDH) assay was performed. To explore whether the suppression of neural damage when pre-treated with CHD is associated with its anti-apoptotic effect, the CHD effect on the expression of Bcl-2 and Bax was analyzed by Western blotting analysis. **Results**: Cytotoxicity of N2a cell line was slightly increased in 42 h hypoxia condition and dramatically increased under the H/R condition. CHD treatment markedly decreased the cytotoxicity in both conditions ($P<0.01$, $P<0.05$). H/R markedly increased the expression of the pro-apoptotic protein, Bax, but slightly increased the expression of the anti-apoptotic protein, Bcl-2, compared with the normoxia or hypoxia group. CHD significantly decreased Bax expression ($P<0.01$) and slightly decreased Bcl-2 expression ($P>0.05$), resulted in a reduction of Bax/Bcl-2 ratio in N2a cells subjected to H/R. **Conclusion**: CHD has neuroprotective effect in N2a cells subjected to H/R, which might be derived at least in part from its ability to decrease the expression of the pro-apoptotic protein, Bax.

KEYWORDS apoptosis, stroke, Chunghyuldan, herbal

Stroke is a major cause of disability and death worldwide. In Korea, stroke is ranked the second leading cause of death, which has increased to 12% of deaths in 2007.[1] Each year in the United States, about 780,000 people experience stroke. On average, one American has a stroke every 40 s. Currently there are approximately 2 million survivors of strokes living in the U.S. with prolonged disability, many unable to work or resume personal relationships.[2]

Strokes can be subdivided into two categories, ischemic and hemorrhagic. Ischemic strokes are more prevalent than hemorrhagic, making up nearly 60%–87% of all cases, and have been the target of most drug trials.[2] Brain damage following ischemic stroke is caused by reduced blood supply to the brain cells, which drastically limits their access to oxygen and glucose. Oxygen-glucose deprivation (OGD) leads to multiple processes that cause cell death: excitotoxicity, acidotoxicity and ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation and apoptosis.[3] Within the core of the ischemic area, where blood flow is most severely restricted, excitotoxic and necrotic cell death occur within minutes. In the penumbra of the ischemic area, where collateral blood flow can buffer the full effects of a stroke, cell death occurs less rapidly via mechanisms such as apoptosis and inflammation.[4]

Apoptosis is one of the main causes of brain damage after an ischemic stroke. Triggers of apoptosis include oxygen-free radicals, death receptor ligation, DNA damage, protease activation and ionic imbalance. The release of cytochrome c from the outer mitochondrial membrane plays a central role in mediating apoptosis in response to ischemia. The release of cytochrome c is caused by ionic imbalance and mitochondrial swelling or the formation of pores in the outer mitochondrial

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membrane. The complex interplay of the B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins either promotes or prevents pore formation.[5]

Chunghyuldan (Qing Xue Dan in Chinese, CHD) is a combinatorial drug consisting of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Gardeniae Fructus*, and *Rhei Rhizoma*. Previous studies revealed that CHD activated endothelial nitric oxide synthase (eNOS) production[6] and had anti-oxidative and anti-inflammatory[7] properties. In clinical studies, CHD alleviated arterial stiffness[8] and inhibited stroke recurrence.[9] Recently, It markedly reduced infarct volume in a focal ischemia-reperfusion rat.[10] Although there is some evidence mentioned above that CHD provides neuroprotection against ischemia, its cellular and molecular mechanisms underlying the neuroprotective effects have not been fully explained as of yet.

To explore the molecular basis of CHD's neuroprotective effect, we examined whether CHD showed a cell-protective activity and has a regulatory effect on Bax and/or Bcl-2 expression in mouse neuroblastoma 2a (N2a) cells subjected to hypoxia-reoxygenation (H/R).

METHODS

Preparation of CHD

CHD is a capsulated 80% ethanol extract (300 mg per capsule) of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Gardeniae Fructus*, and *Rhei Rhizoma* (in weight ratios of 1:1:1:1). Each herbal medicine was extracted with 80% ethanol in boiling water for 2 h. These extracts were filtered and evaporated in a rotary vacuum evaporator and then finally lyophilized with a freezing dryer. To standardize the quality of CHD, berberine in *Coptidis Rhizoma* and *Phellodendri Cortex*, baicalin in *Scutellariae Radix*, geniposide in *Gardeniae Fructus*, and sennoside A in *Rhei Rhizoma* were quantitatively assayed according to the previous methods.[11]

Cell Culture, H/R and CHD Treatment

Mouse N2a cells were cultured in Dulbecco modified eagle medium (DMEM), 10 U/mL penicillin, 10 mg/mL streptomycin, and 5% fetal bovine serum (FBS) at 37 ℃ in a 5% CO_2 incubator for 24 h (normoxia). To induce hypoxia, a plate with 85% confluenced cells was placed in a humidified incubator at 37 ℃ with an atmosphere of 95% N_2/5% CO_2 for 42 h. And then it was returned to normoxic condition for 12 h (reoxygenation). The CHD powder was resolved in the culture medium and was incubated at 37 ℃ for 2 h. Then, 50–400 μg/mL of CHD was arranged to a plate containing 1×10^5 cells for 2 h prior to hypoxia.

MTT Assay

Cell viability was assessed with the method where 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was converted to insoluble formazan via intracellular enzymatic reduction. N2a cells (1×10^4) were seeded on each well in 96-well plates and cultured for 24 h before the CHD treatment. The cells with or without the CHD treatment were subjected to hypoxia or H/R. The measurement of the cell viability was performed by adding MTT to each well followed by additional incubation for 2.5 h and measurement of the formed formazan. The formed formazan crystal in cells was dissolved in DMSO after removal of the medium and the absorbance at 570 nm was determined using ELISA reader (Emax, USA).

Lactate Dehydrogenase Assay

Cytotoxicity was evaluated by measurement of lactate dehydrogenase (LDH) released into the culture medium during the experimental conditions. N2a cells (1×10^5) were seeded on each well in 24-well plates and cultured for 24 h before the variable amount of CHD was administered. After 2 h, they were subjected to hypoxia or H/R. LDH activity from the medium was determined according to the protocol of an LDH kit (Roche, USA).

Western Blotting

Western blot analysis was performed to measure apoptosis-related proteins, such as Bax or Bcl-2. The protein extract (20 or 40 μg) was separated either on 10% or 12% SDS-polyacrylamide gel and the experimental conditions depended on proteins. The separated protein bands were transferred onto a 0.2 μm polyvinylidene difluoride (PVDF) membrane in a transfer buffer using a semidry transfer apparatus, Trans-BlotSD (Bio-Rad). The membrane was treated with a blocking buffer containing 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween (TBS-T) (20 mmol/L Tris-base, pH 7.6, 137 mmol/L NaCl, 0.1% (v/v) Tween-20 for 1 h at room temperature. The primary antibody was placed on the membranes in blocking solution at 4 ℃ for overnight. The membranes was washed with TBS-T and incubated with a horseradish

peroxidase-labeled secondary antibody (anti-goat IgG or anti-rabbit IgG) in blocking buffer for 1 h at room temperature. The immunoreactive bands were detected based on chemiluminescence using WEST-oneTM (iNtRON Biotechnology). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Abcam, UK).

Statistical Analysis

The data was analyzed using two sample *t*-test. A value of $P<0.05$ was considered significant. All statistical calculation was performed by SPSS for Windows, version 13.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Effects of CHD on Cellular Viability Subjected to Hypoxia or H/R

As shown in Figure 1A, the cell viability was decreased in 42 h hypoxic injury relative to that of 42 h normoxia. When the cells in the presence of 50, 100, 200, 400 μg/mL of CHD were subjected to hypoxia for 42 h, the cell viability was gradually decreased but the extent was not significant. This result indicates that the CHD treatment has minor effects on the viability of the cells in the hypoxic condition. Figure 1B shows the effect of CHD on the cell viability under 42 h hypoxia followed by 12 h reoxygenation. Similar to that of cells in hypoxia, the CHD treatment did not show any significant effect on the viability of the cells under H/R.

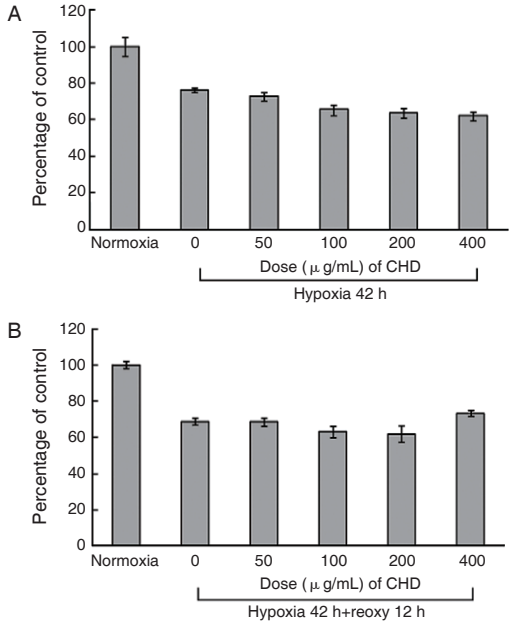

Figure 1. Effects of CHD on Cellular Viability of N2a Cells Subjected to Hypoxia or H/R (n=5, $\bar{x} \pm s$)

Notes: MTT assay of CHD at various concentrations (0, 50, 100, 200, and 400 μg/mL) against N2a cells subjected to 42 h hypoxia (A) or 42 h hypoxia followed by 12 h reoxygenation (B)

Effects of CHD on Neural Damage Subjected to Hypoxia or H/R

Similar to the experiments for MTT assay, the cells were treated with 50, 100, 200, 400 μg/mL of CHD and subjected to hypoxia for 42 h or 42 h hypoxia followed by 12 h reoxygenation (H/R) and the results are shown in Figures 2A and 2B, respectively. Cytotoxicity of N2a cell line was slightly increased in the 42 h hypoxia condition and dramatically increased under the H/R condition. However, the CHD treatment markedly decreased the cytotoxicity in both conditions ($P<0.01$, $P<0.05$).

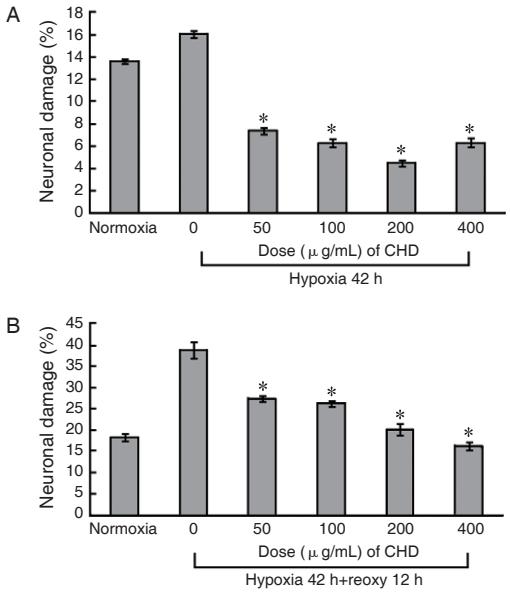

Figure 2. Effects of CHD on Neural Damage of Mouse N2a Cells Subjected to Hypoxia or H/R (n=5, $\bar{x} \pm s$)

Notes: LDH assay of CHD at various concentrations (0, 50, 100, 200, and 400 μg/mL) against N2a cells subjected to 42 h hypoxia (A) or 42 h hypoxia followed by 12 h reoxygenation (B); *$P<0.05$, compared with their untreated controls

Effects of CHD on the Expression of Bcl-2 and Bax

The expression of apoptosis-related proteins in N2a cells subjected to H/R is shown in Figure 3. H/R markedly increased the expression of the pro-apoptotic protein, Bax, but only slightly increased the expression of the anti-apoptotic protein, Bcl-2, compared with the normoxia or hypoxia group. In contrast, CHD significantly decreased Bax expression ($P<0.01$) and slightly decreased Bcl-2 expression ($P>0.05$), resulted in a reduction of Bax/Bcl-2 ratio in the N2a cells subjected to H/R.

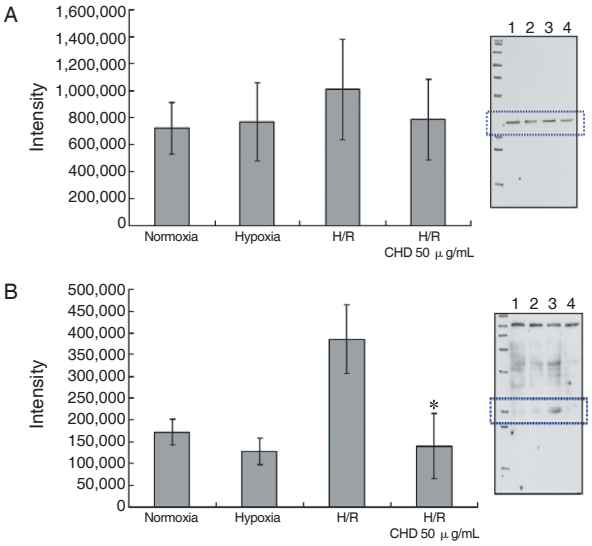

Figure 3. Effects of CHD on the Expression of Apoptosis-Rlated Poteins in Mouse N2a Cells Subjected to H/R (n=3, $\bar{x} \pm s$)

Notes: CHD effect on the expression of Bcl-2 (A) and Bax (B) was analyzed by Western blot analysis. Lane 1: Normoxia; Lane 2: Hypoxia; Lane 3: H/R; Lane 4: H/R with CHD pretreatment. *P<0.05, compared with their untreated controls

DISCUSSION

CHD, a combinatorial drug of Hwangyeonhaedoktang (Huang Lian Jie Du Tang in Chinese, HHDT) and *Rhei Rhizoma*, was developed to treat hyperlipidemia and atherosclerosis.[11] HHDT is a Chinese medicine consisting of four herbs: *Coptidis Rhizoma*, *Scutellariae Radix*, *Phellodendri Cortex*, and *Gardeniae Fructus*. It was reported to increase the cerebral blood flow in the penumbral areas of ischemia, reduce infarction size via reduction of neutrophil infiltration and prevent the progression of atheromatous plaque.[12-14] *Rhei Rhizoma* has strong anti-inflammatory properties,[15] and its constituents have myocardial protective effects due to antioxidant activity.[16] Recently, CHD, the combinatorial drug of HHDT and *Rhei Rhizoma*, is reported to have markedly reduced infarct volume in focal ischemia–reperfusion rat models and also improved the survival rate with edaravone.[17] However, the cellular and molecular mechanisms underlying the neuroprotective effect of CHD have not been fully identified.

In the present study, we demonstrated that CHD could protect neurons from the damage induced by H/R. CHD significantly reduced LDH release in mouse N2a cells subjected to both hypoxia and H/R.

Ischemic neuronal death is roughly categorized into necrosis and apoptosis. Necrosis is acute cell death occurring immediately after ischemic insult, while apoptosis is slowly progressing cell death, which appears in the peri-infarct zone or transient global ischemia. Here, CHD protected neurons from damage subjected to not only H/R but also hypoxia, so it is likely that both necrotic and apoptotic pathways are influenced by CHD. Apoptosis is an active energy-dependent mode of cell death and is regulated by tightly controlled intracellular signaling events. Apoptotic cell death is characterized by a long delay between the insult and the manifestation of major cell damage. Because of this prolonged time window, apoptosis could be a target of medical salvage. So, in this study we investigated whether CHD could influence apoptotic pathways.

There are two apoptotic signaling pathways: intrinsic and extrinsic. Following severe cellular stress such as DNA damage and cell cycle defects, the intrinsic pathway is activated, involving the activation of the proapoptotic members of the B-cell leukemia/lymphoma 2 (Bcl-2) family and the subsequent release of apoptosis inducing factors by the mitochondria. Members of the Bcl-2 family of proteins are key regulators of apoptosis by acting on mitochondria.[17] It is now clear that mitochondria plays a central role in most cases of apoptosis[18] by releasing cytochrome c,[19] and possibly other proteins, from the intermembrane space, and it is clear that adequate cytochrome c can induce apoptotic changes, at least in part by activating caspases.[20,21] There are several pathways by which cytochrome c could be released, including the MPT20. A possible mechanism for release after ischemia is direct interaction of Bax with the outer mitochondrial membrane.[21] Over-expression of Bax induces cytochrome c release from mitochondria in several systems,[22] and recombinant Bax causes release of cytochrome c from isolated mitochondria.[23] Bax is indeed up-regulated in vulnerable cell populations after ischemia. Another frequently occurring feature of apoptotic death is an increase in the ratio of Bax or other pro-apoptotic members of the Bcl family to the anti-apoptotic members of this family, Bcl-2 or Bcl-xL.[21]

This study demonstrated that CHD markedly decreased Bax expression and resulted in a reduction of Bax/Bcl-2 ratio in N2a cells subjected to H/R. This supports the theory that the neuroprotective effects of CHD may result, at least in part, from its regulatory function of the pro-apoptotic protein, Bax. How CHD

regulates proteins associated with apoptosis is not clear at the present time. During H/R injury, return of oxygen to the ischemic tissues is accompanied by an increased production of reactive oxygen species (ROS) in mitochondria, which are involved in the release of cytochrome c, possibly through mechanisms that involve Bcl-2 family proteins, Bcl-2, Bcl-XL, Bax, or Bid.[24] And many antioxidants have been shown to inhibit apoptosis by regulating genes such as Bax and Bcl-2.[25] Based on the report that CHD has anti-oxidative and anti-inflammatory effects,[7] we can speculate that it may regulate apoptosis-related proteins following H/R via antioxidant action.

This study suggests that CHD has neuroprotective effect in N2a cells subjected to H/R, which might be derived at least in part from its ability to decrease the expression of the pro-apoptotic protein, Bax.

Author Disclosure Statement

No competing financial interests exist.

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