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2008 年 2 月

碩士學位論文

Neuroprotective effects of *Tripterygium regelii* extract in human neuroblastoma SH-SY5Y cells

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미역줄나무 추출물이 인간신경세포 SH-SY5Y 에서 신경보호효과

2008 年 2 月 25 日

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2007 年 11 月 日

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ABBREVIATIONS

AAADC ..... Aromatic L-amino acid decarboxylase

AD ..... Alzheimer's disease

BCA ..... Bicinchoninic acid

BDNF ..... Brain-derived neurotrophic factor

BHT 2,6-Di-tert-butyl-4-methylphenol

BHT ..... Butylated hydroxytoluene

BSA ..... Bovine serum albumin

CA ..... Catecholaminergic

DAPI 4,6-diamidino-2-phenylindole

DBH ..... Dopamine β-hydroxylase

DEPC ..... Diethyl pyrocarbonate

DMSO ..... Dimethyl Sulfoxide

DPBS ..... Dulbecco's Phosphate-Buffer Saline

DPPH ..... 1, 1-diphenyl-2-picryl hydrazyl

DPPH ..... 1,1-diphenyl-2-picryl hydrazyl

EDTA ..... Ethylene diamine tetraacetic acid

EtBr ..... Ethidium bromide

FBS ..... Fetal bovine serum

KCLB ..... Korea Cell Line Bank

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

MOPs ..... 3-(N-morpholino) propanesultionic acid

MTT ..... 3-4, 5-Dimethylthiazol-2-yl-2, 5 diphenyltetrazolium bromide

NIH ..... National Institutes of Health

OD ..... Opticaldensity

PCR ..... Polymerase chain reaction

PD ..... Parkinson's disease

PNMT ..... Phenylethanolamine N-methyltransferase

PVDF ..... Polyvinylidinedi fluoride

RIPA ..... radio-immunoprecipitation assay buffer

ROS ..... Reactive oxygen species

RP ..... Reducing power

RT ..... Reverse transcription

SDS-PAGE ..... Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS ..... Sodium dodecylsulfate

SN ..... Substantia nigra

TAE ..... Tannic acid equivalents

TCA ..... Trichloroacetic acid

TFC ..... total flavonoid compounds

TH ..... Tyrosine hydroxylase

TPC ..... total phenolic compounds

ABSTRACT

Neuroprotective effects of *Tripterygium regelii* extract in human neuroblastoma SH-SY5Y cells

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The reality is facing a rapidly aging society in Korea. It means that the aging population will increase continuously. Consequently, geriatric nervous disease Patients also will increase continuously. The nervous diseases are Alzheimer's disease (AD), The Parkinson's disease (PD) and schizophrenia.

PD is characterized pathologically by loss of dopaminergic neurons and low response of dopaminergic neurons in the substantia nigra (SN), but still not entirely clear. The reason of disease is rising on oxidative injure, environmental factors and genetic predisposition as co-factors of the neurodegenerative process.

Tripteryguim regelii Sprague et Takeda is a very general plant at nature. The *T. regelii* has long been used as an adaptogen in traditional Korean medicine. In this study, the extracted from ethanol (TRE) and methanol (TRM) of *T.*

rege/ii were evaluated using various anti-oxidants assays and neuroprotective effects.

I. Introduction

Tripteryguim regelii Sprague et Takeda (Fig. 1) is a very general plant at nature. The herb *T. regelii* has been used in traditional korean medicine for centuries (Ahn, 1998) and it has been reported to have various pharmacological properties including anti-aging, anticancer and anti-inflammation effects (Tao *et al*., 1999; David *et al*., 2000; Byun *et al*., 2003). There is a function which is like this various. So, it is presumed it will be also anti-oxidation effect. In the study reported here we were used ethanol (TRE) and methanol (TRM) extracts isolated from leaves and stems of *T. regelii*.

Autonomic and neuroendocrine systems are controlled also by central catecholaminergic (CA) neurons (Mravec *et al*., 2007). The catecholamine-synthesizing cells are distributed mainly in the midbrain and in the peripheral sympathetic nerves, and also hormones of the adrenal medulla. The biosynthesis of catecholamines proceeds by hydroxylation of tyrosine catalysed by tyrosine hydroxylase (TH), the first and major rate limiting enzyme in catecholamine biosynthesis. Other enzymes involved in catecholamine biosynthesis are: aromatic L-amino acid decarboxylase (AAADC), dopamine β-hydroxylase (DBH), and phenylethanolamine N-methyltransferase (PNMT). In addition there is depletion in catecholaminergic system (Fig. 2) in Parkinson's disease (PD), Alzheimer's disease (AD) and schizophrenia.

Brain-derived neurotrophic factor (BDNF) is implicated in a variety of

Fig. 1. *Tripterygium regelii* plant with leaves and flowers.

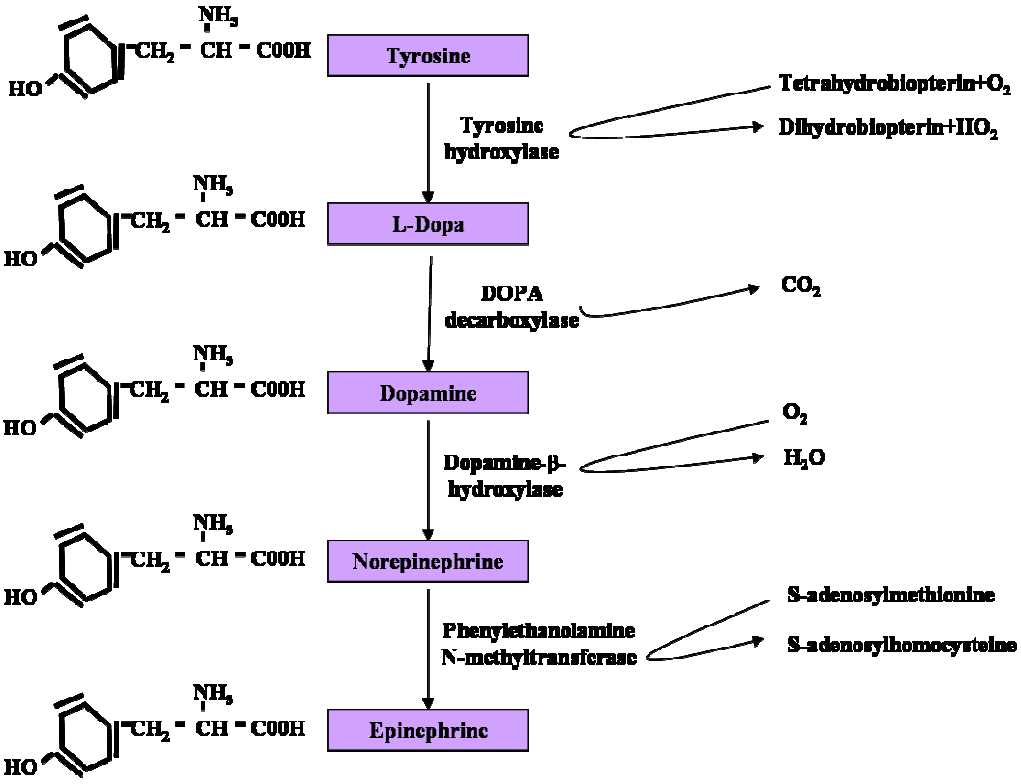

Fig. 2. Biosynthetic pathway of catecholamines.

neural processes as a function of stage of development in both animals and humans. Initially, BDNF is important for neurogenesis (birth of new neurons), neuronal survival, and normal maturation of neural developmental pathways. BDNF have important intracellular and transcriptional effects on a variety of neurochemical systems (Patapoutian and Reichardt, 2001).

Oxidative stress-induced cell damage has long been implicated both in the physiological process of aging and in a variety of neurodegenerative disorders such as PD, AD and schizophrenia (Markesbery, 1997; Finkel and Holbrook, 2000; Choi *et al*., 2006). Oxidative damage, mediated by reactive oxygen species (ROS) which can be generated following cell lysis, oxidative burst, or the presence of an excess of free transition metals, can attack proteins, deoxynucleic acid, and lipid membranes, thereby disrupting cellular function and integrity (Gorman *et al*., 1996; Gardner *et al*., 1997). Hydrogen peroxide (H_2O_2), one of the main ROS, is produced during the redox process and is recently considered as a messenger in intracellular signaling cascades (Rhee, 1999). In addition, it is well known that H_2O_2 could cause lipid peroxidation and DNA damage, thus inducing apoptosis in many different cell types (Halliwell and Aruoma, 1991; Yoshikawa *et al*., 2006). Therefore, therapeutic strategies aimed at preventing or delaying ROS induced apoptosis might be a reasonable choice for the treatment of these diseases. Among various therapeutic strategies, one of the plausible ways is to augment or fortify endogenous defense against oxidative stress through dietary or pharmacological intake of antioxidants. Many

synthetic chemicals such as phenolic compounds have been proven to be strong radical scavengers, but they usually have some severe adverse effects (Heilmann *et al*., 1995). Recently, attention has been focused on searching for natural substances with neuroprotective potential that can scavenge free radicals and protect cells from oxidative damage.

Oxidative stress and mitochondria play an important role in the control of most physiological processes, cell injury and programmed cell death (Fig. 3) (Kehrer and Smith, 1994). A number of reports point to the presence of ongoing oxidative stress and inflammatory processes occurring selectively in the substantia nigra (SN) pars compacta of parkinsonian brains (Levites *et al*., 2002 and Jenner and Olanow, 2003).

In recent years, a worldwide trend towards the use of natural phytochemicals present in herbs. Leaves and stems of *T. regelii* have been described as a useful therapeutic agent in various diseases of the human nervous system including PD. However, at present no information is available regarding the effects of *T. regelii* on catecholamine biosynthetic enzyme and neurotrophic factor gene expression in animal. To explore possible neuroprotective effect of *T. regelii* and related molecular mechanism, we examined the expression of TH and BDNF in rat brain tissue and human neuroblastoma SH-SY5Y cells.

In the present study, we examined the mechanisms by which *T. regelii* extract

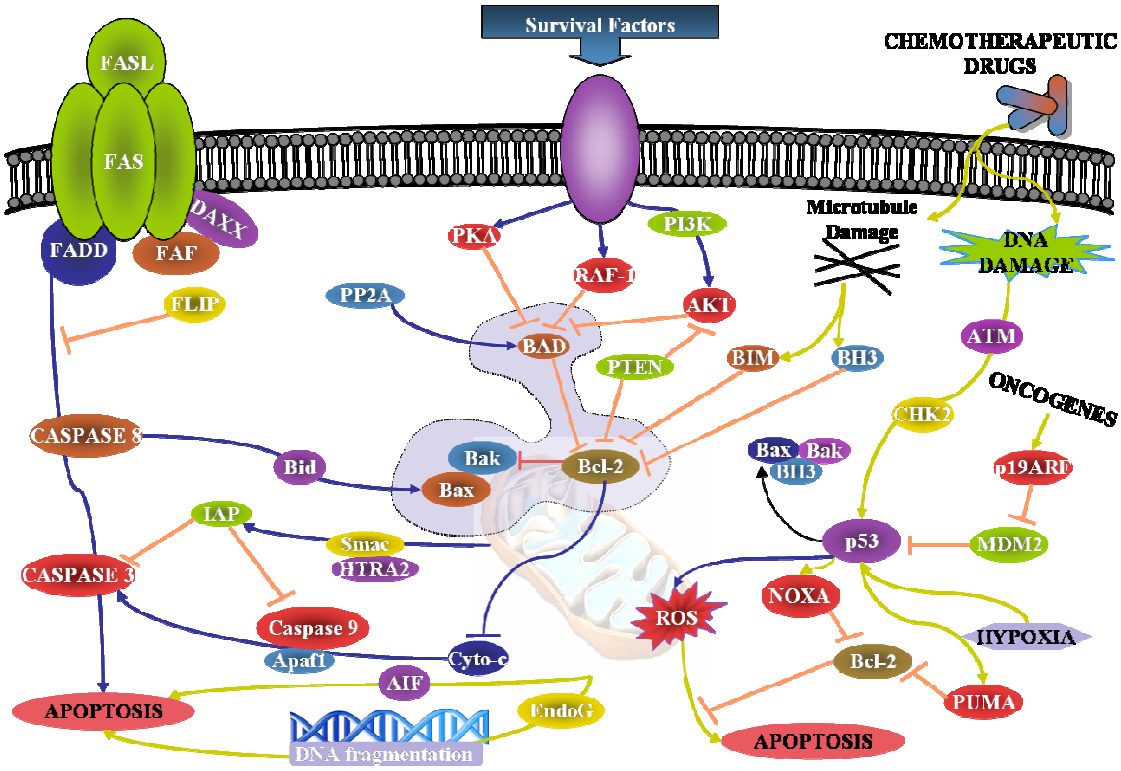

Fig. 3. The general apoptosis signaling pathway.

(TRE and TRM) protects SH-SY5Y cells from H_2O_2 induced oxidative damage. The purpose of the present study was to investigate the effects of pretreatment with TRE on H_2O_2-induced SH-SY5Y cell apoptosis in order to find a possible therapeutic application of these natural compounds to degenerative diseases.

II. Materials and methods

A. Materials

Cell line SH-SY5Y (Korea Cell Line Bank (KCLB), Korea) used for cell culture and toxicity. Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12), fetal bovine serum (FBS), Dulbecco's Phosphate-Buffer Saline (DPBS) and TrypLE™ Express (Trypsin-EDTA solutions) were purchased from the Invitrogen (U.S.A). Dimethylsulfoxide (DMSO), Hydrogen peroxydase (H_2O_2) and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (U.S.A). Tissue culture dishes and multiplex 24 and 96-wells were purchased from Corning (U.S.A).

For RT-PCR, Trizol™ was purchased from the Invitrogen Co. (U.S.A). Isopropanol, chloroform, diethyl pyrocarbonate (DEPC), 3-(N-morpholino) propanesultionic acid (MOPs), formamide, formaldehyde and ethidium bromide (EtBr) were purchased from the Sigma-Aldrich Co. (U.S.A). Agarose was purchased from the Seakem®Cambrex (U.S.A). Moloney murine leukemia virus ribonuclease (M-MLV), oligo deoxythymidine (dT) primer, deoxynucleicacidtriphosphate (dNTPs),

Taq polymerase and specific primers (for TH, AADC, DBH, BDNF and Actin) were purchased from the Bioneer (Korea).

For Western blot assay, Trizma®-Base, sodium chloride (NaCl), glycine, ethylene diamine tetraacetic acid (EDTA), 2-mercaptoethanol, boricacid,acylamide, bis-acylamide, guanidinehydrochloride, sodium dodecylsulfate (SDS), bovine serum albumin (BSA) and ponceau Sredwere purchased from the Sigma-Aldrich (U.S.A). Whatman filter paper and polyvinylidinedi fluoride (PVDF) membrane (Pall life science BioTrace™ PVDF membrance) were purchased from Pall life science. Tween-20 was purchased from the Yakuri pure chemicals (Japan). Prosieve® prestained protein marker and Prosieve® unstained protein marker were purchased from the Cambrex Bioscience RocklandInc (U.S.A). Bicinchoninic acid (BCA) protein assay kit was purchase from the Cabres (U.S.A). WEST-ZOL(plus) was purchase from Intron Biotechnology and X-ray film was purchase from BioMax MS-1, Estman Kodak. Antibodies; Anti-TH Mouse monoclonal (OMA-04051) was purchase from Affinity BioReagents™, Anti-AAADC Rabbit polyclonal (ab3905) was purchase from abcam, anti-BDNF Rabbit polyclonal (SC-546), anti-Bad Rabbit polyclonal (SC-942), anti-Bak Rabbit polyclonal (SC-832), anti-Bcl-2 Mouse monoclonal (SC-7382), HRP conjugated goat anti-mouse IgG (SC-2054), HRP conjugated goat anti-rabbit IgG (SC-2055) were purchase from Santa Cruz Biotechnology. Actin was purchase from Biomeda (U.S.A).

For determination of free radical activity and antioxidant capacity, 1, 1-diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxytoluene (BHT) and ascorbic

acid (Vit C) were purchased from Sigma-Aldrich (U.S.A). Sulfuric acid and sodium phosphate were purchased from Chemical (Japan).

B. Methods

1. Sample extraction

a. Preparation of TRE extract

The plant material used was the lived leaves and stems of *Tripteryguim regelii* Sprague et Takeda which was obtained from the Jiri mountain. Leaves and stems of *T. regelii* were soaked in aqueous absolute ethanol and methanol solution, and extracted at room temperature for 8 weeks. The extracts were filtered, and the filtered extracts were mixed and evaporated to 500 ㎖ in a rotary evaporator under reduced pressure. The ethanol (TRE) and methanol (TRM) extracts were freeze-dried and used in this study as appropriate to the each experiments soluble part. After concentration, the concentrate was centrifuged to remove insoluble materials.

b. Analysis of total flavonoid compounds

The total flavonoid compounds were determined by María method (2000). Flavonoid concentration was determined as follows: The TRE and TRM (100 ㎕) were diluted with 80% aqueous ethanol (900 ㎕). An aliquot of 100 ㎕ was added to test tubes containing 4.3 ㎖ of 10% aluminum nitrate, 1 μM potassium acetate in 80% ethanol. After 40 min at room temperature, the absorbance was

determined spectrophotometrically at 415 nm. The experiment was repeated three times. Total flavonoid concentration was calculated using naringin as standard (Park *et al*., 1997).

c. Analysis of total phenolic compounds

The content of phenolic compounds in the extracts was determined according to the method of Jayaprakasha and his co-workers (Singh, Murthy, & Jayaprakasha, 2002). TRE and TRM solution (10 ㎕) containing 500 ㎍ extract was taken in a 96-well plate, 10 ㎕ Folin-Ciocalteu reagent was added and plate was shaken. After 3 min, 20 ㎕ of solution 10% Na_2CO_3 was added and the mixture was allowed to stand for 1 h with intermittent shaking. The supernatants were transfered to new 96-well plate. Absorbance was measured at 725 ㎚. The experiment was repeated three times. The final results were expressed as ㎎ of tannic acid equivalents (TAE) per g of samples (Robert *et al*., 2008).

d. Determination of DPPH radical scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was used for determination of free radical-scavenging activity of the extracts by Blois (1958) with minor modifications. Samples were dissolved in absolute methanol and then centrifuged to remove insoluble materials. Different concentrations of L-ascorbic acid (Vitamin C) and 2,6-Di-tert-butyl-4-methylphenol (BHT) were used standard. The various concentrations (10, 50, 100, 300, and 500 ㎍/㎖) of TRE and TRM (50 ㎕) were added to 100 ㎕ of 0.4 mM

methanol solution of DPPH. The 96-well plate was allowed to stand at room temperature for 30 min. A control was prepared as described above without TRE, TRM or standards. Methanol was used for baseline correction. The changes in the absorbance of the all the samples and standards were measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following equation:

Radical scavenging activity (%) = $(OD_{control}-OD_{samples})/OD_{control} \times 100$

e. H_2O_2 scavenging activity assay

The hydroxyl radical (H_2O_2) scavenging assay was carried out using the method described by Aebi (1984) with some modifications. 990 ㎕ of 75 mM phosphate buffer (pH 7.0) containing 25 mM H_2O_2 and 10 ㎕ of 0.2 ㎎/㎖ TRE and TRM were mixed. The mixture was incubated at 37℃ for 2 min, and the absorbance was measured at 240 ㎚. Specific absorption coefficient of H_2O_2 used 0.03408 $Cm^{-1}mM^{-1}$ for calculation. Results were determined using the following equation: H_2O_2 scavenging activity = decreasing H_2O_2 (㎛M)/{sample weight (㎎)×reaction time (min)}

f. Determination of reducing power

The reducing power from TRE and TRM were determined according to the methods of Oyaizu (1986). Briefly, 200 ㎕ of TRE and TRM at various concentrations (10, 50, 100, 300, 500 ㎍/㎖) were mixed with 0.2 M phosphate buffer solution (200

㎕, pH 6.6) and 1% potassium ferricyanide (200 ㎕). The mixture was incubated at 50℃ for 20 min. A portion (200 ㎕) of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 12,000 ×g for 10 min. The upper layer of solution (500 ㎕) was mixed with deionized water (500 ㎕) and 0.1% iron(II)chloride (50 ㎕) in eppendorf (EP) tube, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The vitamin C was also assayed at the same concentration for comparison purpose.

2. Animals and treatments

All experiments were carried out on male Sprague-Dawley (SD) rats (180-280 g) approximately 6-8 weeks old were used for this experiment. Animals were housed four or five per cage and maintained under control environmental conditions (12 h light/12 h dark cycle with light on at 08:00 h; temperature, 22 ± 2℃). Food and tap water were available ad libitum. Experiments were performed between 10:00 and 16:00 h. The animals were prevented from all external noises and other stressful stimuli. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH).

Dose response experimental was performed. Before treatment, rats were weighed, and TRE were prepared in saline to achieve the desired dose based on the average weight of the animals. For the dose response study, 5 rats/group

were oral administration with 50, 100, 200, and 400 TRE ㎎/body weight ㎏. Control groups received an equal volume of saline. For the dose course study, animals were oral administration with 1 ㎖ of saline for a nominal dose of 0 (vehicle control). Five animals were treated per dose group for each dose. Rats were killed 4 h after dosing. Brain was rapidly extirpated, cleaned of fat tissue, frozen in liquid nitrogen and stored at -70℃ for the further analysis.

a. Total RNA extraction

Total RNA was extracted from the frozen tissues of rat using TRIzol reagent (Invitrogen, U.S.A) following the company protocol, which is based on the method described by Chomczynski (1993). Tissues (50-100 ㎎) were transferred to 1.0 ㎖ of TRIzol in a 6 ㎖ round bottom tube (Falcon, U.S.A) and homogenized using a tissue homogenizer. 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 (RT) for 10 min. The resulting mixture was then centrifuged at 12,000 ×g at 4℃ for 10 min. After being washed with 75% ethanol in diethyalpyro carbonate (DEPC) treated water the visible RNA pellet was dried and suspended in a 20 ㎕ of DEPC water. The RNA concentration was determined by opticaldensity (OD) at 260 nm (by using an OD_{260} unit equivalent to 40 ㎍/㎖ of RNA). Integrity of the RNA was verified by gel electrophoresis of 4 ㎍ RNA on a 1.5% agarose gel containing 2.2 M formaldehyde.

For intact samples, we were able to visualize both 28S and 18S (ribosomal RNA) bands. Samples were stored at -20℃ until subsequent analyses.

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

Two micrograms of total mRNA samples were reverse transcribed for each sample to be analyzed by incubation with a reverse transcription mixture containing the following constituents: 40 pM oligo(dT) primer, 8 ㎕ of 5× Moloney murine leukemia virus ribonuclease (MMLV) reaction buffer, 4 ㎕ of 0.1 M DTT, 4 ㎕ of 10 mM dNTPs (dATP, dTTP, dGTP, and dCTP), 1 ㎕ of 40 U/㎕ RNase inhibitor and 2 ㎕ of 400 U MMLV. The reaction mixture was incubated for 60 min at 42° C, followed by 5 min at 70℃ to inactivate the RT enzyme. Quality of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -20℃ for further using.

c. Polymerase chain reaction (PCR) and the analysis of PCR products

The cDNA in the RT-PCR product 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 30s, annealing at 64℃ for TH, and at 56℃ for BDNF and β-actin, polymerization at 72℃ for 30 s. The cyclic process was performed 35 times for TH and BDNF and 25 times for β-actin. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide.

Table 1. Primer sequences of tyrosine hydroxylase (TH) and Brain-derived neurotrophic factor (BDNF) genes for rat and SH-SY5Y cell lines

Host	Genes	Primer sequences	Nucleotides Position	PCR product	Gene reference	Annealing temperature
Rat	β-actin	Sense : CCT CTA TGC CAA CAC AGT Antisense : AGC CAC CAA TCC ACA CAG	No: 957-1111	155 bp	BC063166	58℃
	TH	Sense : GCT GTC ACG TCC CCA AGG TT Antisense : TCA GAC ACC CGA CGC ACA GA	No 93-472	380 bp	M10244	61℃
	BDNF	Sense : AAC GAA GAA AAC CAT AAG GA Antisense : GTC TAT CCT TAT GAA TCG CC	No. 244-688	465 bp	AY176065	56℃
Human	TH	Sense : GAG GAG AAG GAG GGG AAG Antisense : TCC AAG TCC AGG TCA GGG TC	No. 332-681	350 bp	NM_199292	50.7℃
	BDNF	Sense : GAT GAC CAT CCT TTT CCT TAC TAT GG Antisense : CTA TCT TCC CCT TTT AAT GGT CAA T	No. 399-114	745 bp	NM_170732	55℃

Intensity of individual bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems, Israel).

d. Total protein isolation

Total protein was separated in the organic phase during the preparation of total RNA and subsequently precipitated with isopropanol, at 12,000 ×g at 4℃ for 10 min. The each pellets were washed three times for 20 min each using 0.3 M guanidine hydrochloride in absolute ethanol and than added 1 ㎖ of 70% ethanol for washing, and dissolved in 1% SDS. Protein concentration was determined using a BCA protein assay kit (Pierce, U.S.A). Bovine serum albumin (BSA) was used as the standard.

e. Western blot analysis

Protein samples (20 ㎍/lane) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking and 12% separating) with a mini protein 3 apparatus (Bio-Rad, U.S.A). For transfer a Mini Trans-Blot Cell (Bio-Rad) was used. After electrophoresis completion the polyacrylamide gel was placed between two sheets of Whatman® paper and PVDF membrane(Pall life sciences BioTrace™ PVDF membrance), all preliminary soaked in transfer buffer. Two additional Whatman filter papers were placed on the top on the membrane and the "sandwich" was placed in the Mini Tans-Blot Cell. A voltage of 100 V was applied for 90 min at 4° C. The membrane was incubated in fresh blocking solution (0.1% Tween-20 in Tris-buffered saline, pH 7.6,

containing 3% BSA) at room temperature for 3 h and then probed with the mouse monoclonal anti-TH antibody (Pall life sciences, U.S.A), rabbit polyclonal anti-BDNF antibody (Santa Cruz Biotechnology, U.S.A) and mouse monoclonal anti-actin antibody (Biomeda, U.S.A) in TBST at 4℃ overnight.

The membrane was washed three times for 15 min each using TBST. After that it was incubated in the appropriate HRP-conjugated secondary antibody at room temperature for another 3 h and washed again three times in TBST. The membrane was incubated with WEST-ZOL® plus substrate solution (iNtRON Biotechnology, U.S.A) for 2 min according to the manufacturer's instructions and visualized with autoradiography film (Kodak or Fuji medical X-ray film, Japan).

3. Cell culture and treatment

The human neuroblastoma SH-SY5Y cells were initially grown in 1:1 mixture of DMEM and F12 Medium supplemented with 10% fetal bovine serum, 1% Antibiotic-Antimycotic (10,000 U/㎖ penicillin G sodium, 10,000 ㎍/㎖ streptomycin sulfate and 25 ㎍/㎖ amphotericin B as Fungizone® in 0.85% saline) (GIBCO™, Invitrogen, U.S.A) in a humid atmosphere (Forma Scientific, U.S.A) of 5% CO_2 and 95% air at 37℃. The medium was replaced after 1-2 days and cells were subcultured after 3 days. SH-SY5Y cells were plated in 24 and 96 wells plate or 100-mm dish. Then cells were pretreated with various concentrations (5, 10, 20, 40 ㎍/㎖) of TRE for 30 min or 2 h, followed by exposure to 100 μM of H_2O_2. To produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each

experiment (Zhang *et al.*, 2007). The control cells were added with the same medium without H_2O_2, TRE.

a. Total RNA extraction

Total RNA was extracted from the SH-SY5Y cell lines using TRIzol reagent following the company protocol. One 100-mm dishes were transferred to 1.0 ㎖ of TRIzol in a EP tube. The pelleted cells were centrifuged at 2,000 ×g for 3 min at RT, and then lysed in TRIzol reagent by repetitive pipetting. Lysed cells were 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 RT for 10 min. The resulting mixture was then centrifuged at 12,000 ×g at 4℃ for 10 min. After being washed with 75% ethanol in DEPC treated water the visible RNA pellet was dried and suspended in a 20 ㎕ of DEPC water. The RNA concentration was determined by OD at 260 nm. Samples were stored at -20℃ until subsequent analyses.

b. RT-cDNA preparation

Two micrograms of total mRNA samples were reverse transcribed for each sample to be analyzed by incubation with a reverse transcription mixture containing the following constituents: 40 pM oligo(dT) primer, 8 ㎕ of 5× MMLV reaction buffer, 4 ㎕ of 0.1 M DTT, 4 ㎕ of 10 mM dNTPs, 1 ㎕ of 40 U/㎕ RNase inhibitor and 2 ㎕ of 400 U MMLV. The reaction mixture was incubated for 60 min

at 42° C, followed by 5 min at 70℃ to inactivate the RT enzyme. Quality of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -20℃ for further using.

c. PCR and the analysis of PCR products

The cDNA in the RT-PCR product 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 30s, annealing at 50.7℃ for TH, and at 55℃ for BDNF and β-actin, polymerization at 72℃ for 1 min. The cyclic process was performed 35 times for TH and BDNF and 25 times for β-actin. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide. Intensity of individual bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems, Israel).

d. Total protein isolation

Treated cells (2×10^6 cells/㎖ in 100-㎜ dish) were collected and washed with PBS. After centrifugation, cells were lysed in 50 ㎕ radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), with 150 mM sodium chloride (NaCl), 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) (Sigma-Aldrich, U.S.A). The lysate was incubated on ice for 30 min and centrifuged at 12,000 ×g for 25 min at 4℃. The supernatant was

collected and followed by protein concentration determination using BCA kit (Pierce, U.S.A).

e. Western blot analysis

Protein samples (20 ㎍/lane) were separated by SDS-PAGE. After electrophoresis completion the polyacrylamide gel was placed between two sheets of Whatman® paper and PVDF membrane, all preliminary soaked in transfer buffer. A voltage of 100 V was applied for 90 min at 4° C. The membrane was incubated in fresh blocking solution at room temperature for 3 h and then probed with the mouse monoclonal anti-TH antibody (Pall life sciences, U.S.A), rabbit polyclonal anti-BDNF antibody (Santa Cruz Biotechnology, U.S.A), mouse monoclonal anti-actin antibody (Biomeda, U.S.A), polyclonal rabbit anti-Bad antibody, monoclonal mouse anti-Bcl-2 antibody and the polyclonal rabbit anti-Bak antibody (Santa Cruz Biotechnology, U.S.A) in TBST at 4℃ overnight.

The membrane was washed three times for 15 min each using TBST. After that it was incubated in the appropriate HRP-conjugated secondary antibody at room temperature for another 3 h and washed again three times in TBST buffer. The membrane was incubated with WEST-ZOL® plus substrate solution for 2 min according to the manufacturer's instructions and visualized with autoradiography film.

f. Determination of cell viability

SH-SY5Y cells were plated at a density of 1×10^4 cells per well in 96-well plates, and the cell viability was determined using the conventional 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan, whose amount is proportional to the number of living cells. SH-SY5Y cells were exposed to various concentrations of H_2O_2 for 24 h. And SH-SY5Y cells were exposed to various concentrations of TRE (5, 10, 20 and 40 ㎍/㎖) for 30 min and 2 h. After pretreatment, cells were treated with 100 μM H_2O_2 for 24 h and cell viability was determined by MTT assay. 10 ㎕ of 5 ㎎/㎖ MTT in PBS were added to each well and the cells were incubated at 37℃ for 4 h. The supernatants were aspirated carefully and 150 ㎕ of dimethyl sulfoxide (DMSO) were added to each well to dissolve the precipitate and the absorbance at 540 nm was measured with a VersaMax microplate reader and SoftMax Pro software (Molecular devices, U.S.A).

g. Determination nuclear staining for morphological changes

For the cellular morphological studies, 12-24 h prior to the treat, cells were plated at a density of 1×10^5 cells/㎖ of a 24-well plate. SH-SY5Y cells were exposed to various concentrations of TRE (0, 5 and 10 ㎍/㎖) for 30 min. After pretreatment, cells were treated with 100 μM H_2O_2 for 24 h. The treated cells were washed three times in PBS and fixed with 1% paraformaldehyde (W/V). Cells were washed again with PBS and then stained with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.3 μM for 10 min at 37℃. The

treated cells displaying DAPI fluorescence were observed under a fluorescence microscope TE 2000-U (Nikon, Japan).

4. Statistical evaluation

Data were analyzed using the Sigma Plot software. The values from individual animals were divided by the mean of the control group to give relative values with mean of control group as 1.0. All data were presented as mean ±S.E.M. Results were evaluated by Student's t-test or by analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered significant.

III. Results

A. Antioxidant activities in two extracts of TRE

1. Analysis of total flavonoid (TFC) and phenolic (TPC) compounds

Although most antioxidant activities from plant sources are derived from phenolic-type compounds (Bravo, 1998 and Cai *et al.*, 2004), antioxidant activity does not always correlate with the presence of large quantities of these polyphenolic compounds, hence both data need to be examined together, confirming the previous report (Hua *et al.*, 2008). For this, the extracts were analyzed for total flavonoids and phenolic content. Table 2 presents the TFC and TPC compounds from extracts of *T. regelii* that have been investigated. The TRE and TRM had high TFC (101.43±2.60 and 86.63±0.65 mg NE/g) and TPC (282.73±10.58 and 237.96±13.94 mg TAE/g) contents. TFC and TPC were found in higher concentrations the TRE than the TRM.

The published Total flavonoid content in the other five citrus peels ranged from 32.7±1.06 to 49.2±1.33 mg/g db (rutin equivalents), the highest levels being present in Ponkan (*C. reticulata* Blanco) and Peiyou (*C. grandis* Osbeck CV) peels, followed by Wenden (*C. grandis* Osbeck CV) peel, confirming the previous report (Wang *et al.*, 2008). By comparison, total flavonoid content of TRE and TRM were much higher. On the other hand, total phenolic content in the five plant materials (Two ecotypes of *C. quinoa*: The Japan sea-level type (JQ) and the Bolivia Altiplano type (BQ) and three varieties of *Amaranthus:*

Table 2. The total flavanoid compounds (TFC), phenolic compounds (TPC), and hydrogen peroxide scavenging activity of TRE and TRM

Sample	Flavonoid compound (mg NE/g)	Phenolic compound (mg TAE/g)	Hydrogen peroxide scavenging activity ($\mu M \times \mu g^{-1}\ min^{-1}$)
TRE	101.43±2.60	282.73±10.58	57.68±3.61
TRM	86.63±0.65	237.96±13.94	36.08±3.28

Amaranthus hypochondriacus (AH) K-343, *Amaranthus cruentus* (AC) R 104 and *Amaranthus cruentus* (AC) M, were similar to the results of Robert *et al*. (2008).

2. Determination of DPPH radical scavenging activity

The role of antioxidant is removal of free radicals. One mechanism through which this is achieved involves donating hydrogen to a free radical and hence its reduction to an unreactive species. Addition of hydrogen removes the odd electron feature which is responsible for radical reactivity (Hua *et al*., 2008). The results of DPPH radical scavenging activities showed (Fig. 4) that TRE and TRM found a similar level of free radical scavenging activity. This activity was TRE with 52.05±0.25%, followed by TRM with 46.17±0.37% and BHT with 27.4787±3.98%, which were greater than that of vitamin C with 92.67±2.40% at the level of 50 ㎍/㎖. Furthermore, the TRE and TRM have radical scavenging activities about 2-fold higher activity levels than BHT.

3. H_2O_2-scavenging activity

Although hydrogen peroxide (H_2O_2) is not very reactive, its high penetrability of cellular membrane leads to OH formation when it reacts with Fe^{2+} or the superoxide anion radical in the cell (Hua *et al*., 2008). The H_2O_2 scavenging activities of TRE and TRM were shown in Table 2. The TRE and TRM exhibited some extent of H_2O_2 scavenging capacity. At the same concentration, TRE was a stronger scavenger than TRM. The scavenging activities of TRE and TRM were 57.68±3.61 and 36.08±3.28 μM/㎍/min, respectively. Both TRE and TRM

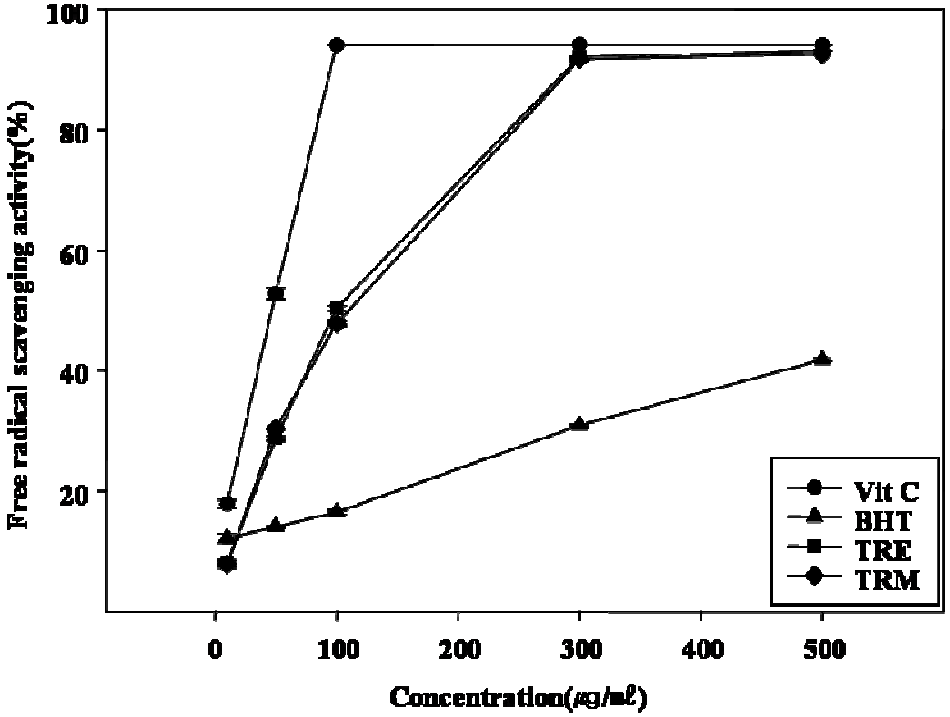

Fig. 4. Effects on DPPH radical scavenging activity of ethanol (TRE) and methanol extracts (TRM) from *T. regelii* were compared with that of vitamin C (Vit C) and that of Butylhydroxytoluene (BHT).

showed a weaker scavenging activity against H_2O_2 as compared to vitamin C (data not shown).

4. Determination of reducing power

An electron-donating reducing agent contributes to antioxidant activity by its capacity to donate an electron to free radicals, which results in neutralization of the radical, and the reduced species subsequently acquires a proton from extracts (Hua *et al*., 2008). The reducing power (RP) of TRE and TRM were concentration-dependent (Fig. 5). The RP of TRE was 0.854±0.015 at 100 ㎍/㎖ and 1.322±0.028 at 300 ㎍/㎖. The TRM was 0.763±0.009 at 100 ㎍/㎖ and 1.267±0.031 at 300 ㎍/㎖. However, L-ascorbic acid only showed slightly higher activity with a reducing power of 1.299±0.028 and 1.477±0.026 at 100 ㎍/㎖ and 300 ㎍/㎖, respectively.

However, the reducing powers of the samples were found to be in the following order: vitamin C (0.456-1.525) > TRE (0.361-1.433) > TRM (0.365-1.369). The published the reducing power in the other *Choerospondias axillaris* fruit (0.601 at 0.625 ㎎/㎖ and 0.828 at 2.5 ㎎/㎖) (Hua *et al*., 2008), *Kappaphycus alvarezii* (methanol, ethanol, ethyl acetate, water and hexane were 0.07-0.74, 0.333-0.44, 0.013-0.467, 0.017-0.193 and 0.017-0.16 at 0.5 to 5.0 ㎎/㎖) (Suresh *et al*., 2008) and *Monascus* fermented soybeans (MFS)-31499 and MFS-31527 (cold water extracts and hot water extracts were both 0.54 at 5 ㎎/㎖ and 0.48 and 0.31 at 5 ㎎/㎖) (Lee *et al*., 2008).

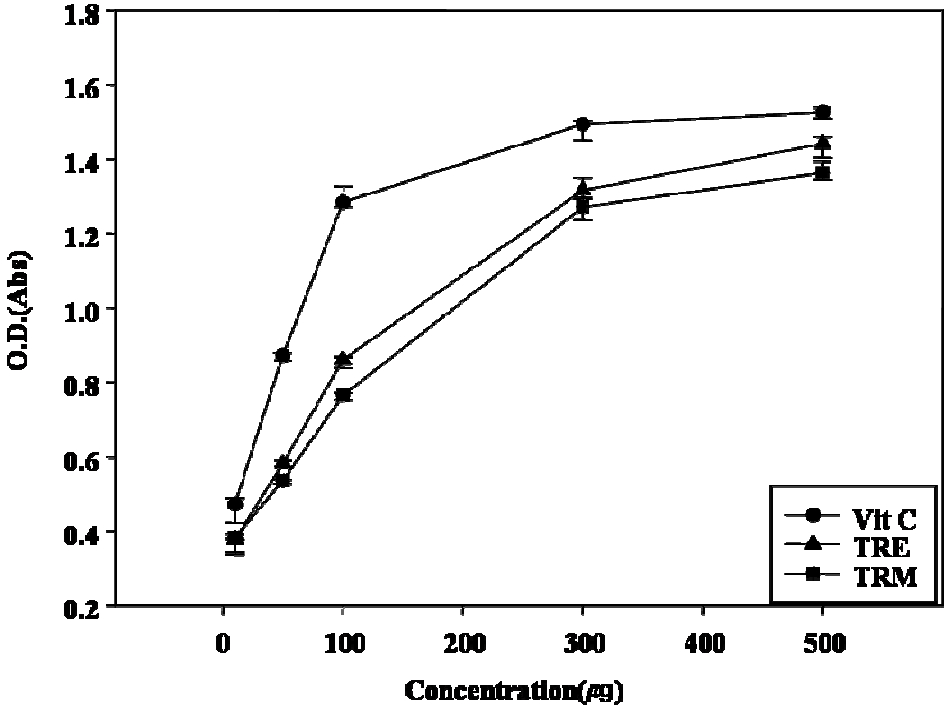

Fig. 5. The reducing power of vitamin C, TRE and TRM from *T. regelii* were compared with that of vitamin C (Vit C).

B. TH induction of TRE treated rat brain and SH-SY5Y

1. TH and BDNF induction of Rat

A dose-dependent increase induced TH and BDNF in rat brain tissue by the administration of 50, 100, 200 and 400 ㎎/㎏. Vertical axis represents the increase of induced genes and horizontal axis shows different control and treated groups. To determine whether TRE induced expression of TH and BDNF is accompanied by an increase in mRNA and protein levels. As shown in Fig. 6A and B. The mRNA levels of TH and BDNF in the brain of rats after 4h treatments with 200 ㎎/㎏ TRE was significantly higher (1.3 and 1.4 fold) than in controls. And the protein levels of TH and BDNF with 200 ㎎/㎏ TRE was significantly higher (1.3 and 1.6 fold) than in controls.

2. TH and BDNF induction of SH-SY5Y

A time and dose-dependent increase induced TH and BDNF in SH-SY5Y cell line by the treatment of 5, 10, 20 and 40 ㎍/㎖. Vertical axis represents the increase of induced genes and horizontal axis shows different control and treated groups. To determine whether TRE induced expression of TH and BDNF is accompanied by an increase in mRNA (Fig. 7A, C) and protein (Fig. 7B, D) levels. The levels of TH and BDNF in the cells after 30 min (Fig. 7A, B) and 2 h (Fig. 7C, D) treatments with 10 ㎍/㎖ TRE were significantly higher than in controls.

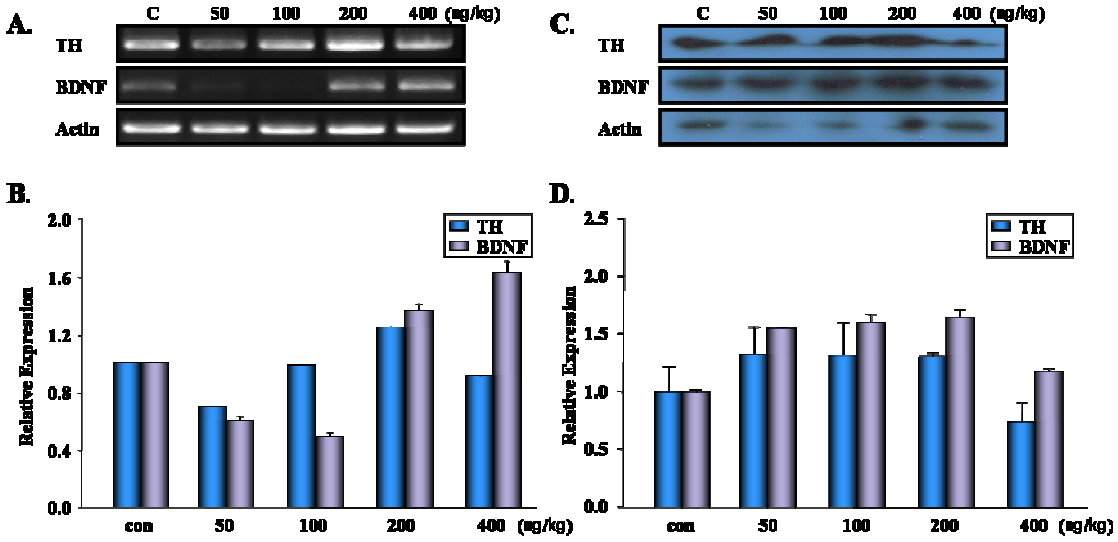

Fig. 6. Changes of TH and BDNF mRNA (A, B) and protein (C, D) level in brain of rats treated with TRE. Rats were treated with various concentrations of TRE (50-400 mg/kg). The levels of TH and BDNF mRNAs and proteins in the rat brain were measured by Gel Quant software, respectively.

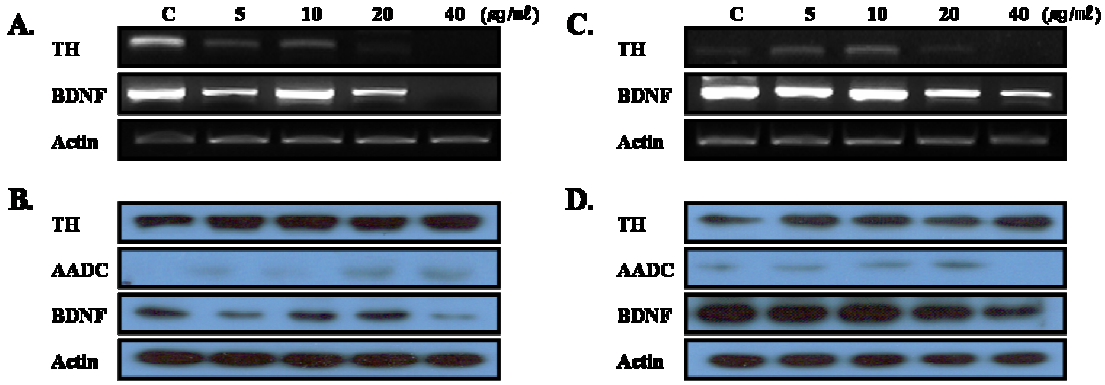

Fig. 7. The levels of TH and BDNF mRNA (A, C) and protein (B, D) in SH-SY5Y cells exposed to TRE for 30 min (A, B) or 2 h (C, D). The SH-SY5Y cells were incubated with various concentrations of TRE (5-40 ㎍/㎖). The levels of TH and BDNF in the cultured cells were measured by RT-PCR and Western blotting, respectively.

C. Neuroprotective effects of TRE in SH-SY5Y

1. 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

SH-SY5Y cells were exposed to H_2O_2 (25-800 μM) for 24 h and cell survival was assessed by MTT assay. As shown in Fig. 8A, 25 to 800 μM of H_2O_2 induced significant decreases in cell survival in a dose-dependent manner. In the presence of 100 μM of H_2O_2, there were only 59.84±3.61% (mean ± S.E.M., n=6) viable cells as compared to control cells. Therefore, the treatment of 100 μM H_2O_2 for 24 h was used to induce SH-SY5Y cell injury in the subsequent experiments.

As illustrated in Fig. 8B (pretreatment for 30 min) and Fig. 8C (pretreatment for 2 h), and than 100 μM of H_2O_2 for 24 h treatment decreased cell viability to 66.54±8.20 or 59.21±9.47%. While 30 min of TRE with 5, 10, 20 and 40 ㎍/㎖ prevented cells from H_2O_2-induced damage, restoring cell survival to 76.08±9.13, 80.97±10.06, 83.49±9.94 and 82.87±10.30%. While 2 h of TRE with 5, 10, 20 and 40 ㎍/㎖ prevented cells from H_2O_2-induced damage, restoring cell survival to 57.38±9.36, 58.18±9.85, 55.40±9.37 and 48.85±7.70%. (mean ± S.E.M., n=3), respectively. There was a significant injury in SH-SY5Y cells after treatment with H_2O_2, including the disappearance of cellular processes, decrease of the refraction and falling to pieces. The damage in groups of TRE treated cells was greatly decreased. TRE at each of concentrations did not cause apparent cytotoxicity (data not shown).

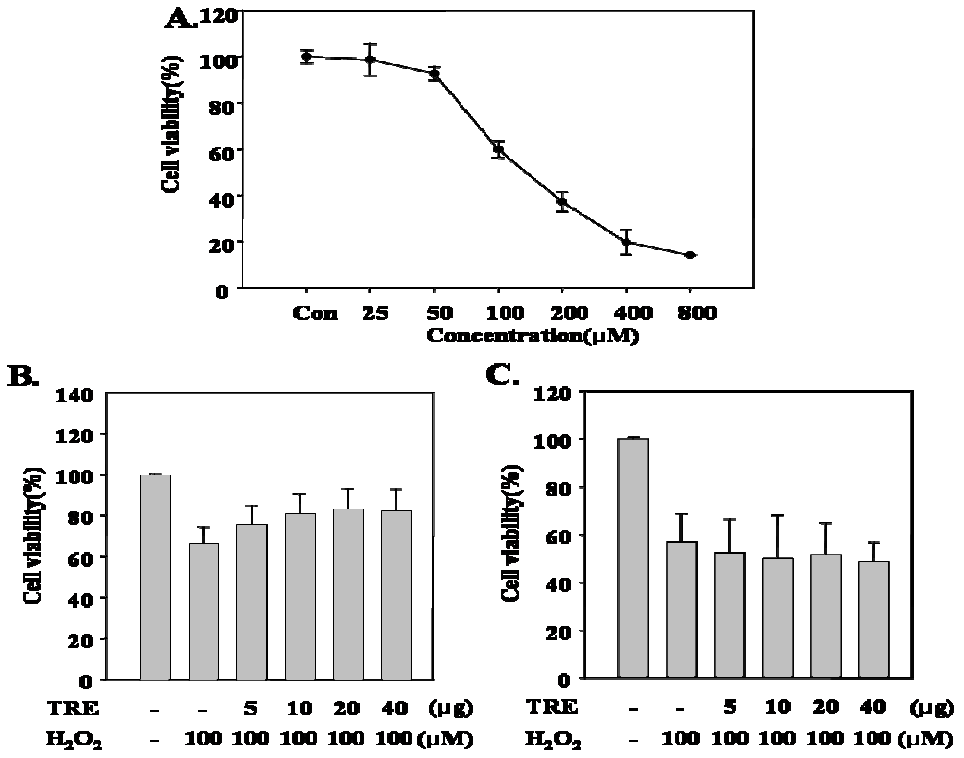

Fig. 8. Effects of TRE on the cell viability in SH-SY5Y cells. (A) H_2O_2 treatment induced SH-SY5Y cell death dose-dependently. SH-SY5Y cells were exposed to different concentrations of H_2O_2 for 24 h. The cell viability was identified by the MTT assay. SH-SY5Y cells were exposed to various concentrations of TRE (5, 10, 20 and 40 ㎍/㎖) for 30 min (B) and 2 h (C). After pretreatment, cells were treated with H_2O_2 (100 μM) for 24 h and cell viability was determined by MTT assay.

2. 4,6-diamidino-2-phenylindole (DAPI)

SH-SY5Y cells were pretreated with TRE at 5 and 10 ㎍/㎖ for 30 min and than treated with 100 μM H_2O_2 for 24 h, and apoptosis was evaluated by DAPI staining by fluorescence microscopy (Fig. 9). Compared with the cells treated with only 100 μM H_2O_2, the pretreatment with 5 and 10 ㎍/㎖ of TRE before exposure to 100 μM H_2O_2 increased the cell viability and decreased of cells displayed morphological changes with typical characteristics of apoptotic cell death, including cell shrinkage, chromatin condensation and crescent. Taken together our results suggest that protection against oxidative stress by pretreated with TRE.

3. Western blot

For investigation the mechanisms of TRE inducing the apoptosis in SH-SY5Y cells, pro-apoptotic gene Bad and Bak, anti-apoptotic genes Bcl-2 protein expression levels were measured by Western blots. As shown in Fig. 10, H_2O_2 enhanced the expression of Bad and Bak and concomitantly decreased the levels of Bcl-2. The effects of TRE on the Bad, Bcl-2 and Bak protein levels were further confirmed by western blotting. Pretreatment of TRE concentration-dependently increased Bcl-2 and, in contrast, decreased Bad and Bak protein level.

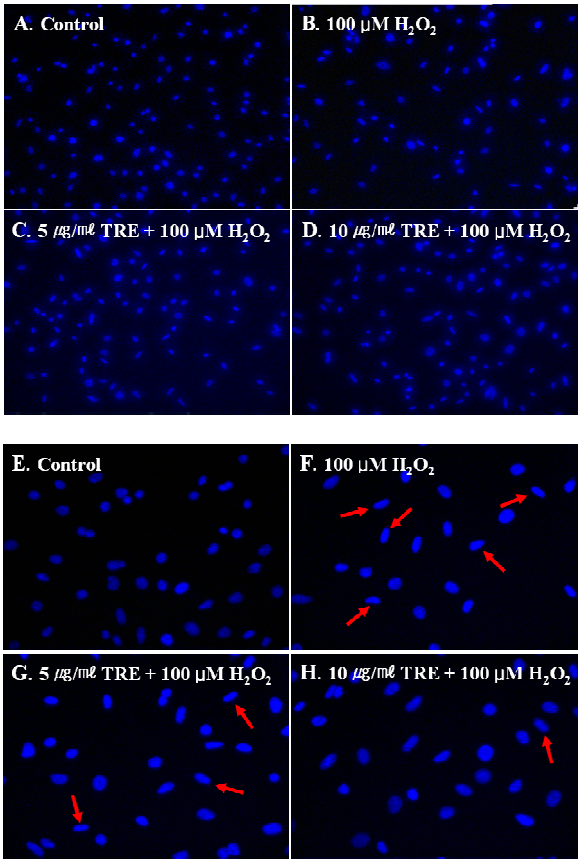

Fig. 9. Nuclear morphological assessment of SH-SY5Y cells by fluorescence microscopy. SH-SY5Y cells were treated with one of the following: positive control (A, E); negative control (B, F); 5 ㎍/㎖ TRE (C, G); 10 ㎍/㎖ TRE (D, H). After pretreatment for 30 min, effects of pretreatment on the H_2O_2-induced changes in nuclear morphology were identified by the staining with DAPI. Magnification is about 200X (A, B, C and D) and 400X (E, F, G and H).

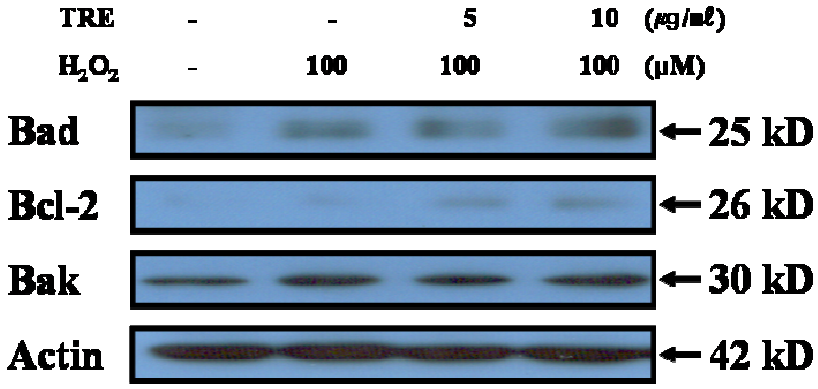

Fig. 10. Changes of Bad, Bcl-2 and Bak protein level in SH-SY5Y pretreated with TRE. The pretreatment of TRE was treated 5 and 10 ㎍/㎖. Changes of Bad, Bcl-2 and Bak protein level in SH-SY5Y pretreated with TRE.

IV. Discussion

Many studies have shown that oxidative stress is a major cause of cellular injuries in a variety of human diseases including neurodegenerative disorders. Reactive oxygen species such as hydrogen peroxide, superoxide anion and hydroxyl radical, which can ultimately lead to apoptotic or necrotic cell death (Gorman *et al*., 1996). Thus, apoptotic cell death to dopamineric neurons of SN is believed to be one of the leading causes of neurodeneration in PD. Recently, many reports have made considerable efforts to search for natural antioxidants with neuroprotective potential.

The ethanol (TRE) and methanol (TRM) extracts from *T. regelii* has antioxidant activity test results. The TRE is higher than the TRM, than it shows the flavonoid compound, phenolic compound, H_2O_2 scavenging activity (Table 1.) and reducing power activity (Fig. 5). However, TRE and TRM found a similar level of free radical scavenging activity (Fig. 4). Taken together our results suggest that protection against oxidative stress by pretreated with TRE.

Before pretreatment, to determine whether TRE induced expression of TH and BDNF is accompanied by an increase in mRNA and protein levels. As shown in the brain of rat (Fig. 6) and SH-SY5Y (Fig. 7). The mRNA and protein levels of TH and BDNF in the brain of rats after 4h treatments with 200 ㎎/㎏ TRE was significantly higher than in controls. The levels of TH and BDNF in the cells after 30 min (Fig. 7A, B) and 2 h (Fig. 7C, D) treatments with 10 ㎍/㎖ TRE were significantly higher than in controls. However, pretreated of 30 min is higher than pretreated of 2 h, the mRNA and protein levels of TH and BDNF.

H_2O_2 has been extensively used as an inducer of oxidative stress *in vitro* model (Satoh *et al*., 1996). The exposure of cultured cells to H_2O_2 results in an imbalance in energy metabolism and the deleterious effects of hydroxyl and peroxyl radicals on membrane lipids and proteins. Our present studies confirmed that treating cells with H_2O_2 resulted in a dose-dependent viability loss (Fig. 8A). However, pretreated for 30 min (Fig. 8B) and 2h (Fig. 8C) with different concentrations (5 and 10 ㎍/㎖) of TRE, greatly decreased the cell viability loss.

Excessive reactive oxygen species ultimately lead to apoptotic or necrotic cell death. Next, we explored whether TRE has a protective effect against neuronal cell apoptosis. H_2O_2-treated cells stained with fluorescent DNA binding dye, DAPI, displayed typical morphological features of apoptosis with sickle shaped-nuclei. TRE pretreatment mitigated these morphological changes (Fig. 9). Studies have shown members of Bcl-2 family are involved in both the positive and negative regulation of cell apoptosis (Reed, 1997). Among them, Bcl-2 and Bcl-X_L are anti-apoptotic, while Bax, Bcl-Xs, Bad, Bid, Bak and Bik are pro-apoptotic. The balance of pro- and anti-apoptotic proteins may be critical to the survival of individual neurons. Pretreatment with TRE concentration-dependently increased Bcl-2 and, in contrast, decreased Bad and Bak protein level (Fig. 10). Taken together, these results strongly suggested that TRE could protect SH-SY5Y cells against H_2O_2-induced apoptosis and the modulation of apoptosis-related gene expression might at least partly contribute to the anti-apoptotic effect of TRE.

In summary, TRE could ameliorate hydrogen peroxide-induced oxidative stress and apoptosis in SH-SY5Y cells. The protective effects of TRE were not only related to modulate endogenous antioxidant enzymes and apoptosis-related gene expression.

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VI. 감사의 글

석사학위를 들어왔던 때가 엊그제 같은데, 어느덧 2 년이라는 시간이 지나버렸네요. 그 동안 많은 일들이 있었지만 힘들 때 마다 주위에서 힘이 되어 주시는 분들이 있어서 석사과정을 무사히 마칠 수 있었던 것 같습니다. 그리고 항상 열심히 할 수 있도록 뒤에서 아낌없이 응원해주시는 부모님과 큰이모님께 감사 드립니다.

학부부터 오랜 시간 동안 아낌없는 관심과 좋은 말씀해주신 김성준 교수님께 깊은 감사의 말씀을 전합니다. 박열 교수님, 양영기 교수님, 정현숙 교수님, 이정섭 교수님, 전홍성 교수님, 박윤경 교수님께도 지난 2 년 동안 많은 관심과 조언을 해주셔서 감사 드립니다. 그리고 논문 지도와 교정을 도와주신 유진철 교수님, 항상 웃음으로 인사를 받아주시던 이숙영 교수님 감사 드립니다.

지금은 각자의 꿈을 향해 달려가고 있을 분자생물학실험실 선배님들 송규영 박사님, 김재성 박사님, 심명화 박사님, Kumar 박사님 그리고 김지은, 이미홍, Hiep. 함께하면서 많은 것을 알려주었던 선배님들과 실험 결과가 안 좋을 때에도 웃음을 잃지 않고 항상 자신감이 넘치는 김승 박사(진)님, 졸업동기인 박세은, 조교로써 고생하고 있는 김지은, 실험실 막내이면서 화초를 잘 가꾸는 회창이 에게도 고맙다는 말을 전합니다.

동기들이 많아서 있어서 물어보기도 편했었고, 술 한잔하자면 바쁜 와중에도 뛰어 나와주던 친구 박으뜸, 실속은 없어도 인기가 많은 조홍석, 항상 열심히 김상용, 힘들다고 하면서도 웃음 잃지 않고 열심히 하고 있는 박영민 함께여서 고마워. 바로 옆방이지만 서로가 바빠서 화장실 갈 때나 얼굴 볼 수 있던 식물분자생물학 사람들. 박사과정으로 마지막까지 열심히 하시고 계신 황인덕 선생님, 네팔에서 와서 말도 안 통하지만 열심히 하시는 기리, 딜리, 항상 웃는 모습이 아름다운 박가영, 열정이

넘쳐서 보기 좋은 재영이 형. 단백질 소재 연구센터에 있어 얼굴보기는 힘들지만 옆집 형 같은 정은이 누나, 목소리가 귀여운 효정이, 신경분자생물학 실험실의 막내면서도 노력하는 모습이 보기 좋던 박정애.

그리고 바쁘신 가운데 논문에 대한 많은 관심을 가져주신 캐러스에 정남준 박사님, 캐러스에서 연구원이자 논문 수정에 도움을 주셨던 분자생물학실험실 출신 서현정에게도 깊은 감사의 말씀을 전합니다.

마지막으로 서로 바쁜 중에도 잊지 않고 연락해주던 전남대병원에 근무하는 박연선, 김지현, 고려대에서 학위중인 김보경, 경희대에 한설희, 노경진 모두들 무사히 학위 마치길 빌면서, 미처 쓰지 못하였지만 항상 함께였던 모든 사람들에게 깊은 감사의 말씀을 전하면서 마치도록 하겠습니다.

우리에게는 끝은 없다 단지 또 다른 시작이 펼쳐질 뿐.

2007 년 12 월

최 봉 석