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淸心蓮子湯의 抗酸化 效果와 機轉에 관한 硏究

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Abstract

Antioxidant effects and its mechanism of Cheongsimyeonjatang in Sasang Constitutional Medicine

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To investigate the antioxidant capacity of traditional Korean herb medicines, water extracts from 42 species which has been used for the Taeum constitution of Sasang Medicine, were tested on their antioxidant activity using radical scavenging effects against ABTS. Some Of them showed strong antioxidant capacities at 50㎍ concentration. And, antioxidant capacities of 2 prescriptions extract of Sasang Medicine, Cheongsimyeonja-tang and Yeoldahanso-tang, in the different concentration (10㎍, 50㎍, and 100㎍) were determined. At the same time, the antiperoxidation effects of these 2 prescriptions extract were determined. Lipid peroxidation in brain homogenates induced by NADPH and ADP-Fe^{2+} was strongly inhibited by Cheongsimyeonja-tang *in vitro*.
The above-mentioned 2 prescriptions are a potent antioxidant capacity and antiperoxidation activity, further investigation into the in vivo antioxidant therapeutic potential for treatment of human disorders in brain tissue.

Key Word: Cheongsimyeonja-tang, Yeoldahanso-tang, Taeum constitution, Antioxidant effects, JNK, ERK, p38 MAPK, caspase-3

Ⅰ. INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (·OH) are potentially transient chemical species and generated in all aerobic metabolism cells. Oxidative damage resulting from an inbalance between production and elimination of ROS has been implicated in the pathogenesis of a variety of human diseases, including cancer, diabetes, cataractogenesis, rheumatoid arthritis, reperfusion injury, as well as degenerative disorders of the neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease.[1-4] The cells have biological system to protect from ROS induced damage by a variety of endogenous ROS scavenging enzymes and chemical compounds.[5] 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.

Hypoxia is a pathophysiological condition characterized by an increase in reactive oxygen species (ROS) and a change in intracellular redox level. The cellular response

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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.[6] Reactive oxygen intermediates, superoxide radical (O2-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH), are produced mainly in mitochondria. Antioxidants may have the potential to protect cells from oxidative damage.[7] Antioxidants (Trolox and ascorbic acid) protect the fetal heart in rabbit fetal hypoxia,[8] and vascular hypoxia,[9] and ameliorate oxidative stress in PC12 cells.[10] Therefore, they are able to protect the human body from several diseases. attributed to the reactions of radicals.[11-12]

Medicinal herbs are the source of healthcare and disease management for a natural antioxidants. Various medicinal plants have been reported as therapeutic drugs for free radical pathologies.[13-15] 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, the present study on the effect of water extracts from 42 species which has been used for the Taeyeum constitution and 2 prescriptions of Sasang Medicine, Cheongsimyeonja-tang and Yeoldahanso-tang, were undertaken to evaluate the antioxidant capacities and antiperoxidation activities and the mechanism of Cheongsimyeonja-tang which showed significant effect of antioxidant. 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, protein kinase cascades was involved in growth, differentiation, and apoptosis induced by numerous extracellular stimuli. Members of the mitogen-activated protein kinase (MAPK) family have been implicated in regulation of cell survival and cell death under numerous conditions.[16-18] 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.[19] So, it was studied whether the protective mechanism of Cheongsimyeonja-tang which showed significant effect as a antioxidant, antioxidants was mediated through the inhibition of MAP kinase and apoptosis pathways in PC12 cells and cortical neuronal cells in rats damaged by hypoxia and H_2O_2 stress.

Ⅱ. MATERIALS AND METHODS

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).

Preparation of medicinal plant extracts

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

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,

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brain was rapidly dissociated and placed on chilled surface. The superficial blood vessels were removed, and washed with ice-cold 50mM Tris-HCl contained 32 mM sucrose buffer (pH 7.4). The brain was homogenized (200 mg tissue/ml buffer) in ice-cold 50mM Tris-HCl buffer (pH 7.4) using a Potter Elvehjehm Teflon homogenizer. These homogenates were used for the determination of antioxidant and lipid peroxidation.

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.[20] 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℃. The antioxidant status of the plant 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.

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℃ for 30min, 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.

Cell Culture

Cultured cortical cells were prepared from the cerebral cortices of 2-day-old Sprague-Dawley rats under pentobarbital anesthesia as described previously (Huang et al., 2002). 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 $2-4\times10^5$ 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

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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%.

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.

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.

Western Blotting

Protein samples containing 50g 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 (0.1M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) to block nonspecific binding, and then incubated with rabbit anti-caspase-3 (1:2000; 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).

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.

MTT Reduction Assay for Cell Viability

Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethylthiazol-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 5×105 cells per well for 24hr, 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

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SpectraMAX 250 (Molecular Devices, USA) microtiter plate reader. Data were expressed as the mean percent of viable cells vs. control.

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.

DAPI Staining

Cortical culture was seeded onto a chamber (Nunc, USA) precoated with 1% poly-D-lysine at a density of 2×105 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) (1g/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.[21]

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)[22] 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.

Statistical Analysis

All data were expressed as the mean ± SEM. For single variable comparisons, Student's 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.

Ⅲ. RESULTS

Determination of antioxidant activity of extracts *in vitro* using brain homogenate of rats

In the present study, 42 water extracts of medicinal herbs which has been used for Taeyeum constitution and 2 prescriptions extract of Taeyeum constitution in Sasang Medicine, Cheongsimyeonja-tang and Yeoldahanso-tang, were tested for antioxidant capacity and antiperoxidation activity by *in vitro* assay. The antioxidant capacity in such extracts was determined using the total antioxidant status kit (Randox Labs) against ABTS$\cdot^+$ radical was determined in biological fluid and human plasma/serum for routine use in clinical chemistry analysis has been proposed,[23-25] and recently, the assessment of antioxidant capacity in plant extracts and medicinal drugs using this assay method has been applied in recently.[14] The antioxidant levels of the above-mentioned extracts are shown in Table 1. Of the extracts which has been investigated, the highest levels of antioxidant activity were obtained from about 10 herbs which has been belonged to 2 prescription, Cheongsimyeonja-tang and Yeoldahanso-tang, respectively, Especially, Cheongsimyeonja-tang significantly showed strong antioxidant activity with comparison to Yeoldahanso-tang. On the other hand, several medicinal herbs appeared to contain little antioxidant capacity. The extracts which showed the highest levels of antioxidant activity were belonged to

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the composition of Taeyeum prescriptions(Table 1).

Measurement of thiobarbituric acid reactive substances (TBARS) using brain homogenate of rats

Malondialdehyde (MDA) is the major oxidative degradation product of membrane unsaturated fatty acid, and has been shown to be biologically active with

Table 1. Comparison of antioxidant activity of herbs and prescriptions used for Taeyeum constitution in Sasang Medicine

生藥名	Species	Herbal Name	Antioxidant activity(mM TE)
葛根	*Pueraria thunbergiana*	RADIX PUERARIAE	1.52±0.14
甘菊	*Chrysanthemum indicum*	FLOS CHRYSANTHEMI	0.44±0.10
乾栗	*Castanea crenata*	FRUCTUS CASTANEAE	1.13±0.11
藁本	*Ligusticum tenuissimum*	RADIX LIGUSTICI TENUISSIMAE	0.84±0.12
瓜蔕	*Cucumis melo*	PEDICELUS MELO	0.51±0.06
款冬	*Tussilago farfara*	FLOS FARFARAE	0.60±0.07
桔梗	*Platycodon grandiflorum*	RADIX PLATYCODI	1.55±0.13
蘿葍子	*Raphanus sativus*	SEMEN RAPHANI	1.41±0.08
鹿茸	*Cervus nippon*	CORNU CERVI PARVUM	1.24±0.11
大豆黃卷	*Glycine max*	LEGUMINOSAE	1.01±0.13
大黃	*Rheum coreanum*	RHIZOMA RHEI	1.25±0.16
麻黃	*Ephedra sinica*	HERBA EPHEDRAE	0.72±0.11
麥門冬	*Ophiopogon japonicus*	RADIX OPHIOPOGONIS	1.67±0.08
白果	*Ginkgo biloba*	SEMEN GINkgO	1.75±0.09
白蘞	*Ampelopsis japonica*	RADIX AMPELOPSIS	1.54±0.11
柏子仁	*Thuja orientalis*	SEMEN BIOTAE	1.34±0.14
白芷	*Angelica dahurica*	RADIX ANGELICAE DAHURICAE	1.01±0.15
浮萍	*Spirodela polyrrhiza*	HERBA SPIRODELAE	0.64±0.15
使君子	*Quisqualis indica*	FRUCTUS QUISQUALIS	0.54±0.11
麝香	*Moschus moschiferus*	MOSCHUS	1.23±0.09
山藥	*Dioscorea batatas*	RHIZOMA DIOSCOREAE	1.78±0.13
酸棗仁	*Zizyphus jujuba*	SEMEN ZIZIPHI SPINOSAE	1.87±0.17
桑白皮	*Morus alba*	CORTEX MORI	1.02±0.09
犀角	*Rhinocers unicornis*	CORNU RHINOCERI	1.30±0.12
石菖蒲	*Acorus gramineus*	RHIZOMA ACORI GRAMINEI	1.74±0.14
升麻	*Cimicifuga heracleifolia*	RHIZOMA CIMICIFUGAE	0.68±0.05
蓮子肉	*Nelumbo nucifera*	SEMEN NELUMBINIS	1.67±0.20
羚羊角	*Saiga tatarica*	CORNU ANTELOPIS	0.46±0.11
烏梅	*Prunus mume*	FRUCTUS MUME	1.48±0.12
五味子	*Schizandra chinensis*	FRUCTUS SCHIZANDRAE	1.25±0.17
龍腦	*Dryobalanops aromatica*	BORNEOL	0.65±0.11
牛黃	*Bos taurus*	CALCALUS BOVIS	1.10±0.19
熊膽	*Selenarctos thibetanus*	FEL URSI1	1.13±0.17
遠志	*Polygala tatarinowi*	RADIX POLYGALAE	1.61±0.12
薏苡仁	*Coix lachryma-jobi*	SEMEN COICIS	0.47±0.10
樗根白皮	*Ailanthus altissima*	CORTEX AILANTHI	0.67±0.08
蠐螬	*Holotrichia diomphalia*	HOLOTRICHIA	0.55±0.08
皁角子	*Gleditsia sinensis*	SPINA GLEDITSIAE	1.12±0.11
天門冬	*Asparagus cochinchinenensis*	RADIX ASPARAGI	1.88±0.09
蒲黃	*Typha latifolia*	POLLEN TYPHAE	0.67±0.16
杏仁	*Prunus armeniaca*	SEMEN ARMENIACAE	1.54±0.12
黃芩	*Scutellaria baicalensis*	RADIX SCUTELLARIAE	1.31±0.09
淸心蓮子湯	*Cheongsimyeonja-tang*	CYT	1.74±0.11
熱多寒少湯	*Yeoldahanso-tang*	YHT	1.31±0.12

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Table 2. Antiperoxidant activity of lyophilized water extracts of 2 prescriptions and herbs which were belonged to those prescriptions on NADPH and ADP-Fe^{2+} induced lipid peroxidation in rat brain homogenates

生藥名	Species	Herbal Name	Antioxidant activity(MDA formation rate of control)
葛根	*Pueraria thunbergiana*	RADIX PUERARIAE	78.5±5.4
甘菊	*Chrysanthemum indicum*	FLOS CHRYSANTHEMI	112.1±12.6
藁本	*Ligusticum tenuissimum*	RADIX LIGUSTICI TENUISSIMAE	96.8±8.3
桔梗	*Platycodon grandiflorum*	RADIX PLATYCODI	75.4±6.7
蘿葍子	*Raphanus sativus*	SEMEN RAPHANI	71.5±7.8
麥門冬	*Ophiopogon japonicus*	RADIX OPHIOPOGONIS	69.4±6.5
山藥	*Dioscorea batatas*	RHIZOMA DIOSCOREAE	65.3±5.8
酸棗仁	*Zizyphus jujuba*	SEMEN ZIZIPHI SPINOSAE	69.1±4..7
石菖蒲	*Acorus gramineus*	RHIZOMA ACORI GRAMINEI	71.5±5.8
升麻	*Cimicifuga heracleifolia*	RHIZOMA CIMICIFUGAE	95.3±9.5
蓮子肉	*Nelumbo nucifera*	SEMEN NELUMBINIS	63.8±8.6
遠志	*Polygala tatarinowi*	RADIX POLYGALAE	68.5±7.9
天門冬	*Asparagus cochinchinenensis*	RADIX ASPARAGI	72.7±6.8
白芷	*Angelica dahurica*	RADIX ANGELICAE DAHURICAE	73.8±6.4
黃芩	*Scutellaria baicalensis*	RADIX SCUTELLARIAE	82.5±8.3
淸心蓮子湯	*Cheongsimyeonja-tang*	CYT	71.8±7.0
熱多寒少湯	*Yeoldahanso-tang*	YHT	81.6±9.2

cytotoxic and genotoxic properties.[26] Quantitation of MDA, one of the product of lipid peroxidation, with thiobarbituric acid reactive substance (TBARS) is the most common assay used for determination of the lipid peroxidation. In the present study, $NADPH^{+}$-Fe^{2+} induced lipid peroxidation in brain homogenates. The antiperoxidative effect was expressed as MDA formation, setting the values obtained without inhibitor, as 100% activation. This experiments showed that NADPH and ADP-Fe^{2+} induced lipid peroxidation was inhibited dose-dependent manner in water extract of 15 herbs which were belonged to 2 Taeyeum prescription, Cheongsimyeonja-tang and Yeoldahanso-tang. And, also 2 extracts of Cheongsimyeonja-tang and Yeoldahanso-tang showed the effective inhibition of MDA formation. The extract of Cheongsimyeonja-tang has been proved to be more effective, with comparison to Yeoldahanso-tang in this system. Among 15 herb extracts, the 5 extracts of RADIX OPHIOPOGONIS, RHIZOMA DIOSCOREAE, SEMEN ZIZIPHI SPINOSAE, SEMEN NELUMBINIS, RADIX POLYGALAE were found to highest inhibition TBARS generated (lipid peroxidation) by the induction of NADPH and ADP-Fe^{2+} in rat brain homogenates, It was supposed that IC_{50} range from 10 ㎍/㎖ and 100 ㎍/㎖, respectively (Table 2). So the hot water extract of these herbs, which might act as scavengers of reactive oxygen species because of confirmed with antioxidant effect, could inhibit lipid peroxidation. Prevention of the TBARS formation and action of antioxidant in the extracts is good health importance in decreasing the risk of neurodegenerative diseases.

Protective effect of Cheongsimyeonja-tang (CYT) 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.[27] 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

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A

B

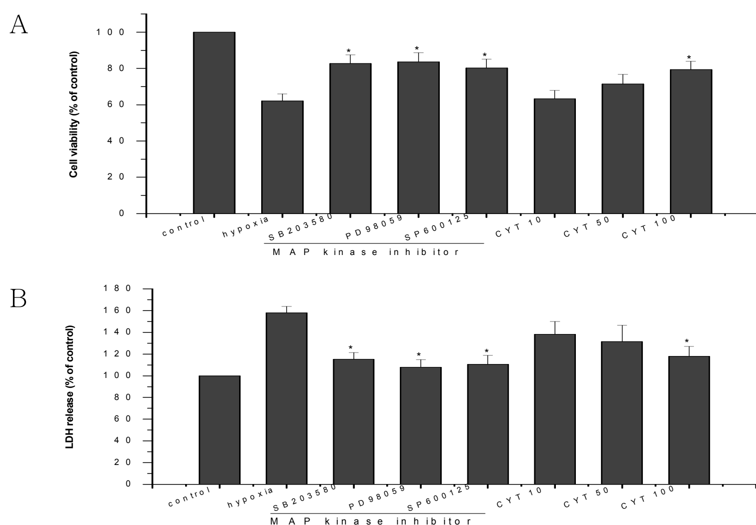

Fig. 1. Antioxidant effect of Cheongsimyeonja-tang(CYT) on the cell viability and cytotoxicity of PC12 cells exposured to hypoxic condition.

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 ㎍/㎖) of CYT 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.

A

B

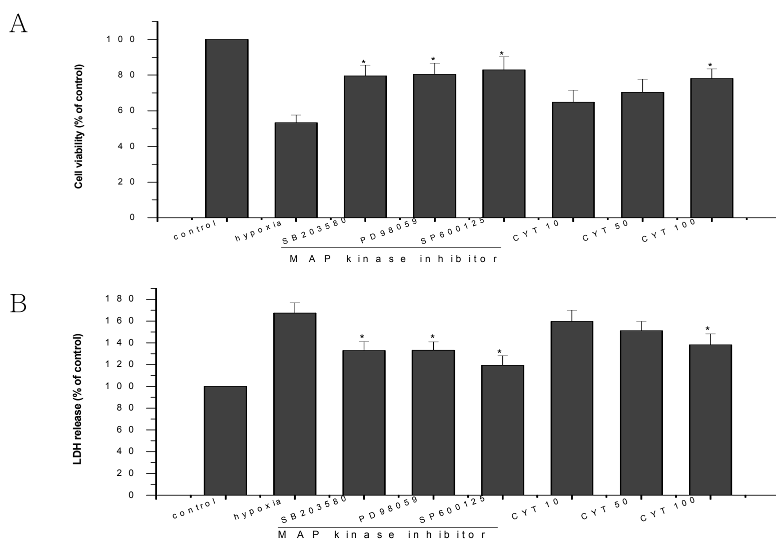

Fig. 2. Protective effect of Cheongsimyeonja-tang(CYT) extract as a antioxidant on the cultured cortical cells exposured to hypoxia.

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 ㎍/㎖) of CYT 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.

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of CYT extract (10, 50, 100 ㎍/㎖), or with 10 μM of SB203580, PD98059, and SP600125 (MAP kinase inhibitors) for 2 hr. CYT 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 CYT 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 CYT extract a concentration of 50 ㎍/㎖ (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 CYT extract in hypoxia-treated cortical cell cultures (Fig. 3). These results demonstrated clearly that CYT extract protected PC12 and primary neuronal culture from hypoxia-induced stress.

Preventive effect of CYT on H_2O_2-induced cytotoxicity in PC12 Cells

The ability of CYT extract 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 CYT extract increased cell viability by about 1-20% when compared to viability in H_2O_2-stressed controls. CYT extract at a concentration of 100 ㎍/㎖ increased cell viability by about 20% after 2 hr of H_2O_2 treatment. CYT extract reduced cell damage 10-30% in H_2O_2-stressed PC12 cells as measured by LDH release. CYT extract at a concentration of 100 ㎍/㎖ 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).

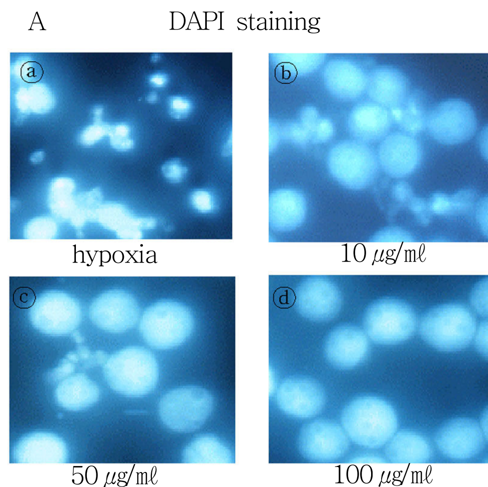

Fig. 3-A. Apoptotic patterns of cortical cell neurons exposured to hypoxia by fluorescent DAPI-stain and DNA ladder patterns.

A was the morphological change of nucleus DNA after the inducement of apoptosis ⓐ was control, ⓑ, ⓒ, ⓓ were the features after the treatment of CYT with in the cells exposured to hypoxia, B was treated with hypoxia or H_2O_2, and 10, 50, 100 ㎍/㎖ of CYT extract.

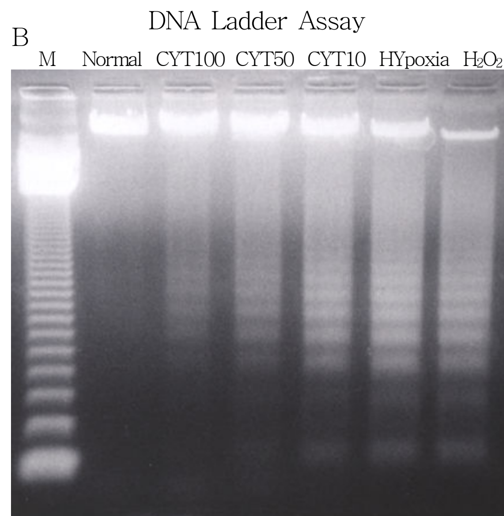

Fig. 3-B. Apoptotic patterns of cortical cell neurons exposured to hypoxia by fluorescent DAPI-stain and DNA ladder patterns.

A was the morphological change of neucleous DNA after the inducement of apoptosis ⓐ was control, ⓑ, ⓒ, ⓓ were the features after the treatment of CYT with in the cells exposured to hypoxia, B was treated with hypoxia or H_2O_2, and 10, 50, 100 ㎍/㎖ of CYT extract.

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A
B

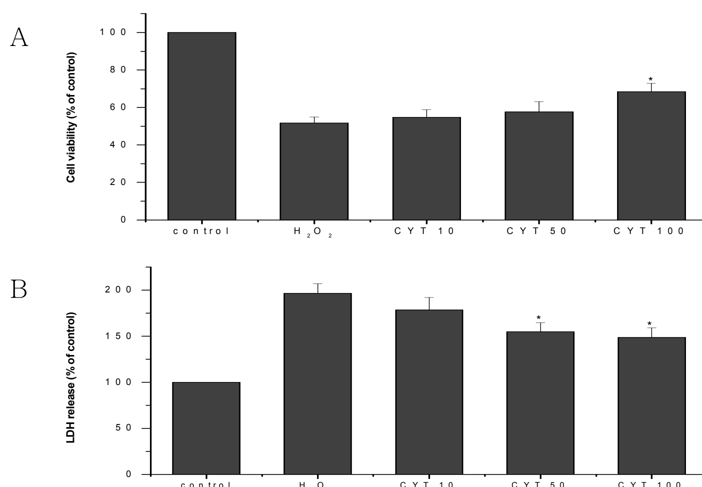

Fig. 4. Antioxidant effect of Cheongsimyeonja-tang(CYT) on the cell viability and cytotoxicity of PC12 cells exposured to H_2O_2-stressed condition.

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 ㎍/㎖) of CYT extract 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.

A
B

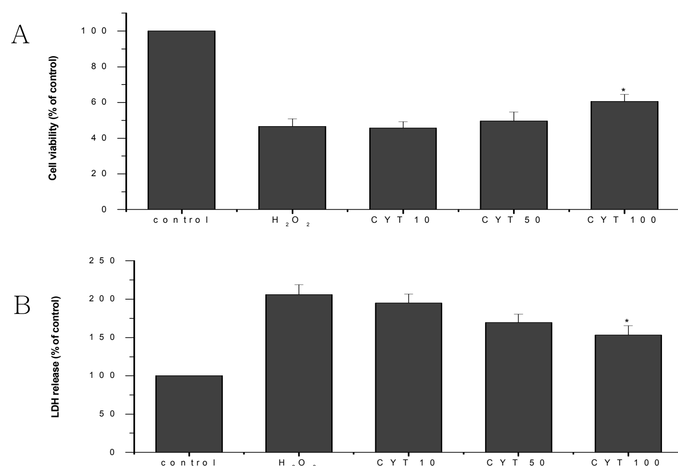

Fig. 5. Protective effect of Cheongsimyeonja-tang(CYT) extract as a antioxidant on the cultured cortical cells exposured to H_2O_2-stressed condition.

The effect were measured by the cell viability(A) and LDH release(B). Cells were treated with H2O2 alone, with various concentrations(10, 50, 100 ㎍/㎖) of CYT extract 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.

Effect of CYT as a antioxidants on the hypoxia-induced ROS in PC12 cells

We 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

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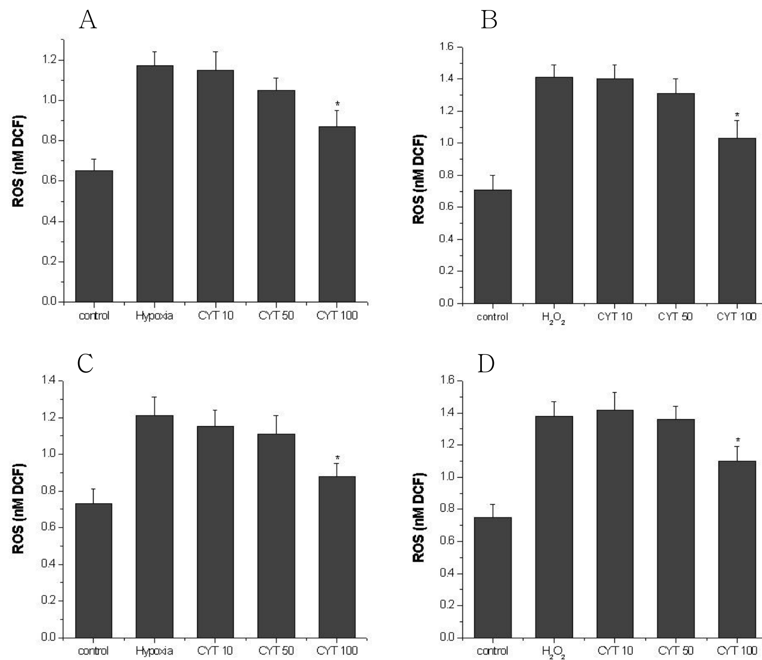

Fig. 6. Effect of Cheongsimyeonja-tang(CYT) extract on ROS generation in PC12 cells and cortical neurons in rats. In this data, ROS formation was as DCF formation in cortical neuronal cells and PC12 cells exposured to hypoxia and H2O2 stress. The cells, PC12 cells(A, B) and cortical neurons of rat(C, D), were treated with hypoxia or H_2O_2 alone, with various concentrations(10, 50, 100 ㎍/㎖) of CYT 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.

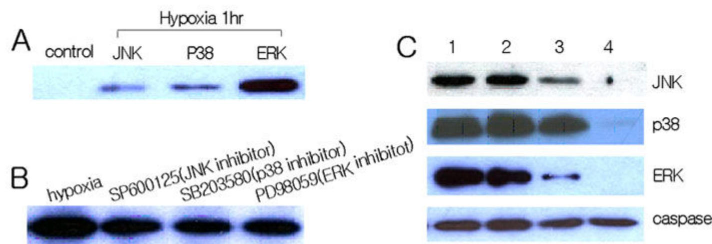

Fig. 7. Antioxidant effect of Cheongsimyeonja-tang(CYT) on MAP kinase and caspase 3 activation on PC 12 cells. (A) : MAP kinase expression after hypoxia for 1 hour. (B) : Expression of MAP kinase, ERK1 after treatment of MAP kinase inhibitor. (C) : Time dependent expression of MAP kinase, Lane 1; hypoxia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

significantly after 30 min to a maximum at 2 hr (data not showen). CYT extract as a antioxidants diminished ROS generation in a dose-dependent manner in PC12 cells and the cortical neuronal cells of rats (Fig. 6).

Inhibitory effect of CYT on the hypoxia-induced MAPK and caspase-3 activation

We found that hypoxia induced MAP kinase activation in cortical cultures, especially activation of ERK MAP kinase (Fig. 7A). MAP kinase inhibitors, U0126 and SB203580 (10 M), were able to suppress ERK MAP kinase in hypoxic cortical cultures (Fig. 7B).

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It was further studied that further the effect of CYT 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 CYT extract at a concentration of 100 ㎍/㎖ significantly decreased hypoxia-activated JNK, ERK, p38 MAPK, and caspase-3 at 1 hr (Fig. 7C). At 10 and 30 min, however, CYT extract had no effect on MAPK and caspase-3 expression compared to expression in controls (data not shown).

Effect of CYT as a antioxidants on SOD and catalase activity

We studied whether ROS scavenging effects of CYT extract antioxidants influence antioxidant enzymes in PC12 cells. SOD and catalase activities were measured with various concentrations of CYT 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 CYT extract at different concentrations (10, 50, 100 ㎍/㎖) 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).

Ⅳ. 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.[28] 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.[29] This hypothesis has prompted research efforts to identify

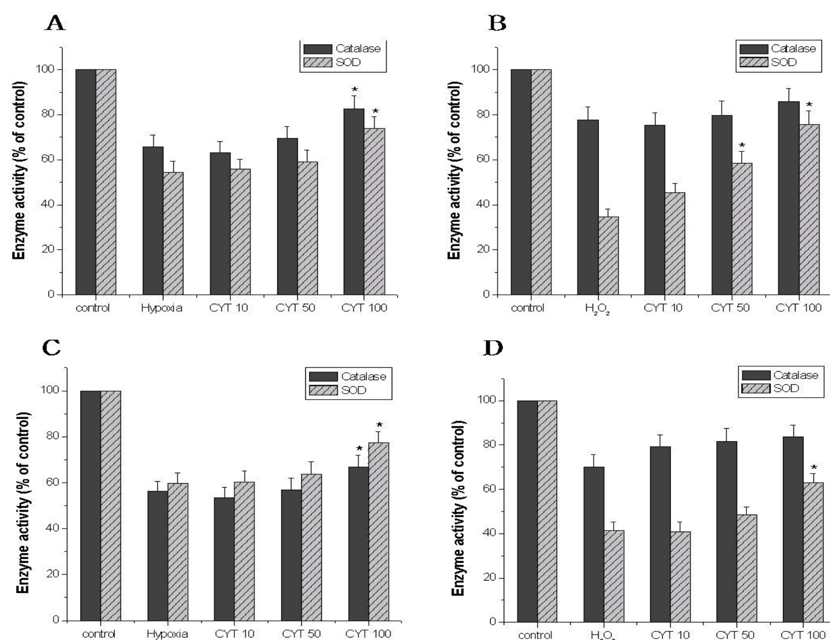

Fig. 8. Effect of Cheongsimyeonja-tang(CYT) extract on antioxidant enzymes, catalase and SOD in PC12 cells and cortical neurons in rats.
In this data, SOD and catalase activity in cortical neuronal cells and PC12 cells exposured to hypoxia and H2O2 stress. The cells, PC12 cells(A, B) and cortical neurons of rat(C, D), were treated with hypoxia or H_2O_2 alone, with various concentrations(10, 50, 100 ㎍/㎖) of CYT extract and hypoxia or H2O2 for 2 hour. * $p<0.05$ as compared to hypoxia and H2O2 stress group. The present data were expressed as mean±SE.

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compounds that might act as antioxidants. The effect of antioxidants in decreasing oxidative damage is believed that contribute to lower the cancer incidence and neurodegenerative diseases.[29] With 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 some herbs and prescriptions which has been used for the constitution of Taeyeum for potential antioxidants and antiperoxidants by preliminary *in vitro* assay screening.

Recently, many herbs have been shown to have strong inhibition of the enzyme acetylcholinesterase in brain tissue *in vitro,* and the protective effect of the erythrocyte membrane against lipid peroxidation,[30-31] In this paper, it was that the extracts of many herbs which has been used for the prescription of Taeyeum constitution have appreciable levels of antioxidant capacity 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 ischemia induced by hypoxia, where there may be a synergistic effect, since free radical induced tissue damage has been implicated in disorder. The 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 great number of studies have suggested that antioxidant nutrient and/or medicines play a protective role in human health.[32]

Also, in the present study, the above-mentioned extracts significantly protected cortical cultured 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).[33] 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.[34-37] ROS also induces apoptotic cell death associated with the loss of mitochondrial membrane potential.[37] 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 cytotoxic neuronal death. In brain ischemia, ROS production occurs during the reperfusion period,[38-39] and free radical scavengers can reduce brain damage after ischemia.[40] Resveratrol has an ROS-reducing effect to protect PC12 cells from oxidative stress-induced cell death.[10,41-42] Antioxidants exhibit the ability to scavenge peroxyl and hydroxyl radicals.[43] It has been shown recently that antioxidants inhibit production of ROS in zymosan-stimulated murine macrophages, human monocytes, and neutro- phils[42] and protect PC12 cells from dopamine-induced cell death through scavenging action of ROS.[44]

Our data 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

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to protect cerebral tissues from ischemic injury[45-47] and global ischemia.[48] In addition, downregulation of SOD or mutant SOD causes neuronal PC12 cell death[49-50] and addition of SOD with catalase inhibits PC12 cell death.[51] 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.[52-53] One major apoptotic path involves caspase-3 activation.[54-55] In addition, chromatin condensation can be evaluated by DAPI staining with a fluorescent microscope as indicator for apoptotic cells.[21] The number of DAPI-stained cells was reduced significantly by the extracts of some herbs and prescriptions 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 Cheongsimyeonja-tang inhibited hypoxia-induced MAPK (JNK, ERK, and p38) and caspase-3 activity in PC12 cells after 1 hr hypoxia. The extracts of Cheongsimyeonja-tang inhibited hypoxia-induced ERK of MAPKs after 1 hr hypoxia in PC12 cells, and 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 Cheongsimyeonja-tang 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 prescriptions which has been used for the Taeyeum constitution, Cheongsimyeonja-tang and Yeoldahanso-tang. These water extracts of some herbs and prescriptions 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. Also, the extracts of some herbs and prescriptions which has been used for the Taeyeum constitution, Cheongsimyeonja-tang and Yeoldahanso-tang protected against acute neuronal death, probably by attenuating ROS production during hypoxia. Furthermore, 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 completely the biological activity of extracts in various animal models.

V. CONCLUSION

1. The extracts which showed the highest levels of antioxidant activity were belonged to the composition of Taeyeum prescriptions

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2. The extracts of Cheongsimyeonja-tang has been proved to be more effective, with comparison to Yeoldahanso-tang, and the 5 extracts of RADIX OPHIOPOGONIS, RHIZOMA DIOSCOREAE, SEMEN ZIZIPHI, PINOSAE, SEMEN NELUMBINIS, RADIX POLYGALAE were found to have highest inhibition effects.

3. CYT extracts, MAPK inhibitors were all effective at protecting PC12 cells and cortical cells from hypoxic damage.

4. CYT extracts showed the protection of cell viability and cytotoxicity in H_2O_2 stressed cells

5. CYT extracts as a antioxidants diminished ROS generation in a dose-dependent manner in PC12 cells and the cortical neuronal cells of rats

6. CYT extracts significantly decreased hypoxia-activated JNK, ERK, p38 MAPK, and caspase-3

7. CYT extracts 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 cortical cells and PC12 cells can be partially prevented by the extracts of some herbs and prescriptions which has been used for the Taeyeum constitution, Cheongsimyeonja-tang and Yeoldahanso-tang.

The assay of antioxidant activity against $ABTS\cdot^{+}$ was described in Materials and Methods. Determination of antioxidant activity was expressed in terms of mmole Trolox equivalent. Data are shown as mean±SD from three independent experiments, each herbal extract was tested (10 ㎕/assay) at 1 mg/ml of the extracts concentration.

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

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