Influence of Daejowhan-gamibang on Antioxidative Effects and Apoptosis Induction in Neuronal Cells

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Daejowhan-gamibang(DJG) is used to prevention and treatment of cerebrovascular disease, heart disease, dementia, hyperlipdemia circulatory disturbance. Korean traditional herbal prescriptions and herb medicines in neuronal cells, which have been used for the treatment of stroke and brain diseases in Korean traditional medicine were screened to study the antioxidant effects and its mechanism. Daejowhan-gamibang water extract(DJGWE) was tested on their antioxidant activity using radical scavenging effects against ABTS. It showed significant antioxidant capacities at 50 μg concentration. The antioxidant activity of DJGWE was determined in the different concentration (10 μg, 50 μg, and 100 μg). At the same time, the antiperoxidation effects was determined. Lipid peroxidation in brain homogenates induced by NADPH and ADP-Fe^{2+} was significantly inhibited by DJGWE in vitro. DJGWE showed a potent antioxidant and antiperoxidative activity, further investigation, in vitro and in vivo, will be needed for the confirm of possibility as an antioxidant therapeutic agents and their optimal treatment of brain diseases in human. In searching the mechanism of antioxidant effects of DJGWE, it showed the inhibition of activity of JNK, p38, ERK and caspase 3 induced by hypoxia. So, DJGWE should be surveyed for the use of the potential therapeutic prescription for stroke and brain degenerative diseases such as pakinson's disease, dementia.

Key words : Daejowhan-gamibang(DJG)

Introduction

Daejowhan is generally used to prevention and treatment of cerebrovascular disease, heart disease, dementia, circulatory disturbance[1-3]. Many chronic human diseases, including cancer, diabetes, the neurodegenerative diseases, such as Parkinson's disease, dementia and Alzheimer's disease have been well known which were induced by oxidative damage resulting from an imbalance between production and elimination of various reactive free radical including oxygen species (ROS)[4-7]. Lipid peroxidation is a complex process occurring in all aerobic organisms and reflects the interactions between molecular oxygen and unsaturated fatty acids. Antioxidants, which act as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes. Therefore, they are able to protect the human body from several diseases which are attributed to the reactions of radicals[13-14].

Altered cellular oxidation and impaired cellular function

occur in many neurodegenerative diseases including Alzheimer's and Parkinson's diseases. ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl ($\cdot$OH) are potentially transient chemical species and generated in all aerobic metabolism cells. Hypoxia increased ROS. The cellular response to hypoxia may provide important clues about impaired cellular function and neuronal cell death. ROS have been proposed to act as second messengers in redox-sensitive signal transduction pathways and can damage biomolecules[9]. Oxygen-derived radicals are implicated in lipid peroxidation events, and are critical in neuronal injury after ischemia[9-10]. Antioxidants may have the potential to protect cells from oxidative damage[11].

Various medicinal plants have been reported as therapeutic drugs for free radical pathologies[19-20]. Recently, natural products acts as potent scavengers have been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury. Therefore, in the present study, DJGWE was undertaken to evaluate the antioxidant capacities and antiperoxidation activities and the mechanism of DJGWE which showed significant antioxidant effects. And, also, it was evaluated that the effect of the above-mentioned herbs and

prescriptions on preventing hypoxic or H_2O_2-stressed death of PC12 cells by analyzing lactate dehydrogenase (LDH) release and viability of PC12 cells.

In many cell types, numerous extracellular stimuli regulate growth, differentiation, and apoptosis through activation of protein kinase cascades. Members of the mitogen-activated protein kinase (MAPK) family have been implicated in regulation of cell survival and cell death under numerous conditions[18-20]. Hypoxia causes the activation of several MAPKs: extracellular signal-regulated protein kinase (ERK1/2), c-jun N-terminal kinase (JNK), and p38 MAP kinase signaling pathways[21]. Therefore, we studied whether the protective effect of the prescription, DJGWE as an antioxidant were mediated through the inhibition of MAP kinase and apoptosis pathways in hypoxic neuronal cells.

Materials and methods

1. Prescriptions of Daejowhan-gamibang

The prescription of Palmultang-gamibang which has been used for the extract of sample in this experiment is shown in Table 1.

Table 1. Prescription of Daejowhan-gamibang(DJG)

韓藥名	Herbal Name	Dose (g)
當　歸	Angellicae Gigantis Radix	75.00
川　芎	Rhizoma Cnidii	75.00
生地黃	Rehmanniae Radix	75.00
龜　板	Testudinis Carapax	56.25
杜　冲	Eucommiae Cortex	56.25
天門冬	Asparagi Radix	56.25
黃　柏	Phellodendri Cortex	56.25
牛　膝	Achyranthis Tuber	45.00
麥門冬	Liriopis Tuber	45.00
丹　蔘	Radix salviae Miltiorrhizae	45.00
天　麻	Gastrodiae Rhizoma	45.00
釣鉤藤	Uncariae Ramulus cum Uncuis	45.00
人　蔘	Ginseng Radix	37.50
五味子	Schizandrae Fructus	18.75
大造丸加味方		731.25

2. Chemicals

Ferrous sulfate, 2-thiobarbituric acid (TBA), ADP and NADPH were purchased from Sigma-Aldrich Chem. Co. (St. Louis, USA), and total antioxidant status (TAS) kit was obtained from Randox Lab. (Crumlin, UK). All other chemicals and reagents were the highest grade of commercially available. MAP kinase inhibitors of SB203580(p38 inhibitor), PD98059(ERK inhibitot), and SP600125(JNK inhibitor) were obtained from Calbiochem (USA), 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) and 46-diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Sigma (USA).

3. Preparation of Daejowhan-gamibang water extract (DJGWE)

The air-dried plants of medical herbs were obtained from College of Oriental Medicine, Wonkwang University, and Plant Extract Bank and and also were purchased commercially. One hundred gram of each herb was extracted with 1,000 ml distilled water for 2 hr at 100°C, respectively, and then centrifuged at 3,000 rpm for 20 min. The supernatant was filtered, dried using freeze dryer, stored at -70°C until used.

4. Brain homogenate

Adult male Sprague-Dawley rats were obtained from Samtako Hi-Quality Laboratory Animal Inc. (Osan city, Kyungki Province, Korea). After decapitation of rat, brain was rapidly dissociated and placed on chilled surface. The superficial blood vessels were removed, and washed with ice-cold 50 mM Tris-HCl contained 32 mM sucrose buffer (pH 7.4). The brain was homogenized (200 mg tissue/ml buffer) in ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Potter Elvehjehm Teflon homogenizer. These homogenates were used for the determination of antioxidant and lipid peroxidation.

5. Determination of antioxidant activity in vitro

The antioxidant activity was determined in vitro by means of scavenging of the ABTS(2,2-azino-bis-3-ethyl-benzthiazoline-6-sulphuric acid) radical generated by a metamyoglobin/hydrogen peroxide system as described previously[25]. The test sample (10 $\mu\ell$) was added to a 1 cm pathlength spectrophotometer cuvette (1 ml capacity) containing 20 mM phosphate buffer (pH 7.4), 2.5 μM metamyoglobin. The reaction initiated by addition of 75 μM hydrogen peroxide and the absorbance change at 734 nm monitored at 30°C. The antioxidant status of the herb extract, determined relative to Trolox (a water soluble vitamin E analogue) antioxidant standards, was expressed in terms of mM Trolox equivalent (mM TE). Corresponding samples of medicinal plant extracts for antioxidant activity as above were dried, and the final antioxidant activity was expressed in terms of mmole TE/mg dried extracts.

6. Measurement of thiobarbituric acid reactive substances (TBARS)

Induced lipid peroxidation in brain homogenates was examined by the detection of malondialdehyde (MDA) production in a medium of 1 ml total volume with a protein content of 0.5 mg ml-1. The medium consisted of 25 mM Tris-HCl buffer (pH 7.4), 5 mM ADP, 0.2 mM $FeSO_4$, 1 mM NADPH, plus various concentrations of medicinal plant lyophilizates. The reaction mixture was incubated at 37°C for 30

min, and reaction was terminated by adding 1 ml of thiobarbituric acid reagent (0.375% thiobarbituric acid and 10% acetic acid). The samples were heated in boiling water bath of 98℃. The malondialdehyde (MDA) and related materials formed were measured at 535 nm and quantitated using an extinction coefficient of $1.56×10^5$ M-1 cm-1. For inhibition studies, the media were preincubated with different concentrations of medicinal plant extracts for 5 min before the initiation of the reaction with ADP-Fe^{2+} and NADPH.

7. Cell Culture

Cultured cortical cells were prepared from the cerebral cortices of 2-day-old Sprague-Dawley rats under pentobarbital anesthesia. After the brains were dissected, blood vessels and meninges were removed under a microscope. The cortices were then placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) and minced. Tissue chunks were incubated with papain solution (100 U/ml papain, 0.5 mM EDTA, 0.2 mg/ml cysteine, 1.5 mM $CaCl_2$, and DNase I) at 37℃ for 20 min to dissociate the cells, then heat-inactivated horse serum (HS) was added to terminate the reaction. After cell suspensions were centrifuged at 6,000 × g, the pellets were resuspended in DMEM supplemented with 10% HS. Cells were plated onto poly-D-lysine-coated 35-mm petri dishes at a seeding density of 2-4 × 10^5 cells per well and incubated at 37℃ in a humidified incubator under 5% CO_2. Two hours after plating, the medium was replaced with neurobasal medium containing 0.5 mM glutamine, 25 M glutamate, and B27 supplement. The medium was changed to neurobasal/B27 medium without glutamate after 4 days. Cell cultures were grown for approximately another 10 days before the experiment.

Rat pheochromacytoma (PC12) cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 5% HS, 100 U/ml penicillin and 100 g/ml streptomycin at 37℃ in a humidified incubator under 5% CO_2. Confluent cultures were passaged by trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red), then treated in serum-free medium. In all experiments, cells were treated with antioxidants before hypoxia or H_2O_2-stress for the indicated times. Sesamin or sesamolin were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO added to cells never exceeded 0.1%.

8. Hypoxia

On the day of experiment, culture media were replaced with glucose-free DMEM, then gassed with 85% N_2, 10% H_2, and 5% CO_2 for various time periods in the absence or presence of various doses of extract of herbs and prescriptions as a antioxidants.

9. Preparation of cell extracts

Test medium was removed from culture dishes and cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped off with a rubber policeman, and centrifuged at 200 × g for 10 min at 4℃. The cell pellets were resuspended in an appropriate volume (4 × 10^7 cells/ml) of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 g/ml aprotinin, 10 g/ml leupeptin, and 5 g/ml pepstain A. The suspension was then sonicated. Protein concentration of samples was determined by Bradford assay (Bio-Rad, Hemel, Hempstead, UK) and samples equilibrated to 2 mg/ml with lysis buffer.

10. Western blotting

Protein samples containing 50 g of protein were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated for 1 hr with 5% dry skim milk in TBST buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) to block nonspecific binding, and then incubated with rabbit anti-caspase-3 (1:2;000; Calbiochem, San Diego, CA), and anti-phospho-MAPKs. Subsequently, membranes were incubated with secondary antibody streptavidin-horseradish peroxidase conjugated affinity goat anti-rabbit IgG (Zymed, USA). Caspase-3 and phosphorylated MAPK proteins were detected by chemiluminescence detection system according to the manufacturer's instruction (ECL; Amersham, Berkshire, UK). The band intensity was quantified with a densitometric scanner (PDI, Huntington Station, NY).

11. Reactive oxygen species generation

Intracellular accumulation of ROS was determined with H2DCF-DA. This nonfluorescent compound accumulates within cells upon deacetylation. H2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). PC12 cells were plated in 96-well plates and grown for 24 hr before addition of DMEM plus 10 M H2DCF-DA, incubation for 60 min at 37℃, and treatment with hypoxia or 1 mM H_2O_2 for 60 or 120 min. Cells were then washed twice with room temperature Hank's balanced salt solution (HBSS without phenol red). Cellular fluorescence was monitored on a fluorometer (Moleculat Devices, USA) using an excitation wavelength of 485 nm and emission wavelength of 538 nm.

12. MTT assay for cell viability

Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-

diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are active only in live cells. PC12 cells were preincubated in 24-well plates at a density of 5×10^5 cells per well for 24 hr, then washed with PBS. Cells with various concentrations of sesamin or sesamolin were treated with H_2O_2 or hypoxia for 1 or 2 hr, and grown in 0.5 mg/ml MTT at 37℃. One hour later, 200 l of solubilization solution was added to each well and absorption values read at 540 nm on an automated SpectraMAX 250 (Molecular Devices, USA) microtiter plate reader. Data were expressed as the mean percent of viable cells vs. control.

13. Lactate dehydrogenase release assay

Cytotoxicity was determined by measuring the release of LDH. PC12 cells with various concentrations of sesamin or sesamolin were treated with H_2O_2 or hypoxia for 1 or 2 hr and the supernatant was used to assay LDH activity. The reaction was initiated by mixing 0.1 ml of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 0.2 ml to 96-well plate. The rate of absorbance was read at 490/630 nm on an automated SpectraMAX 250 microtiter plate reader. Data were expressed as the mean percent of viable cells vs. H_2O_2 or hypoxia control.

14. DAPI Staining

Cortical culture was seeded onto a chamber (Nunc, USA) precoated with 1% poly-D-lysine at a density of 2×10^5 cells/well and grown for 24 hr. Cell cultures were exposed to hypoxic conditions for 60 min, then fixed with 4.0% paraformaldehyde in phosphate buffer for 10 min. Cells were then stained with fluorescent 4,6-diamidino-2-phenylindole (DAPI) (1 g/ml in methanol) for 15 min. The number of nuclei in six fields per well was counted with a fluorescent microscope. Cells with condensed nuclei were considered apoptotic[26].

15. Superoxide dismutase and catalase Assays

Catalase activity was assayed by the method of Beers and Sizer (1952) in which the disappearance of the substrate H_2O_2 was measured spectrophotometrically at 240 nm. Total superoxide dismutase (SOD) activity was determined according to the method of Pattichis et al. (1994) based on inhibition of nitrite formation from hydroxylammonium in the presence of O_2^- generators. One unit of SOD is defined as the amount required for 50% inhibition of the initial level of nitrite formation.

16. Statistical analysis

All data were expressed as the mean ± SEM. For single variable comparisons, Student's t test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. P values less than 0.05 were considered significant.

Results

1. Determination of antioxidant activity of extracts in vitro using brain homogenate of rats

In the present study, the water extract of Daejowhan-gamibang (DJGWE) and its herbs were tested for antiperoxidation activity by in vitro assay. The antioxidant capacity in such extracts was determined using the total antioxidant status kit (Randox Labs) against ABTS radical was determined in biological fluid and human plasma/serum for routine use in clinical chemistry analysis has been proposed[27-29], and recently, the assessment of antioxidant capacity in plant extracts and medicinal drugs using this assay method has been applied in recently.[19] The formula of Daejowhan-gamibang and the antioxidant levels of the prescription and several herbs which have been belonged to the prescription are shown in Table 2 of the extracts which has been investigated, the highest level of antioxidant activity was obtained from the water extract of Daejowhan-gamibang. (DJGWE)

Table 2. Antioxidant activity of Daejowhan-gamibang water extract (DJGWE) and its herbs by the measurement of lipid peroxidation in rat brain homogenate

韓藥名	Herbal Name	Dose (g)
當 歸	Angellicae Gigantis Radix	88.5±4.3
川 芎	Rhizoma Cnidii	91.5±5.8
生地黃	Rehmanniae Radix	85.8±5.4
龜 板	Testudinis Carapax	91.7±6.7
杜 冲	Eucommiae Cortex	84.3±5.9
天門冬	Asparagi Radix	88.2±6.3
黃 柏	Phellodendri Cortex	92.4±7.2
牛 膝	Achyranthis Tuber	86.9±6.5
麥門冬	Liriopis Tuber	89.5±6.1
丹 蔘	Radix salviae Miltiorrhizae	88.3±4.1
天 麻	Gastrodiae Rhizoma	92.1±5.2
釣鉤藤	Uncariae Ramulus cum Uncuis	89.3±5.8
人 蔘	Ginseng Radix	84.7±6.2
五味子	Schizandrae Fructus	92.3±6.4
大造丸加味方		82.5±5.3

Data was tested with 50 µg/ml concentration of extracts. The antiperoxidation activity was expressed as MDA formation and the value was represented as the percentage of control without inhibitor, as 100% inhibition.

2. Protective effect of Daejowhan-gamibang water extract (DJGWE) as a antioxidants on the hypoxia-induced neurotoxicity in primary cultured cortical neuronal cells and PC12 cells

Other results showed that LDH activity increased 2, 5, and 30-fold after cultured cortical cells were treated with glucose-free DMEM under hypoxia for 1, 2, or 4 hr. The

neuroprotective effects of antioxidants in hypoxia-stressed PC12 cells and primary neuron culture were investigated under similar condition. Cells were treated with hypoxia alone, with various concentrations of DJG extract (10, 50, 100 $\mu g/m\ell$), or with 10 μM of SB203580, PD98059, and SP600125 (MAP kinase inhibitors) for 2 hr. DJG extract, SB203580, PD98059, and SP600125 (MAP kinase inhibitors) were all effective at protecting PC12 cells from hypoxic damage. The LDH released was decreased 15-35% in the presence of various concentrations of DJG extract and 40-55% by the MAP kinase inhibitors (Fig. 1). Protective effects were observed in hypoxia-treated rat primary cortical neuronal cells (Fig. 2). After 2 hr of hypoxia, 70% cell death in PC12 cells was observed using trypan-blue dye exclusion under a light microscope. Hypoxia-induced cell death was reduced to about 30% by DJG extract a concentration of 50 $\mu g/m\ell$ (data not shown). Chromatin condensation, as an indication of apoptosis, was visualized by the uptake of DNA-binding DAPI fluorochrome. The number of DAPI-stained cells was reduced significantly by DJG extract in hypoxia-treated cortical cell cultures (Fig. 3). These results demonstrated clearly that DJG extract protected PC12 and primary neuronal culture from hypoxia-induced stress.
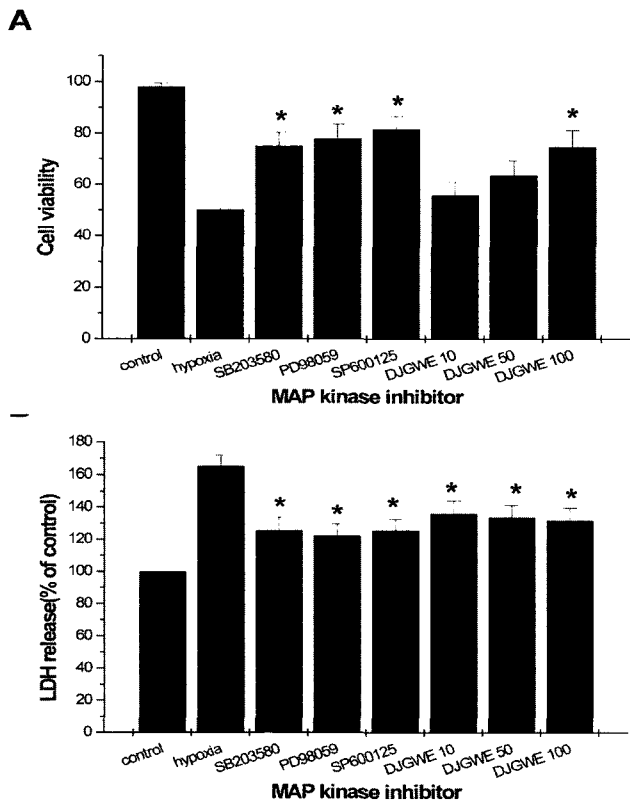

Fig. 1. Antioxidant effect of Daejowhan-gamibang water extract (DJGWE) on the cell viability and cytotoxicity of PC12 cells exposed to hypoxia The effect were measured by the cell viability(A) and LDH release(B). Cells were treated with hypoxia alone, with various concentrations(10, 50, 100 $\mu g/ml$) of DJG extract, or with 10 μM of MAP kinase inhibitors for 2 hour. * p(0.05 as compared to hypoxia group. The present data were expressed as mean±SE which were the % of control.
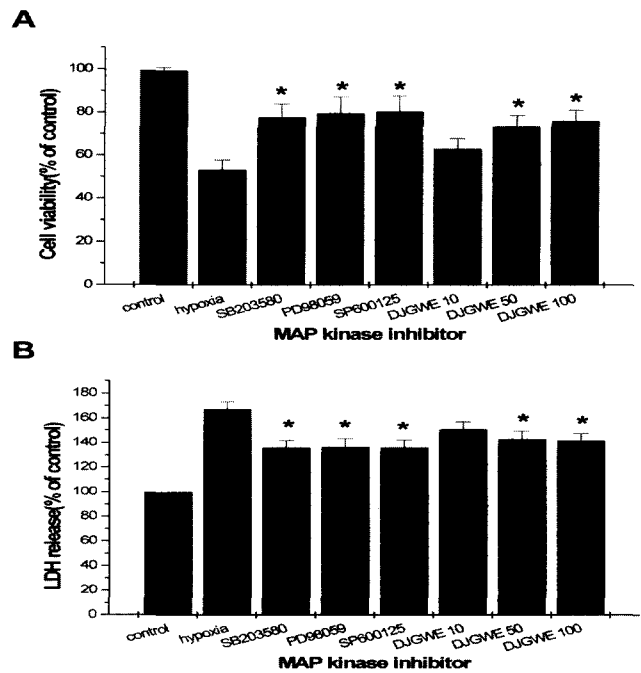

Fig. 2. Protective effect of Daejowhan-gamibang water extract (DJGWE) as an antioxidant on the cultured cortical neuronal cells exposed to hypoxia. The effect were measured by the cell viability(A) and LDH release(B). Cells were treated with hypoxia alone, with various concentrations(10, 50, 100 $\mu g/ml$) of DJGWE, or with 10 μM of MAP kinase inhibitors for 2 hour. * p(0.05 as compared to hypoxia group. The present data were expressed as mean±SE which were the % of control.
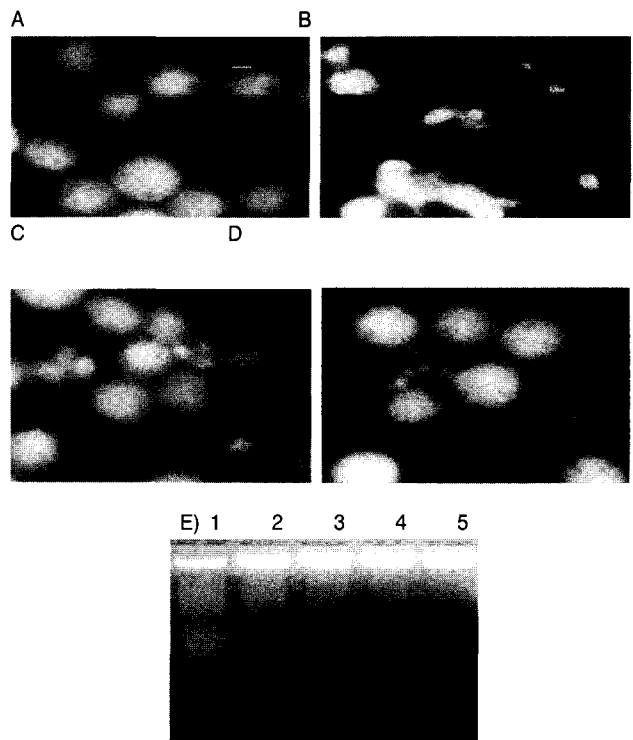

Fig. 3. Apoptotic patterns of cortical neuronal cells exposed to hypoxia by fluorescent DAPI-stain and DNA ladder patterns. A, B were the morphological change of neucleous DNA after the inducement of apoptosis A was control. B, C, D was the features after the treatment of DJGWE 10, 50, 100 $\mu g/ml$ with in the cells exposed to hypoxia. C, DNA ladder assay, was treated with hypoxia(lane 1), H$_2$O$_2$(lane 2) and 10, 50, 100 $\mu g/ml$ of DJGWE (lane 3, 4, 5).

3. Preventive effect of DJGWE on H_2O_2-induced cytotoxicity in PC12 Cells

The ability of DJGWE as a antioxidants to protect cells from oxidative damage was tested further by MTT assay in H_2O_2-stressed PC12 cells. After 2 hr of exposure to 1 mM H_2O_2, different concentrations of DJGWE increased cell viability by about 1-20% when compared to viability in H_2O_2-stressed controls. DJGWE at a concentration of 100 μg/mℓ increased cell viability by about 20% after 2 hr of H_2O_2 treatment. DJGWE reduced cell damage 10-30% in H_2O_2-stressed PC12 cells as measured by LDH release. DJGWE at a concentration of 100 μg /mℓ showed the best protection of cell viability and cytotoxicity in H_2O_2-stressed cells at 2 hr (Fig. 4). In the primary cortical neuronal cells, the results of cell viavility and LDH release was similiar to the results in PC12 cells (Fig. 5).
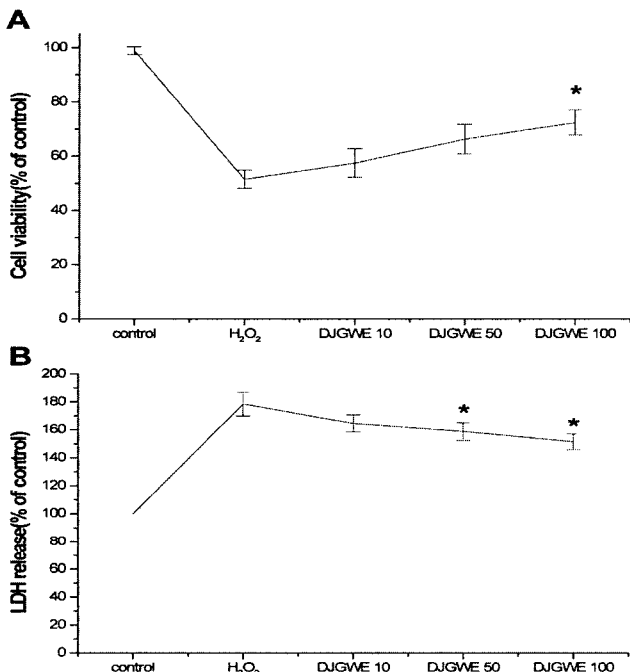

Fig. 4. Antioxidant effect of Daejowhan-gamibang water extract (DJGWE) on the cell viability and cytotoxicity of PC12 cells exposed to H_2O_2-stress. The effect were measured by the cell viability(A) and LDH release(B). Cells were treated with H_2O_2 alone, with various concentrations(10, 50, 100 μg/ml) of DJGWE and H_2O_2 for 2 hour. * p<0.05 as compared to hypoxia group. The present data were expressed as mean±SE which were the % of control.

4. Effect of DJGWE as a antioxidants on the hypoxia-induced ROS in PC12 cells

It was tested whether ROS generation contributed to the effect of hypoxia on PC12 cell death. Hypoxia induced ROS generation in a time-dependent manner. Under hypoxia, ROS generation in PC12 cells increased significantly after 30 min to a maximum at 2 hr (data not showen). DJGWE as a antioxidants diminished ROS generation in a dose-dependent manner in PC12 cells and the cortical neuronal cells of rats (Fig. 6).
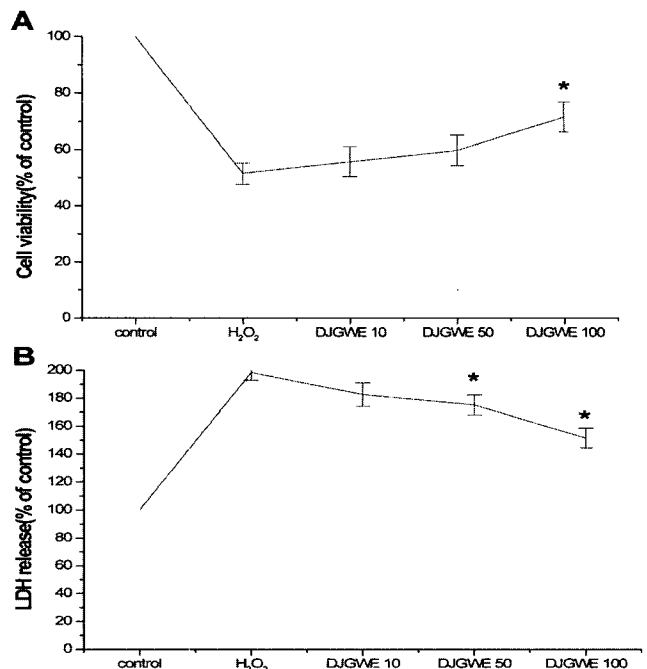

Fig. 5. Protective effect of Daejowhan-gamibang water extract (DJGWE) as an antioxidant on the cultured cortical cells exposed to H_2O_2-stress. The effect were measured by the cell viability(A) and LDH release(B). Cells were treated with H_2O_2 alone, with various concentrations(10, 50, 100 μg/ml) of DJGWE and H_2O_2 for 2 hour. * p<0.05 as compared to hypoxia group. The present data were expressed as mean±SE which were the % of control.
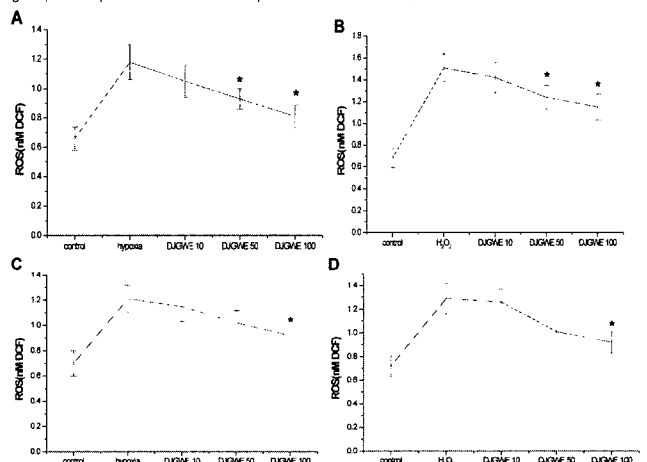

Fig. 6. Effect of Daejowhan-gamibang water extract (DJGWE) on ROS generation in PC12 cells and cortical neuronal cells exposed to hypoxia and H_2O_2-stress. In this data, ROS formation was as DCF formation in cortical neuronal cells and PC12 cells exposed to hypoxia and H_2O_2 stress. The cells, PC12 cells(A, B) and cortical neurons of rat(C, D), were treated with hypoxia or H_2O_2 alone, with various concentrations(10, 50, 100 μg/mℓ) of DJGWE and hypoxia or H_2O_2 for 2 hour. * p<0.05 as compared to hypoxia and H_2O_2 stress group. The present data were expressed as mean±SE.

5. Inhibitory effect of DJG on the hypoxia-induced MAPK and caspase-3 activation

It was found that hypoxia induced MAP kinase activation in cortical cultures, especially activation of ERK MAP kinase (Fig. 7A). MAP kinase inhibitors, SP600125, SB203580, and PD98059(10 μM), were able to suppress ERK MAP kinase in

hypoxic cortical cultures (Fig. 7B). It was further studied that further the effect of DJG extract on MAPKs or apoptosis signaling pathways in PC12 cells stressed by 1 hr hypoxia. Western blot data showed that JNK, ERK, p38 MAPKs, and caspase-3 expression were activated in PC12 cells under hypoxia, however DJG extract at a concentration of 100 μg/mℓ significantly decreased hypoxia-activated JNK, ERK, p38 MAPK, and caspase-3 at 1 hr (Fig. 7C).

of antioxidants in decreasing oxidative damage is believed that contribute to lower the cancer incidence and neurodegenerative diseases[32]. The increasing acceptance of traditional medicines as an alternative form of healthcare, the screening of medicinal plants for biological active compounds is very important. Therefore, this study was designated to investigate whether some herbs and prescriptions have potential antioxidants and antiperoxidants by preliminary in vitro assay screening.
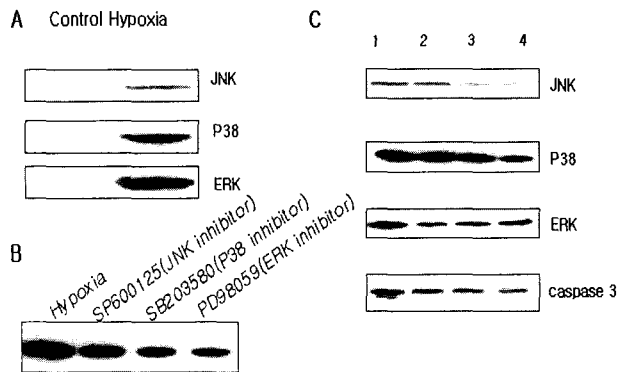

Fig. 7. Antioxidant effect of Daejowhan-gamibang water extract (DJGWE) on MAP kinase and caspase 3 activation on PC 12 cells exposured to hypoxia. (A) : MAP kinase expression after hypoxia for 1 hour. (B) : Expression of MAP kinase, ERK1 after treatment of MAP kinase inhibitor. (C) . Time dependent expression of MAP kinase, Lane 1: hypoxia for 30 minutes, Lane 2: 30 min hypoxia with treatment of DJGWE 10 μg/ml, Lane 3: 30 min hypoxia with treatment of DJG 50 μg/ml, Lane 4: 30 min hypoxia with treatment of DJG 100 μg/ml.
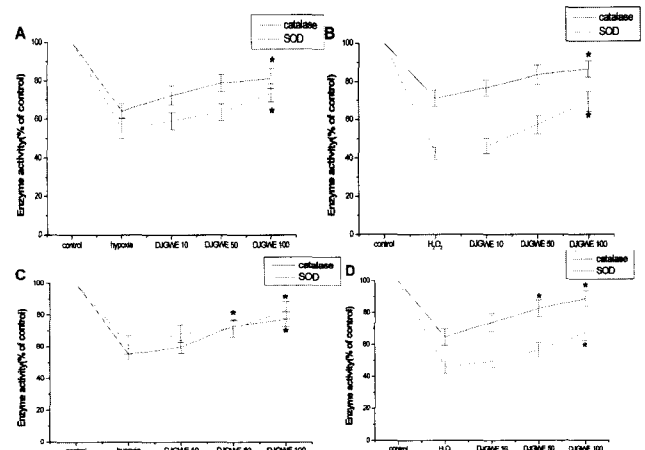

Fig. 8. Effect of Daejowhan-gamibang water extract (DJGWE) on antioxidant enzymes, catalase and SOD in PC12 cells and cortical neuronal cells in rats. In this data, SOD and catalase activity were measured in cortical neuronal cells and PC12 cells exposured to hypoxia and H_2O_2 stress. The cells, PC12 cells(A, B) and cortical neurons of rat(C, D), were treated with hypoxia or H_2O_2 alone, with various concentrations(10, 50, 100 μg/ml) of DJG extract and hypoxia or H_2O_2 for 2 hour. * p<0.05 as compared to hypoxia and H_2O_2 stress group. The present data were expressed as mean±SE.

6. Effect of DJG as a antioxidants on SOD and catalase activity

It was researched whether ROS scavenging effects of DJG extract antioxidants influence antioxidant enzymes in PC12 cells. SOD and catalase activities were measured with various concentrations of DJG extract in PC12 cells and the primary culture of cortical neuronal cells of rats under hypoxia or H_2O_2 stress for 2 hr. The results demonstrated that DJG extract at different concentrations (10, 50, 100 μg/mℓ) dose-dependently maintained SOD and catalase activities in PC12 cells and the primary culture of cortical neuronal cells of rats induced by hypoxia and H_2O_2 for 2 hr (Fig. 8).

Discussion

The formation of reactive oxygen species from different sources, such as enzymatic reaction and xenobiotic metabolism, may lead to lipid peroxidation, subsequent cell injury and toxicity[31]. Oxidative stress is considered to be of major pathophysiological relevance for a variety of pathological processes, such as cancer, Parkinson's disease, and Alzheimer disease[6,32]. This hypothesis has prompted research efforts to identify compounds that might act as antioxidants. The effect

Recently, many herbs have been show to strong inhibition the enzyme acetylcholinesterase in brain tissue in vitro, and the protective effect of the erythrocyte membrane against lipid peroxidation[33-34], In this papaer, it was revealed that the extracts of each herbs which have been used for the treatment in Oriental Medicine and the prescription showed appreciable levels of antioxidant activity and antiperoxidation activity in vitro (Table 1, 2). On the basis of the above, it may be worthwhile undertaking clinical trials of these prescriptions and herbs in neurodegenerative diseases, such as Alzheimer's disease, stroke, and brain ischemia induced by hypoxia, where there may be a synergistic effect, since free radical induced tissue damage has been implicated in disorder. Human body, however, possesses defense mechanisms to reduce the oxidative damage and such mechanisms include using both enzymes and antioxidant nutrients or medicine to arrest the damaging properties of exited oxygen species. A number of studies have suggested that antioxidant nutrient and medicines play a protective role in human health[35].

Also, in the present study, the above-mentioned extracts significantly protected neuronal cells and PC12 cells from

hypoxia. The results showed that LDH release and ROS levels increased significantly after cells were treated with hypoxia for 2 hr, and suggest a role of ROS generation in hypoxia-induced cell death. The results suggest that the protective mechanism of the sample for hypoxic neuronal cells might be through the suppression of ROS generation and MAPK activation.

ROS are generated from mitochondrial respiration. Under physiological conditions, excessive ROS are neutralized by endogenous antioxidants (e.g., ascorbate, -tocopherol, and glutathione) and antioxidant enzymes (e.g., superoxide dismutase, catalase, and glutathione peroxidase)[36]. Increased production of ROS has been implicated in degenerative diseases, ischemia, or trauma, by inducing neuronal damage. In cultured neurons, increased ROS production under conditions, such as hypoxia, induced neuronal cell death with morphological and biochemical features characteristic of apoptosis[37-40]. ROS also induces apoptotic cell death associated with the loss of mitochondrial membrane potential[40]. Oxygen free radicals are highly reactive species that promote damage to lipids, DNA, carbohydrates, and proteins, and induce production of several immune/inflammatory proteins that contribute to the process of excitotoxic neuronal death. In brain ischemia, ROS production occurs during the reperfusion period[41-42], and free radical scavengers can reduce brain damage after ischemia[43]. Resveratrol has an ROS-reducing effect to protect PC12 cells from oxidative stress-induced cell death[15,44-45]. Antioxidants exhibit the ability to scavenge peroxyl and hydroxyl radicals[46]. It has been shown recently that antioxidants inhibit production of ROS in zymosan-stimulated murine macrophages, human monocytes, and neutrophils[45] and protect PC12 cells from dopamine-induced cell death through scavenging action of ROS[47].

The data in this paper showed that ROS generation in PC12 cells under hypoxia or H_2O_2 treatment was reduced by the extracts. The extracts dose-dependently maintained SOD and catalase activity of PC12 cells under hypoxia or H_2O_2 stress. SOD and catalase are two important enzymes that clear free radicals and have been shown to protect cerebral tissues from ischemic injury[48-51] and global ischemia[51]. In addition, downregulation of SOD or mutant SOD causes neuronal PC12 cell death[52-53] and addition of SOD with catalase inhibits PC12 cell death[54]. Therefore, the effect of the above-mentioned extracts as antioxidants might be due to their ROS-scavenging effect that spared SOD and catalase in hypoxia-stressed PC12 cells. SOD catalyzes ROS, forming hydrogen peroxide, which is then detoxified to H_2O_2 and oxygen by catalase. It is reasonable that the effect of these herbal extracts on SOD is stronger than catalase in hypoxia or H_2O_2-stressed PC12 cells.

Ischemic stroke is a major leading cause of morbidity and mortality in developed countries. At present, it is known that many mechanisms are related to the complex brain response after ischemic stroke. Traditionally, neuronal death in the ischemic brain has been attributed to necrosis. Based on morphological and biochemical evidence, however, recent studies suggest that apoptosis is involved in ischemic neuronal damage[55-56]. One major apoptotic path involves caspase-3 activation[57-58]. In addition, chromatin condensation can be evaluated by DAPI staining with a fluorescent microscope as indicator for apoptotic cells[26]. The number of DAPI-stained cells was reduced significantly by the extracts of some herbs and the prescription in primary cortical cell cultures after 2 hr of hypoxia (Fig. 3). The present study also showed that hypoxia activated MAPKs, increasing immunoactivity mainly of phospho-p44 (ERK1) and to a lesser degree JNK and p38 MAPK. MAP kinase inhibitors of SB203580(p38 inhibitor), PD98059(ERK inhibitot), and SP600125(JNK inhibitor) blocked hypoxia-induced ERK1 MAPK activity of PC12 cells after 2 hr of hypoxia and significantly protected cells from hypoxic death. The extracts of Daejowhan-gamibang water extract (DJGWE) inhibited hypoxia-induced MAPK (JNK, ERK, and p38) and caspase-3 activity in PC12 cells after 1 hr hypoxia. Their effects were not better than MAP kinase, but were similar to the effects of MAP kinase inhibitors of SB203580(p38 inhibitor), PD98059(ERK inhibitot), and SP600125(JNK inhibitor) . The extracts of Daejowhan-gamibang prevented phosphorylation and activation of JNK, ERK1, p38 MAPKs, and caspase-3, and reduced the level of LDH release after hypoxia. The results suggest that MAPKs activation is involved in hypoxia-induced PC12 cells.

In conclusion, our study provides evidences that hypoxia-induced death of primary cortical cells and PC12 cells can be partially prevented by the extracts of some herbs and the prescription which have been used for brain damage. These water extracts of some herbs and the prescription exhibited interesting antioxidant and antiperoxidation properties for scavenging of reactive oxygen species. These effects may be useful in the treatment of diseases in which free radical oxidation plays a fundamental role. Daejowhan-gamibang water extract (DJGWE) might protect the brain damage induced by neuronal death, probably by attenuating ROS production during hypoxia. Furthermore, the effect of protection against delayed neuronal death by attenuation of apoptosis was concomitant with downregulation of MAPKs and caspase-3. Further delineation of the mechanisms of ROS generation MAPKs and apoptosis regulation are desirable. This may lead to additional strategies to protect neuronal cells

against hypoxic insults. At the same time, a great deal of work remains to be carried out in order to confirm more biological activities of extracts in various animal models.

Conclusion

Daejowhan-gamibang water extract (DJGWE), MAPK inhibitors were all effective at protecting PC12 cells and cortical cells from hypoxic damage. Also, Daejowhan-gamibang water extract (DJGWE) showed the protection of cell viability and cytotoxicity in H_2O_2 stressed cells. T extracts as antioxidants diminished ROS generation in a dose-dependent manner in PC12 cells and the cortical neuronal cells of rats. Daejowhan-gamibang water extract (DJGWE) significantly decreased hypoxia-activated JNK, ERK, p38 MAPK, and caspase-3. Daejowhan-gamibang water extract (DJGWE) dose-dependently maintained SOD and catalase activity of PC12 cells under hypoxia or H_2O_2 stress.

In conclusion, our study provides evidences that hypoxia-induced death of primary normal cells and PC12 cells can be partially prevented by the extracts of Daejowhan-gamibang and some herbs which have been belonged to the prescription. Therefore, Daejowhan-gamibang might be used used for the treatment of brain damage, however the mechanism of effect in cellular level and also should be studied clinically.

Acknowledgement

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