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Molecular mechanisms of neuroprotection by extract from *Rosmarinus officinalis* (Rosemary) in dopaminergic cell line, SH-SY5Y

朝 鮮 大 學 校 大 學 院

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인간 도파민성 세포주 SH-SY5Y에서 로즈마리

추출물에 의한 신경세포보호효과 기작

2007 年 12 月 日

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朴 世 恩의 碩士學位論文을 認准함.

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ABBREVIATIONS

EGCG.....(-)-epigallocatechin gallate
AADC..... Aromatic amino acid decarboxylase
AD.....Alzheimer’s disease
ALS .....Amyotrophic Lateral Sclerosis
ATCC.....American Type Culture Collection
ALS .....Amyotrophic Lateral Sclerosis
APAF-1..... Apoptotic protease-activating factor 1
ATP..... Adenosine 5’-triphosphate
BCA.....Bicinchoninic acid
Bcl-2 ..... B-cell leukaemia/ lymphoma 2
BDNF ..... Brain derived neurotrophic factor
BSA ..... Bovine serum albumin
Caspase..... Cysteine-requiring aspartate protease
CAT ..... Catalase
CNS ..... Central nervous system
cDNA .....Complement DNA
Cyt ..... Cytochrome
DA ..... Dopamine
DArgic..... Dopaminergic
DMEM ..... Dulbecco's modified Eagle's medium
DMSO ..... Dimethyl Sulfoxide
dNTPs..... Deoxynucleic acid triphosphate
DPBS..... Dulbecco’s Phosphate Buffered Saline
DPPH.....1, 1-diphenyl-2-picryl hydrazyl
EDTA.....Ethylenediamine tetraacetic acid
ETC ....Electron transport chain

EtBr .....Ethidium bromide

FBS..... Fetal bovine serum

GDNF..... Glial cell line derived neurotrophic factor

H_2O_2 .....Hydrogen peroxide

L-DOPA.....Levodopa

LBs .....Lewy Bodies

LDH..... Lactate dehydrogenase

MANEB .....Manganese ethylenebisdithiocarbamate

M-MLV .....Moloney-murine leukemia virus ribonuclease

MMP..... Mitochondria membrane potential

MPP^+ 1-methyl-4-phenylpyridinium

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTT .....3- (4, 5 dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide

NaCl ..... Sodium chloride

NADH .....Nicotinamide adenine dinucleotide

PAGE.....Polyacrylamide gel electrophoresis

PBS.....Phosphate buffered saline

PD.....Parkinson's disease

PI .....Propidium iodide

PVDF.....Polyvinylidine difluoride

Rh 123 ..... Rhodamine 123

RIPA .....Radio-Immunoprecipitation Assay

ROS .....Reactive Oxygen Species

RT-PCR .....Reverse transcription Polymerase chain reaction

rTdT..... Recombinant terminal deoxynucleotidyl transferase

SDS..... Sodium dodecyl sulfate

SN..... Substantia nigra

SNc..... Substantia nigra pars compacta

SOD.....Superoxide dismutase

TH..... Tyrosine hydroxylase

TUNEL..... Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling

ABSTRACT

Molecular mechanism of neroprotection by extract from *Rosemarinus officinalis* L. (Rosemary) in dopminergic cell line, SH-SY5Y

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Parkinson's disease (PD) is characterized by a profound loss of dopaminergic neurons in the substantia nigra. Even though the cause of PD is still unknown, many studies suggest that oxidative stress and mitochondrial dysfunction play a major role in the neurodegenerative process of the diseases. Oxidative stress and/or mitochondrial dysfunction are believed to culminate in the activation of an apoptotic cascade which ultimately results in the loss of dopaminergic cells. Hence regulation of intracellular ROS and modification of the apoptotic cascade may prevent pathological apoptosis in PD.

Many drugs have been used for remedy of PD. But these drugs have many side-effects, and can't use for long-term. So, many natural antioxidants have been tested in both *in vivo* and *in vitro* models of PD. For instance, *Ginkgo biloba*, huperzine A, salvianic acid A, Ginseng and tea catechins have been shown to have

neuroprotective.

Rosemainus officinalis (Rosemary) is one of the most common traditional medicinal herbs. It has been reported to have various pharmacological properties including exceedingly powerful anti-oxidative, liver supportive, anti-cancer, pain relieving, memory-enhancing agent and anti-bacterial activities.

In this study, our results showed that rosemary extracts at the effective concentrations (10 ㎍/㎖) was not cytotoxic and the viability of SH-SY5Y cells was significantly increased by treatment with rosemary extracts. Treatment with rosemary extracts induced the expression of tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC). Furthermore, rosemary extract increased the mRNAs and protein levels of Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in SH-SY5Y cells. We observed that the viability of the cells was enhanced by treatment with rosemary extract compared with only H_2O_2 (150 μM)-treated cells. We found that H_2O_2 caused a significant decrease in cell viability. In contrast, cells exposed to the same amount of H_2O_2 in the presence of rosemary extract appeared remarkably retained virbility, indicating that rosemary extracts prevented neuronal death. We found that treatment of cells with H_2O_2 increased Caspase-3, Caspase-9, Bax and Bak. However, treatment with rosemary extracts decreased the caspase-3, caspase-9, Bax, and Bak activities compared with only H_2O_2-treated cells. Also, treatment of rosemary extract increased mitochondrial memebrane potential, and inhibited nucleus morphological changes.

In conclusion, these data indicate that rosemary extract partially inhibit the neuronal death in the early stage of the mitochondrial apoptotic pathway. Therefore, it suggests that rosemary extract acts on reactive oxygen species to inhibit apoptosis in human dopaminergic neuronal cells.

I. INTRODUCTION

According to increase of the aged ratio, stress and environmental toxin, neurodegenerative disorders are regarded as a social problem. Because neurodegenerative disorders are taken sick in the aged, these diseases were regarded aging-related disorders. But recently, neurodegenerative disorders are increased outbreak in the rising generation.

Parkinson's disease (PD), one of neurodegenerative disorders was first described by James Parkinson in 1817 (Parkinson J, 1817), and is the most common neurodegenerative disorder after Alzheimer disease. PD is a progressive neurodegenerative movement disorder that is taken in approximately 1% of the population older than 65 years of age (Lang & Lozano 1998a, b). PD includes motoric symptoms such as tremor of the hands, bradykinesia, rigidity of muscle and postural instability. These intractable motoric symptoms lead to the disability of increasing immobility and balance difficulties. In extreme cases, PD cause medical disability as daily life is impossible. Moreover, it include non-motor symptoms such as hypochondria, elethism, sleep disturbance and congnitive disturbance.

PD is characterized by two factors: deficiency of dopamine and Lewy bodys (LBs) presents in substantia nigra pars compacta (SNc) (Fig. 1). First is deficiency of dopamine by selective degeneration of dopaminergic (DArgic) neurons, Dopamine is made in substantia nigra pars compacta (SNc) of the striatum and one of three main neurotransmitters called catecholamines. The biosynthesis of catecholamines is proceed by hydroxylation of tyrosine to catalysed levodopa (L-DOPA) by tyrosine hydroxylase (TH), the first and major rate limiting enzyme in catecholamine biosynthesis. L-DOPA is converted by aromatic amino acid decarboxylase (AADC) to dopamine (Fig. 2). When ability of dopamine biosynthesis of DArgic in substatia nigra was reduced below the normal level (below 80%), PD was caused (Schapira, 1999). Second is LBs presence in SNc of the striatum. α–synuclein, one of component of LBs, is soluble neuronal cytoplasmic protein, and is localized to presynaptic ter-

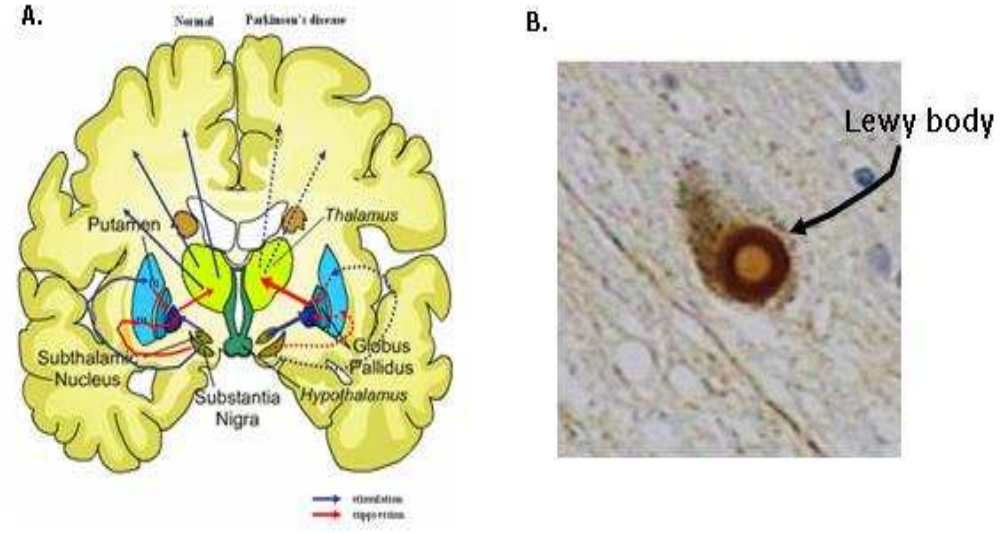

Fig. 1. Pathology of Parkinson's disease. (A) Dopaminergic pathways of the human brain in normal condition (left) and Parkinson's disease (right). (B) Formation of proteinaceous cytoplasmic inclusions called Lewy bodies in SNpc of doparminergic neuron.

minals in the central nerve system (CNS) (Cookson, 2005). Mutatant in α-synuclein display an increased propensity to self-aggregate to form oligomeric species and LBs-like fibrils in vitro compared with normal α-synuclein (Conway et al. 1998). Aggregates of α-synuclein are precursors to cytotoxic events in neurons (Goedert, 1999).A number of causative factors have been reported to be associated with PD. PD is caused by exposure to neuronal toxins such as MPTP, rotenone, paraquat and Maneb (manganese ethylenebisdithiocarbamate) (Goldman and Tanner, 1998). Genetics played little role in PD. Ten monogenic forms of PD, labeled PARK 1-10, have been identified, with genes identified in five (PARK1 and PARK4: α-synuclein gene, PARK2: the parkin gene, PARK5: ubiquitin-carboxy-terminal-hydrolase L1 (UCHL1), PARK7: the DJ-1 gene). Several environmental agents also cause nigrostriatal damage such as metals-iron, manganese, copper, lead, amalgam, aluminium, zinc (Lai et al., 2002), solvents (Davis and Adair, 1999; Hageman et al., 1999; Pezzoli et al., 1996; Seidler et al., 1996; Uitti et al., 1994), and carbon monoxide (Klawans et al., 1982).

Although the underlying mechanism of selective degeneration in DArgic neurons and formation of LBs are not known completely, oxidative stress (Beal, 2003; Zhang et al., 2000), mitochondrial dysfunction (Greenamyre et al., 2001; Orth and Schapira, 2002), protein misfolding (Dawson and Dawson, 2003; McNaught and Olanow, 2003) and others have been reported to play important roles.

PD is taken by selective degeneration of DArgic neurons in the SNc, is thought that resulted from apoptosis by chronic oxidative stress. Although the source of increased oxidative stress is not known completely, it has been reported that is taken by environmental factor, excitotoxin, dopamine homeostasis and others. Oxidative stress may induce mitochondrial dysfunction, genetic mutation and protein aggregation, and ultimately cause cell death. Much evidence suggests that oxidative stress plays a major role for mitochondrial dysfunction in the pathogenesis of PD, and in particular, defects in mitochondrial complex I of the respiratory

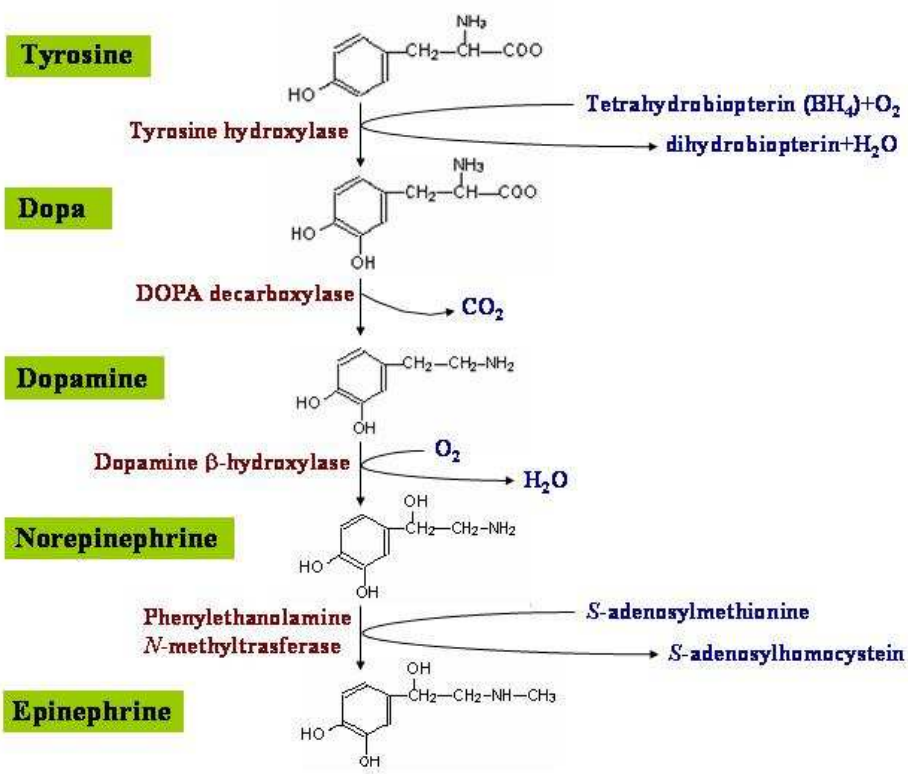

Fig. 2. Biosynthesis of catecholamines.

chain. For example, 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-ducphenyl-1,2,3,6-tetrahydropyridine (MPTP) inhibit complex I (NADH: ubiquinone oxidore-uc tase) of the electron transport chain in DArgic neurons (Nicklas et. al., 1985). Rotenone is insecticide and high-affinity specific inhibitor of complex I. It is extremely hydrophobic and crosses biologic membranes easily. Unlike MPTP, rotenone does not require a dopamine transporter for access to the cytoplasm and therefore is likely to produce systemic inhibition of complex I (Betarbet et al. 2000).The inhibition of complex I has many harmful effects including increase of oxidantproduction (reactive oxygen species (ROS)) and decrease of ATP production. In mitochondria, electrons move along the respiratory chain to complex I, II, III, and IV, along with cytochrome c (cyt c) and ubiquinone. But, increase of ROS production inhibits of complex I and II, so that ubiquinone is unable to carry electrons to complex III and continue to complex IV. If complex IV is unable to carry electron, oxygen is not reduced to water and remains saturated in the cell. Induced ROS cause upregulation of Bax. Bax, a member of Bcl-2 family protein, release cyt c from inner membrane of mitochondria. Released Cyt c can then bind APAF-1 (apoptotic protease-activating factor 1), which then binds to the inactive form of caspase-9. APAF-1 and caspase-9 activate caspase-3, followed by activation of caspases further downstream, ultimately resulting in the hallmarks of apoptosis (condensation of nuclear, DNA fragmentation, and cell blebbing). Mitochondria-mediated activation of caspase-9 can also occur by extracellular receptor-mediated signals such as TNF, growth factor deprivation and etc, thereby causing cyt c release and binding of APAF-1. Decrease of ATP production changes calcium homeostasis, and cause decrease of sodium ion transport to the outside of the cell. Change of calcium homeostasis increases ROS production by the mitochondria in cell, and ROS induce the release of cyt c to the cytosol.

PD is difficult to make a diagnosis and is incurable completely. It has been developed drug therapy, surgery, gene therapy for only relief of symptoms. L-dopa is used with dopa decarbo-

xylase inhibitor for inhibit dopa decarboxylase. Although L-dopa decrease symptoms such bradykinesia, it is caused side-effects such as ventricular extrasystole, atrial fibrillation and anorexia. Moreover, dosage of L-dopa for long-terms is caused motor symptoms such as muscular spasm and tremor and alienation such as melancholia and insomnia. Because clinically symptoms are caused by dosage for long-terms, these medications are restricted. Drug such as dopamine receptor agnosists, anticholinergic, and Eldepryl, had been developed, but these dugs are restricted to prescribe by many side-effects.

Recently, natural substance extracted from medicinal plants is being studied for minimization of side-effect and relief of symptoms or cure by PD-related gene induction. Roots of *Panax ginseng* influence in CNS and have anti-psychotic activity, anti-spastic activity and tocolytic activity. Also, it had been reported protective effects against degeneration of neuron such as PD. Ginseng and ginsenoside, one of ginseng compound have reported various effect such as decrease of oxidative stress and preventive effects of ischemia in brain, spine damage and Amyotrophic Lateral Sclerosis (ALS). Kampen et al (2003) studied about protective effects of DArgic neuron by the ginseng extracts G115 in animal models of PD. Catechins, main compound of green, have been identified as potent free radical scavengers that can inhibit lipid peroxidation. Catechin in the treatment of PD and Alzheimer's disease (AD) has been primarily attributed to their anti-oxidant and anti-inflammatory activity. (-)-epigallocatechin gallate (EGCG), one of eight polyphenolic flavonoid-type compounds have been reported that increase nNOS *in vitro* (Frank S et al., 1999) and *in vivo* in hypoxic rats, and prevent oxidative stress by inhibiting the influx of calcium ions into the cell despite high levels of ROS, which has been shown *in vitro* (Chen L et al., 2003; Ishige K et al., 2001).

Herbs call all medicinal plant that have aroma and is useful for people. Herbs is over 2500 different species in all over the world and had used as treatment, insecticide and cosmetic from ancient. Through many studies, various physiological properties have found such as anti-oxidative activity, anti-microbial activity, anti-cancer activity, anti-tumor activity and raise of

immunity.

Rosemainus officinalis L. (Rosemary) is one of the most common traditional medicinal herbs. The fresh and dried leaves are used frequently in traditional Mediterranean cuisine as a flavoring agent and as a food. Historically, rosemary has been used as a medicinal agent to treat renal colic and dysmenorrhea. It has also been used to relieve symptoms caused by respiratory disorders and to stimulate the growth of hair. Extracts of rosemary are used in aromatherapy to treat anxiety-related conditions and to increase alertness. It have been reported various properties including anti-nociceptive effect (Gonalez-Trujano et al., 2007), anti-ulcerogenic effect (Corre^a Dias et al., 2000), treatment effect for diabetes mellitus and hyperpiesia (Tagraoui et al., 2007), anti-hepatotoxic activity (Galisteo et al., 2000), anti-thrombotic activity (Yamamoto J et al., 2005), anti-microbial activity (Bozin B et al., 2007), and anti-oxidative activity (Cheung S et al., 2007). Representative property of rosemary is antioxidant activity. Antioxidant activity of rosemary had reported more potent than Vitamin E. Phenolic compounds such as carnosol, carnosic acid and rosmarinic acid isolated from rosemary have various properties. Carnosol and carnosic acid have been reported potent anti-oxidative activity. Their anti-oxidative activity accounts for 90 % of anti-oxidative activity of rosemary. These compounds inhibit tumor initiation by chemical carcinogens *in vivo* (Huang et al., 1994; Singratary et al., 1991) and have radioprotective effect (Del Bano M.J., et al 2006). Carnosic acid induces nerve growth factor. Carnosol inhibit production of nitric oxide (NO) (Lo et al., 2002) and have anti-microbial effect (Moreno et al., 2006), anti-platelet activity (Lee et al., 2006), anti-carcinogen effect (Huang et al., 1994). It also has the protective effects against rotenone-induced neurotoxicity in cultured dopaminergic neuronal cells (Kim et al., 2006).

The purpose of this study was to investigate the protective effects of rosemary extract on H_2O_2-induced oxidative stress in human neuroblastoma SH-SY5Y cells in order to find a possible therapeutic application to degenerative disease, as well as provide new strategies for prevention and treatment of neurodegenerative disorders such as parkinson’s disease. In this

study, we examined the effects of neuronal protection by which rosemary extract protects SH-SY5Y cells from H_2O_2-induced oxidative damage. Therefore, we observed that rosemary-mediated neuronal cell protection in SH-SY5Y cell was involved in the attenuation of pro-apoptotic factor, which was induced by H_2O_2. Furthermore, we revealed that the increase of TH and AADC by rosemary extract were responsible for the dopamine produce in catecholamine biosynthesis. And finally, we observed that the increase of BDNF and GDNF, which were neurogrophic factor for neuronal cell survival and growth.

II. MATERIALS AND METHODS

II-A. Materials

Rosemarinus officinalis L. (Rosemary) was purchased from the Sangsoo herb land (480 Oicheon-ri, Buyong-myun, Cheongwon-gun, Chungbuk, Republic of Korea 363-944).

For cell culture, cytotoxicity and staining, human neuroblastoma cell line SH-SY5Y and from human primary transformed embryonal kidney cell line 293 obtained from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium:Nutrient Mix F-12 (1:1, D-MEM/F-12), Dulbecco's Phosphate Buffered Saline (DPBS), Fetal bovine serum (FBS) and Trypsin-EDTA solutions were purchased from the GIBCO-BRL (USA). Dimethyl Sulfoxide (DMSO), 3-4,5-Dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT), Hoechst 33324 and Rhodamine 123 (Rh 123) were purchased from Sigma-Aldrich Co. (USA). Cytotoxicity Detection Kit (LDH assay) was purchased from Roche Applied Science (Switzerland). DeadEnd^{TM} Fluorometric TUNEL System was purchased from Promega coporation (USA). Tissue culture dishes, flasks, multiplex 96 wells plate were purchased from the Nunc (Denmark).

For RT-PCR and PCR, Trizol^{TM} was purchased from the Invitrogen Co. (USA). Isopropanol, chloroform, diethyl pyrocarbonate (DEPC), 3-(N-morpholino) propanesulfionic acid (MOPs), formamide, formaldehyde, and ethidium bromide (EtBr) were purchased from the Sigma-Aldrich Co. (USA). Agarose was purchased from the Seakem® Cambrex (USA). Moloney-murine leukemia virus ribonuclease (M-MLV), oligo dT (deoxythymidine) primer, dNTPs (deoxynucleic acid triphosphate), Taq polymerase, specific primers (for TH, Actin, AADC, GDNF, BDNF, and Actin), and 100 bp DNA ladder were purchased from the BioNEER Co.

(Korea).

For Protein isolation and Western blot assay, Protease inhibitor cocktail was purchased from Calbiochem® (Germany). BCA protein assay kit was purchased from Pierce Co. (USA). RIPA buffer, Trizma®-Base, sodium chloride (NaCl), glycine, 2-mercaptoethanol, acylamide, bis-acylamide, and sodium dodecyl sulfate (SDS) and Bovine serum albumin (BSA) were purchased from the Sigma-Aldrich Co. (USA). Polyvinylidine difluoride (PVDF) was purchased from Pall life science (USA). Tween-20 was purchased from the Yakuri pure chemicals Co., Ltd (Japan). Prosieve® color protein marker and Prosieve® unstained protein marker were purchased from the Cambrex Co (USA). WEST-ZOL (plus) was purchased from Intron Biotechnology (Korea). X-ray film was purchased from Kodak (Japan). Anti-TH, Mouse monoclonal (0MA-04051) was purchase from Affinity BioReagents, Inc. (USA). Anti-BDNF, Rabbit polyclonal IgG (sc-546), Anti-GDNF, Rabbit polyclonal IgG (sc-7890), Anti-human caspase-9 p10, Rabbit polyclonal (sc-7885), Anti-human Bcl-2, Mouse monoclonal (sc-7382), Anti-Bax, Rabbit polyclonal (sc-6236), Anti-Bak, Rabbit polyclonal (sc-832) and secondary antibodies : HRP-conjugated Goat-anti-mouse IgG (sc-2054), HRP-conjugated Goat-anti-rabbit IgG (sc-2055) were purchase from Santa Cruz Biotechnology. Anti-Actin, Mouse monoclonal (V10275) was purchase from Biomeda crop (USA). Anti-Bak, Mouse monoclonal (AM03) was purchase from Oncogene. Anti-human caspase-3, mouse monoclonal (6110322) was purchase form BD bioscience (USA).

II-B. Methods

1. Preparation of rosemary extract

Rosemary leaves were extracted with 70% EtOH at room temperature (1 week). The

extracted solution was filtered and evaporated on a rotatory evaporator under vaccum and further freeze-dried. Rosemary extract were dissolved in phosphate buffered saline (PBS) at a concentration of 50 ㎎/㎖, and filtered through 0.2 ㎛ membrane filter (Millipore, Bedford, MA, USA) and then stored at 4°C.

2. Cell culture

Human neuroblastoma cell line SH-SY5Y was grown in mixture of Dulbecco's Modified Eagle Medium:Nutrients Mix F-12 (1:1, D-MEM/F-12) and human transformed embryonal kidney cell line 293 was grown in Dulbecco's Modified Eagle Medium (DMEM). These media contain 1.5 g/ℓ sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids and 15 mM HEPES. This medium were supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/㎖ penicillin, and 100 ㎍/㎖ streptomycin. All cells were incubated in 37 ℃, 5% CO_2, 90% humidity.

3. Cell Viability

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay was used for determination of cell viability (Altman et al., 1993). The cells were first cultured in T-75 culture flasks. After cells grown up to 70% confluence, cells were seeded at 1×10^4 cells/well into 96 well. After incubation one day, cells were treated with rosemary extract (0, 1, 10, 50, 100 ㎍/㎖) for 24, 48 and 72 hr, and Hydrogen peroxide (0, 1, 10, 50, 100, 150, 300 μM) for 24 hr. After incubation, MTT reagent (final concentration is 0.5 ㎎/㎖) was added to each well. The plate was incubated for 3 hr at 37°C. The medium was removed and then DMSO 100 ㎕ was added into each wells to dissolve the formazan crystal. The absorbance was measured at 540 nm using VERSAmax Microplate Reader (Molecular Devices, USA). To

determine the protection effect by rosemary extract, cells were treated with H_2O_2 and rosemary extract for 24 hr, and the cell viability was determined by MTT assay.

4. Total RNA Extraction

Total RNA was extracted from SH-SY5Y cells using TRIzol reagent (Invitrogen, Life Technologies, USA) following the protocol provided by company, which is based on the method described by Chomczynski (1993). After havesting, cells were lysed in TRIzol reagent by repetitive pipetting. Homogenization was followed by phase separation by addition of 200 ㎕ of chloroform and centrifugation at 12,000 ×*g* at 4℃ for 15 min. The aqueous phase was then transferred to a centrifuge tube containing 500 ㎕ isopropanol and incubated at room temperature for 10 min. The resulting mixture was then centrifuged at 12,000 x*g* at 4℃ for 10 min. After being washed with 75% ethanol the visible RNA pellet was dried and suspended in a small volume of DEPC-treated water. The RNA concentration was determined by optical density (OD) at 260 nm. Integrity of the RNA was verified by gel electrophoresis of ~ 4 ㎍ RNA on a 0.8% agarose denaturing gel. Isolated intact samples were visualized both 28S and 18S (ribosomal RNA) bands. Samples were stored at -20 ℃ until subsequent analyses.

5. Reverse transcription (RT)-Complement DNA (cDNA) preparation

For the cDNA synthesis, 4 µg of total RNA samples were added to a reaction containing 0.1 pmol oligo (dT) primer, 1 X buffer, 0.1 M DTT, 10 mM dNTPs, 20 units of RNase inhibitor (Amersham) and 200 units of MM-LV, the volume of reaction is up to 40 µℓ volume by DEPC water. The reaction mixture was incubated for 60 min at 42°C, followed by 5 min at 70°C to inactivate the reverse transcriptase enzyme. Quality of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -20^{0}C for futher using.

6. Polymerase chain reaction (PCR)

The cDNA was amplified using Taq DNA polymerase. PCR reaction was performed in 20 ㎕ of the total volume using 10 pmol of corresponding primers (Table 1) The cDNA was amplified under the following reaction conditions: denaturation at 94℃ for 30 sec, annealing at 64℃ for TH, and denaturation at 94℃ for 30 sec, annealing at 64℃ for 45 sec for TH, 56℃ for BDNF, at 62℃ for GDNF, and AADC and at 56℃ for β-actin, polymerization at 72℃ for 30 sec. The cyclic process was performed 35 times for TH, AADC, BDNF, and GDNF and 25 times for β-actin. PCR products were analyzed on 1.2% agarose gel and visualized by EtBr. Staining intensity of individual bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems Ltd.).

7. Cytotoxicity assay by measurement of LDH

Lactate dehydrogenase (LDH) release was calculated for cellular toxicity. The cells (1×10^4 cells/well) were seeded in 96 well plates and were incubated with Hydrogen peroxide and roseemary extracts for 2, 4 and 8 hr. The cell medium was transferred into new 96 well plate. To calculate the LDH release, add 100 ㎕ reaction mixture to each well according to manual. Absorbance was measured at 490 nm using Versamax Microplate Reader (Molecular Devices, USA).

8. Total protein isolation and protein concentration determination

For isolate proteins, we were harvested cells using scraper. The cell pellets were lysed for 30 min at ice in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% NP-

40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). After incubation, lysates were centrifuged for 15 min at 13000 x g at 4℃. The supernatants was used as protein fractionation

Protein concentration was measured using a BCA protein assay kit (Pierce Co., USA). Bovine serum albumin (BSA) (2 ㎍/㎕) was used as the standard. 50 parts of BCA solution A was mixed with 1 part of BCA solution before using 10 min. BSA (0, 2, 4, 6, 8 ㎍/㎕) and 2 ㎕ of each sample was inserted into 96 well plate wells. 200 ㎕ of the working reagent was added to each well. Plate was covered with foil and incubated for 30 min at room temperature in the dark. The absorbance was measured at 562 ㎚ on Versamax Microplate Reader (Molecular Devices, USA).

9. Western blot analysis

After SDS-electrophoresis (12% separating gel, Proteins were transferred to PVDF membrane for 2 hr at 4°C. Membrane was blocked with 3% BSA in TBS-T for overnight at 4°C. Primary antibody was used for 2 hr at 4°C and secondary antibody was used for 2 hr at room temperature. The membrane was washed 6 x 5 min with TBS-T between primary and secondary antibody. After incubation with secondary antibody conjugated with HRP, the membrane was washed 4 x 5 min with TBS-T. To detect the bands, the membranes were developed with WEST-ZOL® (plus) Western blot detection system (Intron Biotechnology, Inc., Korea) and were exposed on X-ray film (BioMax MS-1, Eastman Kodak, USA). Quantification of band was performed using Gel Quant (DNR Bio-Imaging Systems Ltd., USA).

10. Antioxidant enzyme assays

After treatment, the cells were washed twice with PBS and lysed in 200 ㎕ of lysis buffer

(50mM Tris–HCl (pH 7.5) and 0.5% Triton X-100). The supernatants were obtained by centrifugation at 12000 rpm at 4°C for 15 min, and antioxidant enzyme activity assays were performed on the resulting supernatants. For all the enzymatic calculations, protein was determined by the BCA protein method, using bovine serum albumin (BSA, Sigma, USA) as the standard.

10.1. Assay of SOD (superoxide dismutase) activity

The activity of SOD was determined by the method of Beauchamp and Fridovich. The reaction mixture contained 50 mM carbonic buffer (pH 10.2), 0.1 mM EDTA, 0.1 mM Xanthine and 0.025 mM NBT. The absorbance was read as decrease at 560 nm against the blank

10.2. Assay of CAT (Catalase) activity

The activity of CAT was measured according the method of Beers and Sizer. The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2 and enzyme extracts. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm.

11. Staining of nuclear DNA with Hoechst 33342

Hoechst 33342 is substitute for nucleic acid staining (Lieberthalet al., 1998), and is used for demonstrates nuclear condensation in apoptosis-induced cell. Hoechst 33324 was dissolved in PBS. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and then washed with PBS 2 × 5 min. Hoechst 33342 (final concentration : 10 ㎍/㎖) was added to the cells. After incubated for 30 min at 37℃, the cells was washed with PBS, and then absorbed by

fluorescence microscopy (Nikon, Eclipse TE 2000-U, Japan) at 480 nm.

12. Mitochondria membrane potential measurement with Rhodamine 123

Rhodamine 123 (Rh 123), a mitochondria-specific fluorescent probe is used for measurement of mitochondrial membrane potential change (Johnson et al., 1980). Rh 123 was dissolved in PBS. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and then washed for 2 × 5 min with PBS. Rh 123 (final concentration: 1 ㎍/㎖) was added to the cells. After incubated for 1 hr at 37℃, the cells was washed with PBS, and then absorbed by fluorescence microscopy (Nikon, Eclipse TE 2000-U, Japan) and quantitated by Spectra Max Gemini EM fluorometer (Molecular Devices, USA).

13. Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling (TUNEL) and Propidium iodide staining

TUNEL assay was used for measure the fragmented DNA of apoptotic cells, important biochemical hallmark of apoptosis (Gavrieli Y. et al., 1992). Recombinant terminal deoxy-nucleotidyl transferase (rTdT) binds the fragmented DNA of apoptotic cells. PI is membrane impermeant and generally excluded from viable cells. PI bind to DNA and is used to detect dead cells in population. This assay was performed using DeadEnd™ Fluorometric TUNEL System (Promega coporation, USA) and propidium iodide (PI). The cells (1 x 3 cell/well) were seeded in 8 well chamber slide and were incubated for 1 day. After treated Hydrogen peroxide and rosemary extract for 4 and 8 hr. The cells were fixed by 4 % paraformaldehyde. The slides were washed by PBS for 5 min at room temperature. To permeabilize cells, the slides were immersed in 0.2 % Triton X-100 in PBS for 5 min. After rinse slide in PBS, staining using rTdT and PI was performed according kit manual. The cells absorbed by fluorescence

microscopy (Nikon, Eclipse TE 2000-U, Japan).

14. Statistical evaluation

Data were analyzed using the Sigma Plot software. All data were expressed as mean ± standard errors of mean (S.E.M.) of three independent experiments. Results were evaluated by Student's t-test or by ANOVA. A value of $P \leq 0.05$ was considered significant.

Table 1. List of primers used for PCR

Genes	Primer sequences	Nucleotides Position	RT-PCR product	Gene reference
TH	Forward: GCTGTCACGTCCCCAAGGTT Reverse: TCAGACACCCGACGCACAGA	No. 93~472	380 bp	M10244
AADC	Forward: CTTCAGATGGCAACTACTCC Reverse: CTTCGGTTAGGTCAGTTCTC	No. 684~1028	345 bp	U31884
BDNF	Forward: AACGAAGAAAACCATAAGGA Reverse: GTCTATCCTTATGAATCGCC	No. 244~688	465 bp	AY176065
GDNF	Forward: CAGAGAATTCCAGAGGGAAA Reverse: TACATCCACACCGTTTAGCG	No.348~681	334 bp	L15305
β-actin	Forward: CCTCTATGCCAACACAGT Reverse: AGCCACCAATCCACACAG	No.957~1111	155 bp	BC063166

Ⅲ. RESULTS

Ⅲ-A. Effects on cell viability of rosemary extract in SH-SY5Y

The effect of rosemary extract on cell viability was determined by MTT assay in two different types of cell lines; 293, which is originated from human primary transformed embryonal kidney cells and SH-SY5Y, which is human neuroblastoma cells. The cells were exposed to different concentrations (0, 1, 10, 50 and 100 ㎍/㎖) of rosemary extract for 72 hr. The viability of 293 and SH-SY5Y cells was decreased in dose-dependent. The viability of cells were sharply decreased at 100 ㎍/㎖ of rosemary extract. We also examined the time-dependent effects of rosemary extract on the cell viability. The cells were treated with 10 ㎍/㎖ rosemary extract for 24, 48 and 72 hr and cell viability was not decreased par·ticularly (Fig. 3). Rosemary extract had no considerable cytotoxic effect on 293 and SH-SY5Y cells.

Ⅲ-B. Effect of rosemary extract on gene induction and protein expression of catecholamine enzymes and neurotrophic factor

1. Induction of TH and AADC mRNA level

As shown in Fig. 4, we examined the dose-dependent and time-dependent effects of rosemary extract on TH and AADC mRNA levels in SH-SY5Y. The cells treated with 10, 20, 40 and 100 ㎍/㎖ for 4 hr. TH was induced by 20, 40 ㎍/㎖ rosemary extract, but AADC was not induced particularly. Because tyrosine is converted to L-dopa by TH, and L-dopa is converted to dopamine by AADC, it was inferred from this result that AADC mRNA expression was induced later than TH expression. Therefore, we investigated the time-dependent effects of rosemary extract. The cell was treated with 10 ㎍/㎖ of rosemary extract

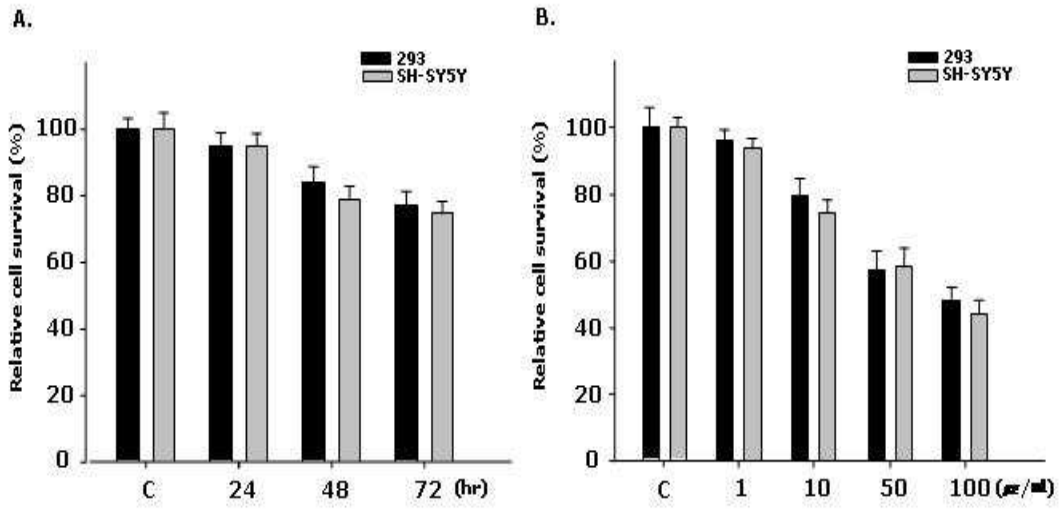

Fig. 3. Effect of rosemary extract on cell viability. The effect of rosemary extract on cell viability was determined by MTT assay in two different types of cell lines; 293 cells and SH-SY5Y. We examined the time-dependent effects of rosemary extract. The cells were treated with 10 ㎍/㎖ rosemary extract for 24, 48 and 72 hr (A). Also, to evaluate dose-dependent effect of rosemary extract, the cells were exposed to different concentrations (0, 1, 10, 50 and 100 ㎍/㎖) for 72 hr (B). The viability of untreated control cells was defined as 100%.

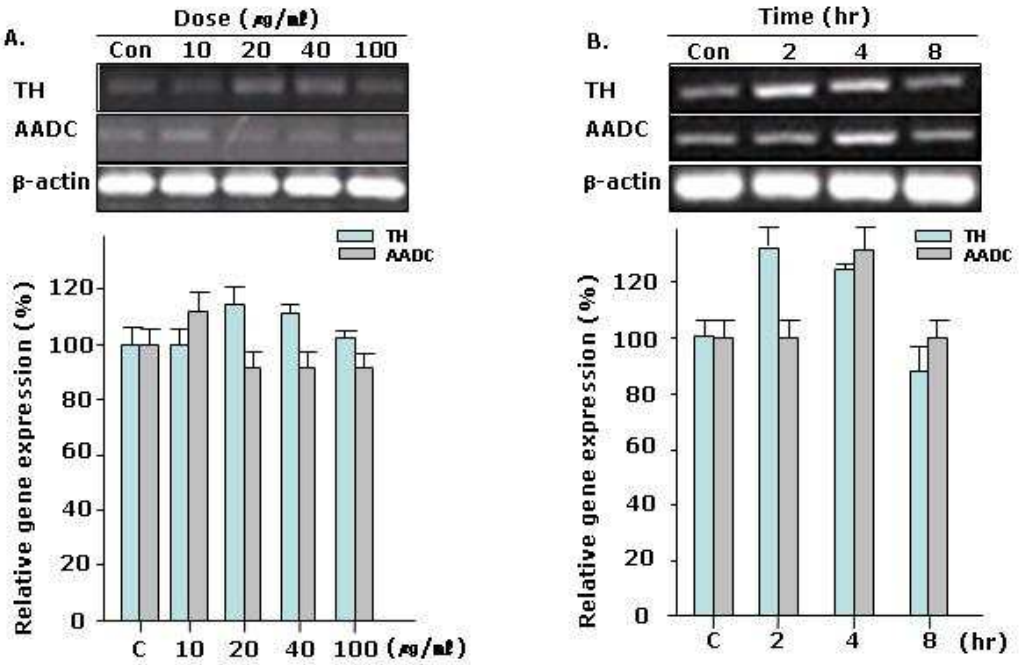

Fig. 4. Induction of genes encoding of TH and AADC by rosemary extract treatment. SH-SY5Y cells were treated with rosemary extract (A) dose-dependently (0, 10, 20, 40, 100 ㎍/㎖) for 4 hr and (B) time-dependently (2, 4 and 8 hr) with 10 ㎍/㎖ of rosemary extract. RNA isolated from individual cells was analyzed separately by RT-PCR. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of TH and AADC mRNA.

for 2, 4 and 8 hr. TH was highly induced After 2 and 4 hr treatment. AADC was induced after 4 hr. But after 8 hr treatment, TH and AADC were not induced particularly compared with control group.

2. Effect of rosemary extract on BDNF and GDNF gene induction

As shown in Fig. 5, we examined the dose-dependent effects of rosemary extract on BDNF and GDNF mRNA levels in SH-SY5Y. The cells treated with 10, 20, 40 and 100 ㎍/㎖ for 4 hr. BDNF and GDNF were induced at the dose of 10 ~ 40 ㎍/㎖. Maximum level of induction was observed at the dose of 40 ㎍/㎖. But BDNF and GDNF were decreased at the dose of 100 ㎍/㎖ compared with control group. This result was inferred that BDNF and GDNF gene induction was inhibited by high concentration of rosemary extract in cell. GDNF was induced lower than BDNF induction level after 2, 4 and 8 hr.

Also, we examined the time-dependent effects of rosemary extract. The cell were treated with 10 ㎍/㎖ of rosemary extract for 2, 4 and 8 hr. BDNF was induced over 40% after 2 hr, and induction was highly continued after 4 hr. GDNF was induced over 10% after 2 hr, and induction was continued after 4 hr. After 8 hr treatment, the induction of BDNF and GDNF were decreased sharply. These results suggest that BDNF and GDNF gene expression was up-regulated only in the early stage of treatment.

3. Effect of rosemary extract on TH and BDNF protein expression

To determine rosemary-induced expression of TH and BDNF in protein levels, western blot analysis was performed. Because AADC and GDNF were induced lower than TH and BDNF, TH and BDNF were analyzed on protein lelvel. The cells were treated with 10, 20, 40 and 100 ㎍/㎖ for 4 hr. As shown in Fig. 6, protein level of TH was increased over 30% at the dose of 10

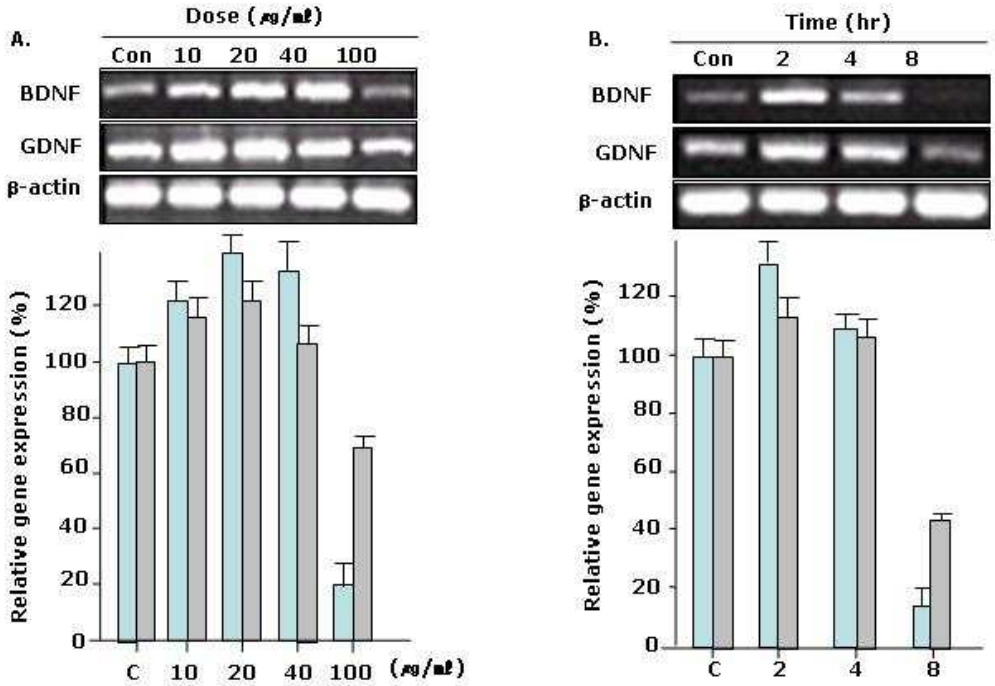

Fig. 5. Induction of genes encoding of BDNF and GDNF by rosemary extract treatment. SH-SY5Y cells were treated with rosemary extract (A) dose-dependently (0, 10, 20, 40, 100 ㎍/㎖) for 4 hr and (B) time-dependently (2, 4 and 8 hr) with 10 ㎍/㎖ of rosemary extract. RNA isolated from individual cells was analyzed separately by RT-PCR. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of BDNF and GDNF mRNA.

㎍/㎖, BDNF was increased over 20% at the dose of 20 ㎍/㎖. Both TH and BDNF protein levels were decrease at the dose of 40 and 100 ㎍/㎖. The protein level expression of TH and BDNF is consistent with the results from mRNA induction (Fig. 6).

Ⅲ-D. Effects on cell viability of H_2O_2 in SH-SY5Y

The effect of H_2O_2 on cell viability was determined by MTT using SH-SY5Y, which is human neuroblastoma cell. The cells were exposed to different concentrations (0, 1, 10, 50, 150, 300 μM) for 24 hr. For 10 μM H_2O_2 treated cells, a significant decrease in cell viability was seen after 24 hr exposure. Cell viability was decreased to 60 ~ 50% at 150 μM, and to 10 ~ 20% at 300 μM H_2O_2 (Fig. 7).

Ⅲ-E. Effect of rosemary extract on H_2O_2 –induced cytotoxicity

To evaluate the protective effects of rosemary against H_2O_2–induced loss of cell viability, SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4, 8 and 12 hr and cell viability was measured by MTT assay. Treatment of SH-SY5Y cells with H_2O_2 caused cell death in a time dependent manner. Following incubation of the cells with H_2O_2, cell viability was reduced from 90% to 68%. Cells treated with rosemary extract showed protecting effects on the cell viability against the damage caused by H_2O_2. Cell viability was increase from 75% to 92% compared with H_2O_2-exposed cell group. Rosemary extract rescued the viability of cells against the neurotoxicity induced by H_2O_2 (Fig. 8).

To further investigate the protective effect of rosemary extract, the release of LDH was measured. LDH release is increasesd as the number of dead cells increases. Release of LDH was observed after 2 hr exposure to 150 μM H_2O_2. Compared with H_2O_2-exposed cell group, rosemary extract-treated cells was decreased release of LDH (Fig. 8). The protective effect of

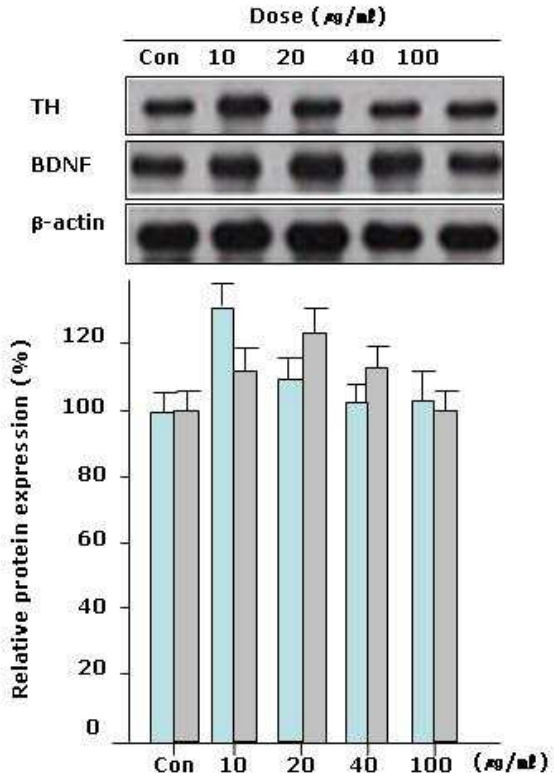

Fig. 6. Protein expression of TH and BDNF by rosemary extract treatment. SH- SY5Y cells were treated with rosemary extract (A) dose-dependently (0, 10, 20, 40, 100 ㎍/㎖) for 4 hr. Protein was isolated from individual cells was analyzed separately by Western blot. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of TH and BDNF protein.

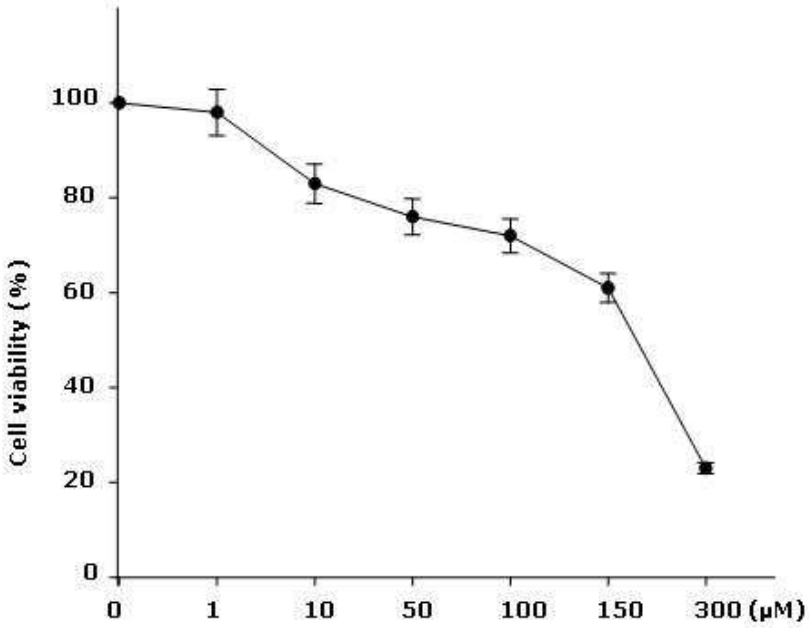

Fig. 7. Effect of H_2O_2 on cell viability in SH-SY5Y. The cells were exposed to different concentrations (0, 1, 10, 50, 150, 300 μM) of H_2O_2 for 24 hr. Cell viability was by MTT assay. The viability of untreated control cells was defined as 100%. Results shown are means ± SD (n=3).

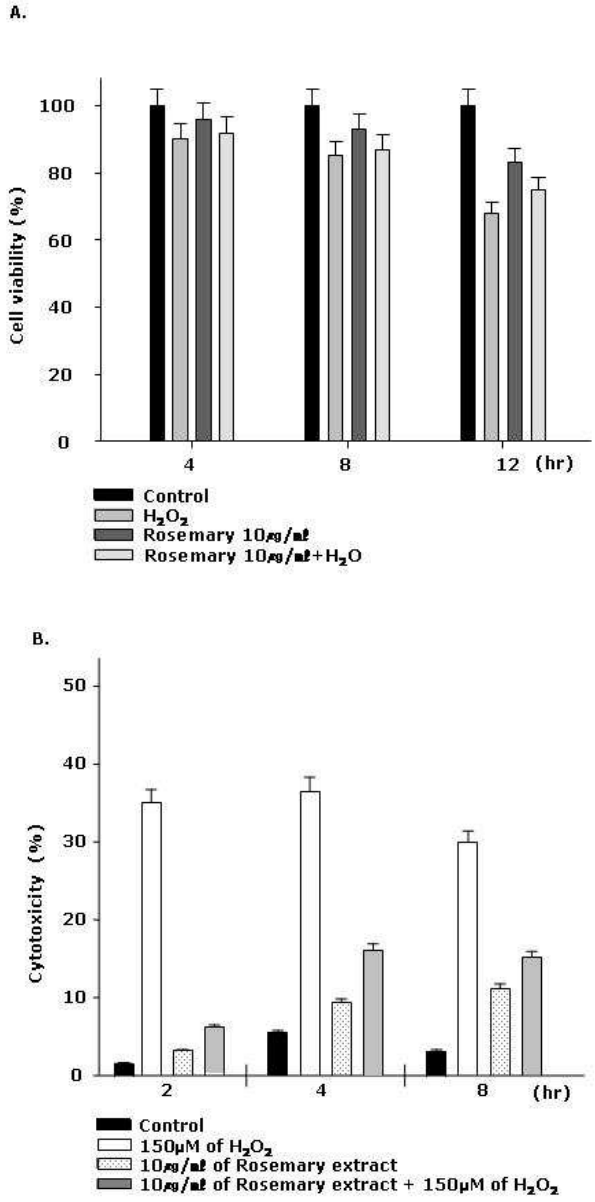

Fig. 8. Effect of rosemary extract on H_2O_2 –induced cytotoxicity. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4, 8 and 12 hr and cell viability was measured by MTT assay (A) and LDH assay (B). The viability of untreated control cells was defined as 100%. Results shown are means ± SD (n=3).

rosemary extract on H_2O_2–induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay

Ⅲ-F. Effects of rosemary extract on antioxidant enzyme activities in H_2O_2-treated cells

After H_2O_2 treatment, the activities of decreased to 65% and 53%, respectively. Treatment of rosemary extract increased SOD and CAT activity to 78% and 81% (Fig. 9). Our results have shown that rosemary extract decreases H_2O_2-induced cell death in SH-SY5Y cells by inducing antioxidant enzymes. The inhibitory effect of rosemary extract by SOD and CAT activity suggests that the cytotoxic effect of H_2O_2 may be mediated by oxidative stress in SH-SY5Y cells. Based on these results, we postulate that the anti-oxidative properties of rosemary extract may contribute to the protection of SH-SY5Y cells from H_2O_2-induced cell death.

Ⅲ-G. Protective effect of rosemary extract on H_2O_2 –induced apoptosis

1. Effect of rosemary extract on protein expression of catecholamine enzyme

We examined the effect of rosemary extract on TH and AADC expression. The cells were treated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr. Treatment of H_2O_2 decreased TH and AADC expression level. However, rosemary extract treatment increased TH and AADC expression level over 40% and 20% compared with H_2O_2-treated group (Fig. 10).

2. Inhibition and induction of pro-apoptotic factor and anti-apoptotic factor

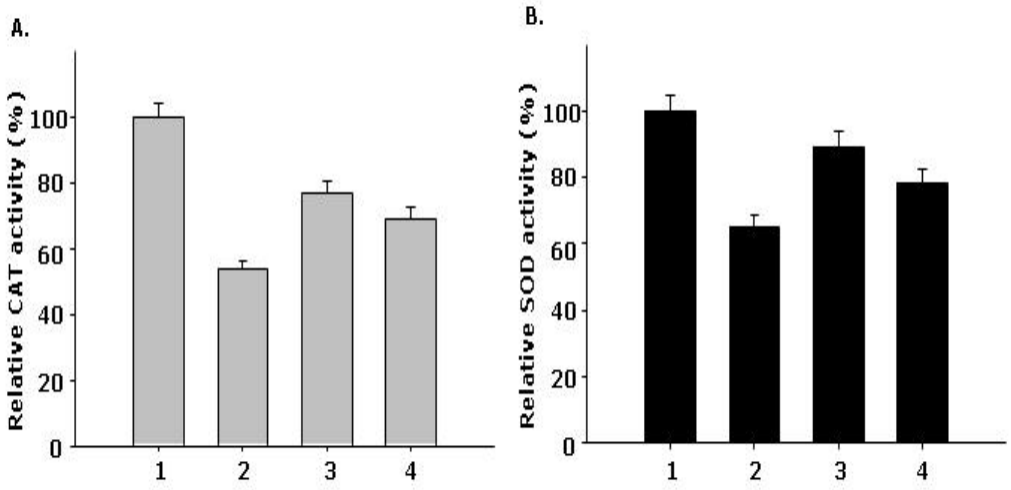

Fig. 9. Effects of rosemary extract on antioxidant enzyme activities in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr and then mearused the SOD activity and CAT activity. The acitivity of untreated control cells was defined as 100%. 1: control, 2: H_2O_2, 3: rosemary extract, 4: rosemary extract / H_2O_2.

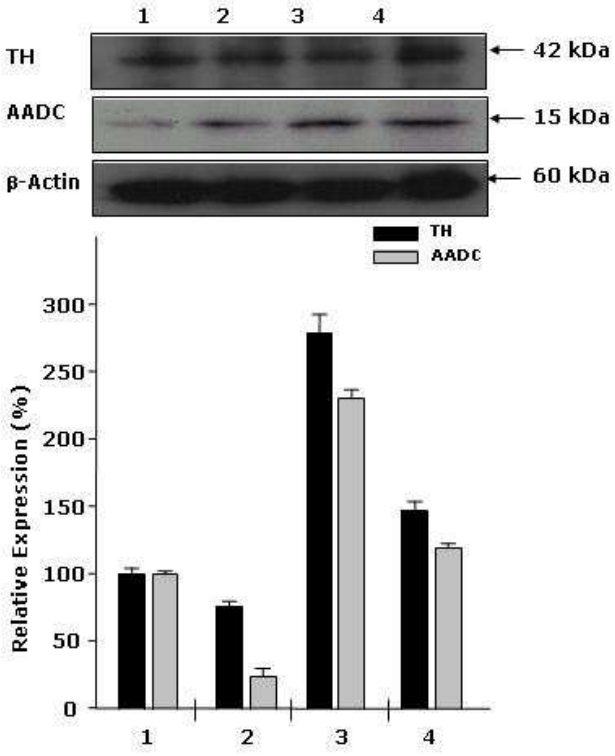

Fig. 10. Effects of rosemary extract on the expression of TH and AADC in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr. Expression of proteins were analyzed by western blotting. The values were normalized to β-actin and expressed as mean ±SD (n=3). A representative result from three independent experiments is shown. 1: control, 2: H_2O_2, 3: rosemary extract, 4: rosemary extract/ H_2O_2

We investigated whether pro-apoptotic and anti-apoptotic factor gene expression were affected by rosemary extract. Pro-apoptotic factor Bax and Bak, and anti-apoptotic factor Bcl-2 protein levels were measured by western blot. As shown in Fig. 11, H_2O_2 increased level of Bax and Bak compared with control group. However, the treatment with rosemary extract decreased the Bax and Bak protein level. On the contrary, treatment with rosemary extract more increased level of Bcl-2 than the level of Bcl-2 in the H_2O_2-treated cell (Fig. 12).

Because caspase-3 and -9 is an important role in apoptosis, its expression level was examined. As shown in Fig. 6. Caspase-3 and -9 was increased by treatment with H_2O_2 expression. Addition of rosemary extract attenuated H_2O_2-induced caspase-3 and -9 expression (Fig. 13).

3. Effect on H_2O_2-induced nuclei morphological change

Changes in nuclear morphology after H_2O_2 treatment were assessed by staining with the membrane-permeable DNA-binding dye Hoechst 33342. Most of control cells had uniformly stained nuclei. After treatment 150 μM H_2O_2, the cells were induced nuclei fragmentation with condensed chromatin and bright staining in morphology. However, the addition of rosemary extract prevented the changes of nuclei morphology induced by H_2O_2 (Fig. 14).

4. Effect on decrease of H_2O_2-induced mitochondrial membrane potential

As shown in Fig. 15, when SH-SY5Y cells were exposed to 150 μM H_2O_2 for 4 and 8 hr, the cells of mitochondrial membrane potential was rapidly reduced, which was detected by a mitochondrial specific probe, Rh 123. H_2O_2 treatment decreased in the percentage of cells with Rh 123 staining from 71% to 77%. But, treatment with rosemary extract increased from 83% to 88% of mitochondrial membrane potential as compared with H_2O_2–treated cells. This result showed that

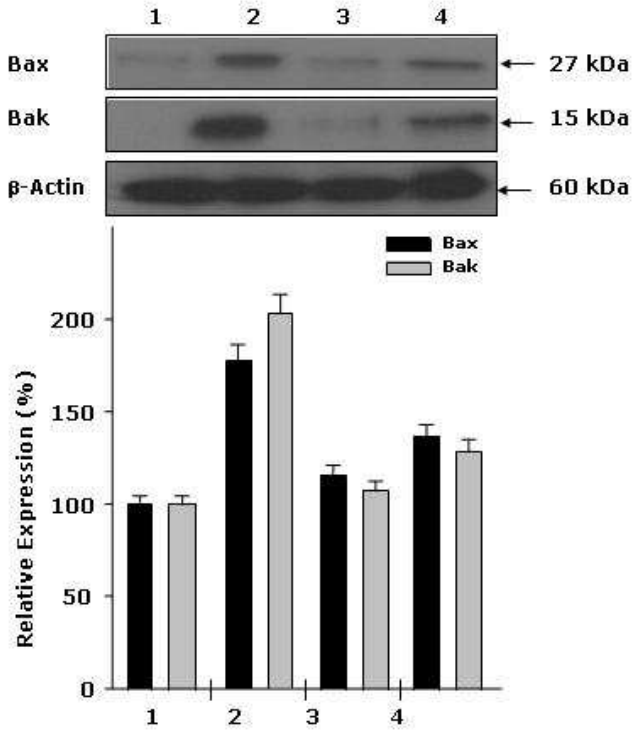

Fig. 11. Effects of rosemary extract on the Bax and Bak protein expression in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr. Proteins were analyzed by western blotting. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of Bax and Bak protein in the control group taken as 100%. A representative result from three independent experiments is shown. 1: control, 2: H_2O_2, 3: rosemary extract, 4: rosemary extract/ H_2O_2

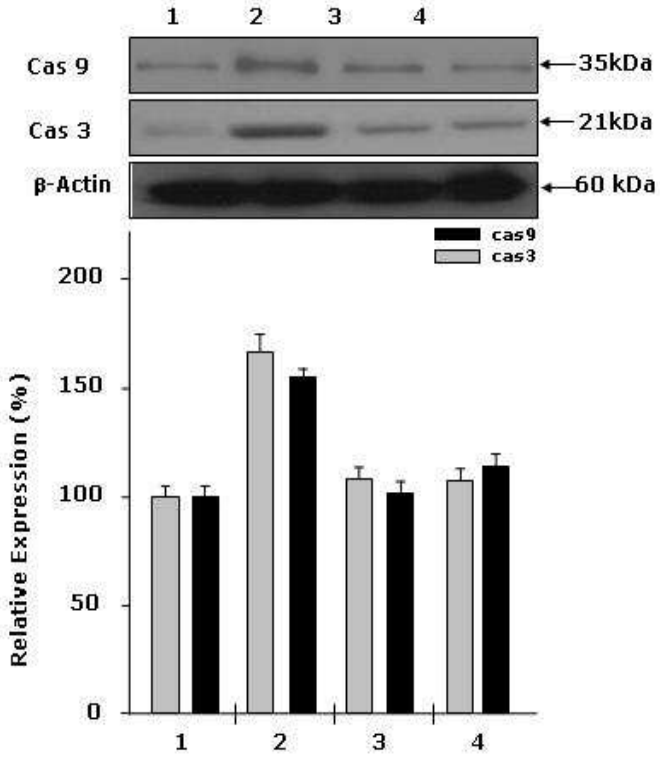

Fig. 12. Effects of rosemary extract on the caspase-3 and -9 protein expression in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr. Proteins were analyzed by western blotting. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of caspase-3 and -9 protein and the control group was taken as 100%. A representative result from three independent experiments is shown. 1: control, 2: H_2O_2, 3: rosemary extract, 4: rosemary extract / H_2O_2

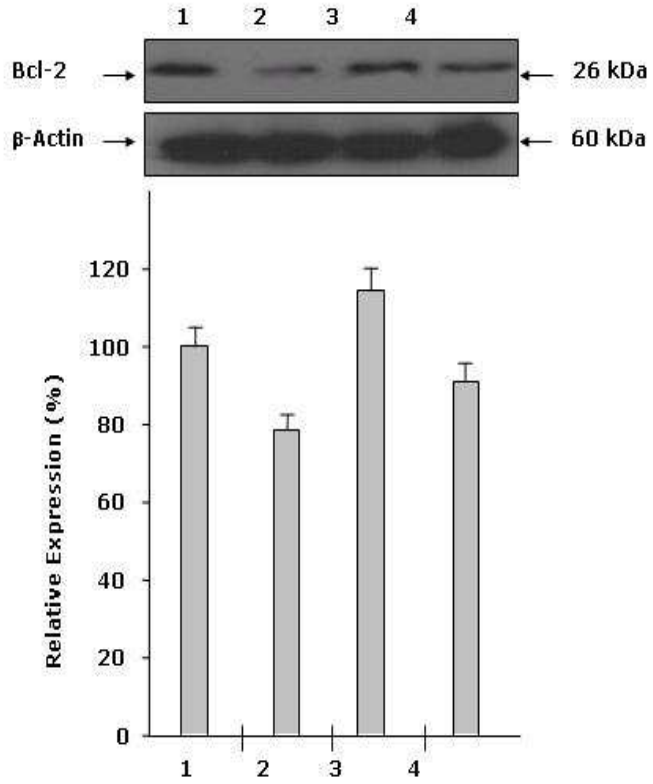

Fig. 13. Effects of rosemary extract on the Bcl-2 protein expression in H_2O_2-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 150 μM H_2O_2 with or without 10 ㎍/㎖ rosemary extract for 4 hr. Proteins were analyzed by western blotting. The values were normalized to β-actin and expressed as mean ±SD (n=3) with levels of Bcl-2 protein and control group taken was as 100%. A representative result from three independent experiments is shown. 1: control, 2: H_2O_2, 3: rosemary extract, 4: rosemary extract/ H_2O_2

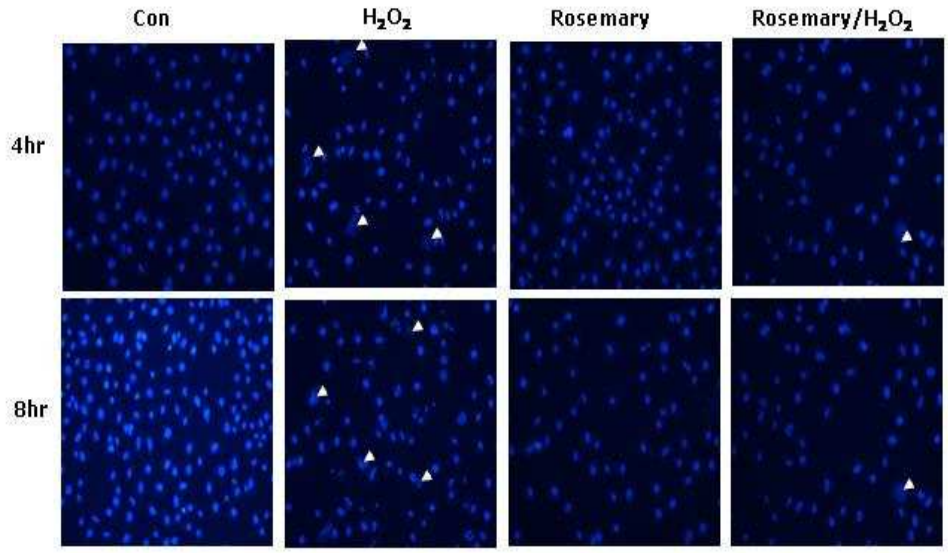

Fig. 14. Inhibition of H_2O_2-induced nuclei morphology changes. Cells were treated with 10 ㎍/㎖ rosemary extract followed by 150 μM H_2O_2 for 4 and 8 hr respectively. The cells were stained with Hoechst 33342 and analyzed by fluorescent microscopy.

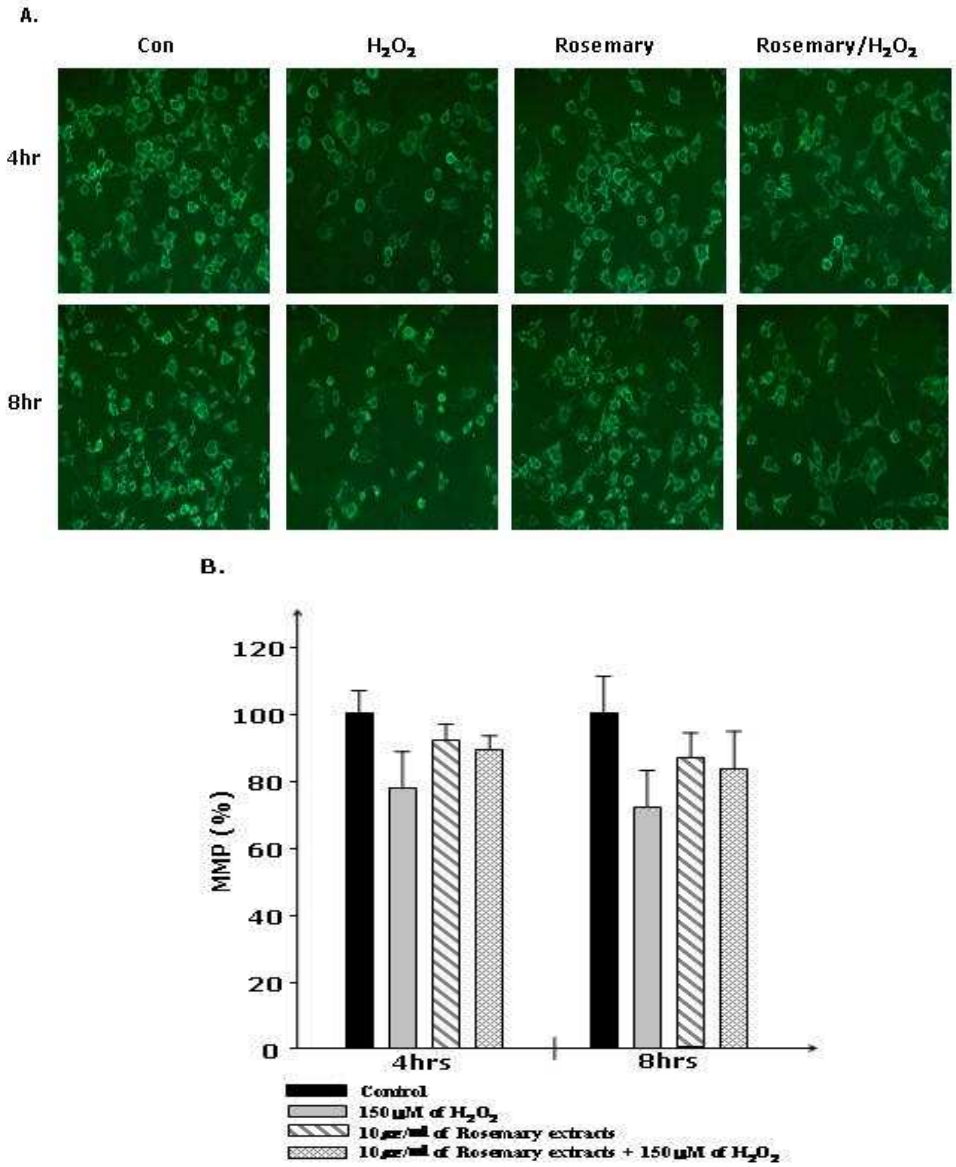

Fig. 15. Effects of rosemary extract on H_2O_2-induced reduction of mitochondrial membrane potential. (A) SH-SY5Y cells were treated with 10 ㎍/㎖ rosemary extract followed by 150 μM H_2O_2 for 4 and 8 hr repectively. The reduced fluorescence of Rh 123 was determined as the reduced mitochondrial membrane potential. The results were quantified after count the reduced MMP cells versus total cells and expressed as percentage (B).

rosemary extract suppressed the H_2O_2–induced decrease of mitochondrial membrane potential.

5. Effect on TUNEL positive-staining in H_2O_2-treated cells

Using the TUNEL assay, we observed apoptotic cell in treated cells. TUNEL assay is a common method for detecting DNA fragmentation that results from apoptosis. We investigated apoptosis following 4 hr and 8 hr incubation with 150 μM H_2O_2. Treatment with only H_2O_2 increased TUNEL-positive cells after 4 hr and 8 hr. However, treatment with rosemary extract reduced the number of TUNEL-positive cells (Fig. 16). These studies demonstrated that rosemary extract decreased levels of cell death and DNA fragmentation of cells

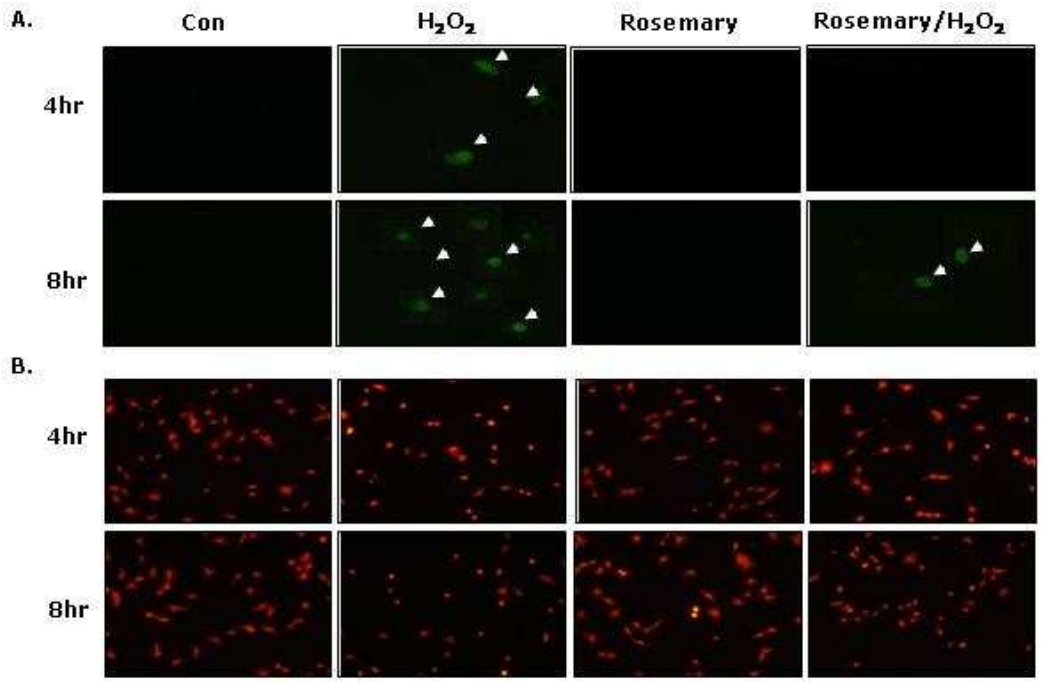

Fig. 20. TUNEL staining of H_2O_2-treated cell with/without rosemary extract. Cells were treated with 10 ㎍/㎖ rosemary extract respectively followed by 150 μM H_2O_2 for 4 and 8 hr. Cells were then washed and fixed before a TUNEL assay was performed as manual. The nuclei of apoptotic cells were labeled by TUNEL with green fluorescence (A) and for identifying dead cells in a population, cells were labeled by PI with red fluorescence (B).

Ⅲ. DISCUSSION

Many studies have shown that the oxidative stress is a major cause of neurodegenerative disorders. Recently, many studies demonstrated that oxidative stress occurs PD. Oxidative stress induces ROS such as hydrogen peroxide and superoxide anion. These induce mitochondrial dysfunction, protein misfolding and genetic mutation, and finally cause cell death. So, suppression of ROS by antioxidant may be effective in inhibition of oxidative stress-induced cell death. So, many researchers have been studied to research for natural antioxidant with neuroprotective effects without side-effects. *Rosemarinus officinalis* L. (Rosemary) is one of the most common traditional medicinal herbs and has been reported to be a strong oxidant. In this study, we investigated protective effects using antioxidant activity of rosemary against H_2O_2–induced cell death.

PD is characterized by a profound loss of DArgic neurons in the SNc. Loss of DArgic neurons decreased catecholamine pathway-related enzyme such as TH and AADC and ultimately decreased dopamine in SNc. Dopamine is made in SNc of the striatum and one of three main neurotransmitters called catecholamines. TH converted tyrosine to L-DOPA. L-DOPA is converted by AADC to dopamine. So, TH is important enzyme for dopamine biosynthesis. In this study, rosemary extract demonstrated that induced gene of catecholamine enzyme and neurotrophic factors. Gene induction of TH and AADC was increased for 2 and 4 hr. However, after 8 hr treatment, TH and AADC were not induced. BDNF and GDNF were induced for 2 hr and induction was continued for 4 hr. After 8 hr treatment, the induction of BDNF and GDNF were decreased sharply. Protein level of TH and BDNF were increased after treatment of rosemary extract, and these results were consistent with the results from mRNA induction. These results suggest that these gene expressions were up-regulated only in the early stage of treatment.

Distinct cytotoxicity was not found in the SH-SY5Y cells and 293 cells treated with rosemary extract. The treatment of rosemary extract didn't decrease significantly cell viability in dose- and time-dependent. However, after treatment with 150 μM H_2O_2 alone, cell viability was reduced. Treatment of rosemary extract increased SH-SY5Y cell viability compared with only H_2O_2-treated cells. We observed decrease of H_2O_2-induced cell death by measurement of the release of LDH on same condition with MTT assay. Release of LDH is consistent with the results from MTT assay. We suggest that rosemary extract have protective effect against H_2O_2-induced cytotoxicity and is worth further research to determine its therapeutic potential. We also investigated neuroprotective effect of rosemary extract thorough the expression level of TH and AADC in H_2O_2-treated cells using western blot. TH and AADC expression level is increased by treatment of rosemary extract compared with H_2O_2-treated cells.

ROS such as hydrogen peroxide and superoxide anion induce cell damage, and lead to apoptotic cell death. The cells have several antioxidant mechanisms for a detoxifying system to prevent damage caused by ROS. In this respect, CAT and SOD maintain low-level intracellular superoxide. CAT protects the cell from hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. SOD detoxifies peroxides such as hydrogen peroxide using ascorbate. In this study, Relative activities of SOD and CAT were decreased after H_2O_2 treatment. The inhibitory effect of H_2O_2 on SOD and CAT expression suggests that the cytotoxic effect of H_2O_2 may be mediated by oxidative stress in SH-SY5Y cells. But after rosemary extract treatment, SOD and CAT increased compared with H_2O_2-treated group cells. Our present results have shown that rosemary extract decreases H_2O_2-induced cell death in SH-SY5Y cells by inducing antioxidant enzymes. Based on these findings, we postulate that the anti-oxidative properties of rosemary extract may contribute to the protection of SH-SY5Y cells from H_2O_2-induced toxicity.

Apoptosis is process of cell death characterized by cell shrinkage, DNA fragmentation and membrane blebbing. In addition, H_2O_2-treated cells stained with Hoechst 33342 were found out

morphological change in the nuclei. But, treatment of rosemary extract prevented the morphological changes in the nuclei. Mitochondrial dysfunction, resulting from hydrogen peroxide, decreased mitochondrial membrane potential, and this has long implicated in the process of apoptosis. We observed change of mitochondrial membrane potential using Rh 123. The treatment of rosemary extract prevented depolarization of mitochondrial membrane potential induced by H_2O_2. Furthermore, apoptotic cells were detected by TUNEL and PI staining specific for DNA fragmentation. The observation that rosemary extract significantly reduced the ratio of TUNEL-positive cells indicates that rosemary extract may possess an inhibitory effect on H_2O_2-induced apoptosis.

Apoptosis is mediated by extrinsic pathway by death receptor and intrinsic pathway by mitochondria. Ultimately, these pathways activate caspases, and activated caspases induce cell death. Bcl-2 family consist of two groups; anti-apoptotic group (Bcl-2 and Bcl-xL) and pro-apoptotic group (Bak, Bax, Bid), and play important roles in Mitochondrial-related apoptosis pathway. Caspase-3 and -9, a member of the CED-3 subfamily of caspases, is activated by caspase 2, 6, 7, and 9, and induce apoptosis (Denault and Salvesen, 2002). Bcl-2, one of anti-apoptotic factors, resides in the mitochondrial outer membrane and inhibits cyt c release (Borner, 2003). Bax and Bak, one of pro-apoptotic factors, reside in the cytosol. Translocation of Bax to the mitochondrial membrane might lead to loss of mitochondrial membrane potential and an increase of mitochondrial permeability. Increased mitochondrial permeability results in the release of cyt c from the mitochondria (Chinnaiyan et al., 1996). Released cyt c triggers activation of caspase-9. Caspase-9 activates caspase-3, and activated caspase-3 induces cell death. In this study, we investigated whether rosemary extract has any effect on the expression of Bak, Bax and Bcl-2 in H_2O_2-treated cells by using western blot. After treatment of rosemary extract, Bak and Bax was decreased, but Bcl-2 was increased compared with only H_2O_2-treated cells. Also, we investigated caspase-3 and -9 expressions using western blot. This result is consistent with the results from Bax. The treatment of rosemary extract decreased caspase-3

and -9 compared with only H_2O_2-treated cells. Therefore, the effect of rosemary extract on H_2O_2-treated cell may be mediated by regulation of Bcl-2, Bak, Bax, caspase-3 and -9 expression and regulation of antioxidant enzyme. In summary, these results show that rosemary extract decreased H_2O_2-induced cell death, morphological change of nuclei, apoptosis-related gene expression (Bak, Bax, caspase-3 and -9), and increased mitochondrial membrane potential, Bcl-2 expression and antioxidant enzyme activities in SH-SY5Y cells.

Based on results, we hypothesized that rosemary extracr present protective effects thorough modulation of endogenous antioxidant enzyme and inhibition of apoptosis-related gene expression. This study may offer a new clinical strategy for anti-PD though further research into neuroprotective mechanisms of rosemary extract will be necessary.

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감사의 글

대학을 입학한지가 엊그제 같은데 벌써 6년이라는 시간이 흘러 대학원 과정을 졸업하게 되었습니다. 오랜 시간 한 곳만 바라보면서 쉼 없이 달려왔습니다. 때로는 힘들고 지쳐서 포기하고 싶은 적도 있었지만 그래도 포기하지 않고 여기까지 버텨온 보람이 있는 것 같습니다.

논문을 마치며 여기까지 올 수 있도록 도와주신 분들께 감사의 말을 전하고 싶습니다.

먼저 이 논문이 완성될 수 있도록 격려와 성원을 보내주시며 끊임없이 지도를 해주신 지도 교수님이신 김성준 교수님께 감사 드리며, 미약한 완성할 수 있도록 논문을 여기까지 쓸 수 있게 지도해 주신 박열 교수님 전홍성 교수님, 그리고 학부 때뿐만 아니라 대학원 과정 동안에도 많은 가르침을 주셨던 양영기 교수님, 정현숙 교수님, 이정섭 교수님, 박윤경 교수님께 감사 드립니다.

학부생때 뿐만 아니라 대학원 과정 중에도 많은 것들을 가르쳐줬던 김재성 선생님 정말 고마워요. 이야기 하는 거 들어주며 많은 힘이 됐던 은진 언니, 가영이. 두 사람이 없었으면 어떻게 견녀낼 수 있었을지. 정말 고마워. 졸업해도 계속 연락하고 지내. 그리고 이번에 같이 졸업하는 김승 선생님, 봉석오빠, 영민오빠, 홍석오빠, 상용오빠, 효정이 모두 졸업 축하하고 가는 길마다 좋은 일 있길 바랍니다.

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언제나 날 즐겁게 해줬던 경진이와 설희. 너희들 덕분에 실험실에 들어올 수 있었고 같이 생활하면서 즐거웠어. 남은 대학원생활 잘 견뎌내고 좋은 결과가 있기를 바래.

비싼 통화료에도 불구하고 힘들때마다 전화해도 잘 받아주던 오사카에 있는 선화..일본 생활 힘들어도 잘 참고..건강 조심해…그리고 언제나 날 챙겨주는 엄마 같은 착한 보령이, 주말이나 시간날 때 놀고 싶을 때 항상 같이 놀아주던 혜정이. 메신저로 고민 들어주고 상담해주던 동네 친구 인경이, 도쿄에서 열심히 공부하는 승혜, 지금까지도 그다

지 친해지지 못한 인경이, 너희들 모두 옆에 있는 것만으로도 큰 힘이 됐어. 모두모두 고마워..취업 잘되고 좋은 일들만 있었으면 좋겠어.

마지막으로 무엇보다도 소중한 우리 가족. 언제나 날 믿어주시고 힘이 되어주신 사랑하는 부모님. 여기까지 올 수 있었던 건 모두 두 분이 있었기에 가능했어요. 언제나 건강하게 오래오래 사세요. 그리고 착한 우리 언니, 하나뿐인 동생 기찬이, 둘에게는 항상 고마워하고 있어. 사랑해~~♡

그 외에도 대학원 과정 동안 도움을 주신 모든 분들께 감사 드리며 지난 2년간의 시간을 마루리 합니다.

2007년 12월 어느 날

박 세 은 올림