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. 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.

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. 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. 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.

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 membrane.
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 antioxidative and anti-inflammatory\(^{(7)}\) properties. In clinical studies, CHD alleviated arterial stiffness\(^{(6)}\) and inhibited stroke recurrence.\(^{(8)}\) Recently, it markedly reduced infarct volume in a focal ischemia-reperfusion rat.\(^{(9)}\) 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.\(^{(10)}\)

**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 °C in a 5% CO₂ incubator for 24 h (normoxia). To induce hypoxia, a plate with 85% confluenced cells was placed in a humidified incubator at 37 °C with an atmosphere of 95% N₂/5% CO₂ 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 °C for 2 h. Then, 50–400 μg/mL of CHD was arranged to a plate containing 1 × 10⁵ 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 × 10⁵) 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 × 10⁵) 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 semidy 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 °C for overnight. The membranes was washed with TBS-T and incubated with a horseradish