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碩士學位論文

Molecular Analysis of Catecholamine Biosynthetic Enzyme Genes Induced by *Mucuna pruriens*

朝 鮮 大 學 校 大 學 院

遺 傳 子 科 學 科

Nguyen Thi Hiep

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***Mucuna pruriens*에 의한 카테콜아민 생합성 효소유전자 유도발현에 대한 분자생물학적 분석**

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Nguyen Thi Hiep

Nguyen Thi Hiep의碩士學位論文을
認准함.

委員長 朝鮮大學校　　敎授　　印

委　員 朝鮮大學校　　敎授　　印

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Molecular Analysis of Catecholamine Biosynthetic Enzyme Genes Induced by *Mucuna pruriens*

A thesis submitted to the Graduate School
of the Chosun University in partial fulfillment of the requirements for
the Master's degree
by

Nguyen Thi Hiep

Department of Biotechnology, Graduate School,
Chosun University, Gwangju, Korea

December 6 2006

Approved by:

Prof. Yeal Park, Ph.D. _____

Prof. Young-Ki Yang, Ph.D. _____

Major Advisor
Prof. Sung-Jun Kim, Ph. D. _____

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ABBREVIATIONS

AADC ..... Aromatic L-amino acid decarboxylase

AIDS ..... Acquired immune deficiency syndrome

BCA ..... Bicinchoninic acid

BHT ..... Butylated hydroxytoluene

BSA ..... Bovine serum albumin

CA ..... Catecholamine

DEPC ..... Diethyal pyrocarbonate

DMEM ..... Dulbecco's modified Eagle's medium

DMSO ..... Dimethyl sulfoxide

DPBS ..... Dulbecco’s phosphate buffered saline

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

dNTPs ..... Deoxynucleic acid triphosphate

DTT ..... Dithiothreitol

EDTA ..... Ethylenediaminetetraacetic acid

FBS ..... Fetal bovine serum

EtBr ..... Ethidium bromide

KCLB ..... Korea cell line bank

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

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

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

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

MP .....*Mucuna pruriens*

L-dopa ..... L-dihydroxyphenylalanine

NADH ..... Nicotinamide adenine dinucleotide

OD ..... Optical density

6-OHDA..... 6- Hydroxydopamine

PD..... Parkinson’s disease

PVDF ..... Polyvinylidene fluoride

RT-PCR .....Reverse trancriptase polymerase chain reaction

TH .....Tyrosine hydroxylase

SDS .....Sodium dodecyl sulphate

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

SNpc..... Substantia nigra pars compacta

ABSTRACT

Molecular Analysis of Catecholamine Biosynthetic Enzyme Genes Induced by *Mucuna pruriens*

Nguyen Thi Hiep
Advisor: Prof. Kim, Sung-Jun, Ph. D.
Department of Biotechnology
Graduate School of Chosun University

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of catecholamines, which are neurotransmitters involved in a wide variety of important physiological functions. The regulation of TH protein level and activity is a key step for controlling catecholamine synthesis. TH is regulated in a complex fashion by transcriptional and post-transcriptional mechanisms and is critically modulated by multiple regulatory mechanisms. TH is key molecule of the functioning of the dopaminergic system. Changes in TH expression generally reflect altered activity of catecholaminergic neurons in brain. The pathogenesis of some catecholaminergic neuron disorders, such as Parkinson's disease (PD) may be related to change in TH.

In recent years, a worldwide trend towards the use of natural phytochemicals

present in herbs to apply for the neurodegenerative disorders. Seeds of *Mucuna pruriens* (MP) 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 MP on catecholamine biosynthetic enzymes in animal. To explore possible neuroprotective effects of MP and related molecular mechanisms, we examined the expression of TH and AADC in rat brain.

Cell viability of SH-SY5Y neuroblastoma cell line and 293 human kidney cell line was determined with different doses (0, 100, 200, 400, 800, 1000 ㎍/㎖) of MP seed treatment for 12, 24 and 48hrs. MP did not induce any cell cytotoxicity to both cell lines even with the higher doses (1000 ㎍/㎖) of MP incubated for longer time (48hrs). To determine the neuroprotective effect of MP on SH-SY5Y cells, cells were treated with rotenone or MP or MP plus rotenone, and then processed MTT assay. The result showed that the MP increased cell viability and had neuroprotective effect against rotenone.

To dertermine the effects of MP on the catecholamine biosynthetic enzyme encoding genes expression, we determined changes in mRNA levels of TH and AADC, the first rate-limiting and the second enzyme in the catecholamine biosynthesis pathway. TH and AADC mRNA levels were examined in rat brain tissue by RT-PCR and real time PCR. Protein levels were determined by Western blot analysis. Rats were treated with different doses of MP (0, 100, 200, 300, and 400 ㎎/㎏) for 2, 4 and 8 hrs, respectively.

The results showed that the MP significantly increased the levels of TH and AADC mRNA and protein at a dose of 400 ㎎/㎏ for 2hrs and at a dose of 200 ㎎/㎏ for 4hrs. However, mRNA and protein levels of both genes were not change

at different doses for 8h. The mRNA and protein levels of AADC were induced by MP at different doses and times. These results indicate that MP has a complex and differential effect on TH and AADC gene expression and it depends on the treatment dose and duration of administration.

In conclusion, MP did not show any cell cytotoxicity to human neuroblastoma SH-SY5Y and human kidney 293 cell lines and had the neuroprotective effects against rotenone. Futhermore, both catecholamine biosynthetic enzymes encoding genes (TH and AADC) were up regulated by the treatment of MP. Therefore, it could have therapeutic value in the treatment and improvement of PD.

I. INTRODUCTION

Parkinson's disease (PD) is described for the first time by James Parkinson in 1817 in his monograph "Essay on the Shaking Palsy". PD is the second most common age related after Alzheimer's disease, effects about 2% of all people over age 65. PD is a degenerative disease of the nervous system due to the progressive loss of nigrostriatal dopaminergic neurons and decrease in striatal dopamine. The typical syndromes of PD are tremor at rest, rigidity, absence of normal unconscious movements, slowness, impaired balance and postural instability. Although PD was discovered over a century ago but the exact cause of the disease is unknown (Corti et al., 2005), despite the known role of oxidative stress, free radical formation (Jenner and Olanow, 1996), genetic susceptibility (Bandmann et al., 1998), programmed cell death (Ziv et al., 1998), and other unknown factors, which might be endogenous or exogenous (Calne and Langston, 1983). PD symptom progresses slowly and can ultimately produce complete akinesia. The neuropathology of the disease is based on the depigmentation and cell loss in the dopaminergic nigrostriatal tract of the brain (Fearnley and Lees, 1991), with a corresponding decrease in striatal dopamine content. The potential points of interaction of the pathogenic factors cited above are diagrammed in Fig. 1. The interactions between these pathways are keys to the demise of substantia nigra pars compacta (SNpc) dopaminergic neurons.

A number of studies have identified a link between certain genes and a predisposition to early onset PD, including α-synuclein, parkin and ubiquitin CH-L1 (Corti et al., 2005). The functions of genes implicated in inherited forms of PD suggest two major hypothesis posits that misfolding and aggregation of proteins are

instrumental in the death of dopaminergric neurons in the SNpc, while the other proposes that the culprit is mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized dopamine species. Several studies have shown that variants of the mitochondrial genes may cause the onset of PD. The main characteristics of PD are dopaminergic cell degeneration and the presence of neuronal, intercellular, sphere-shaped structures in the brain cells called Lewy bodies. Lewy bodies are made up of microscopic, dense deposits of abnormal protein, formed as products of the mutated α-synuclein gene (William et al., 2003).

Recent research has identified a hereditary link where close relatives of PD patients are twice as likely to develop PD as people with no family history of PD (William et al., 2003). The extent to other environmental factors may also play a part in genetic predisposition to remains unclear. The environmental toxin is one of the factors that cause PD such as pesticide rotenone, toxic chemicals (paraquat, dieldrin) and endogenous toxins. Long-term exposure to these toxic chemicals cause brain cell death, especially dopaminergic neurons, and the development of symptoms characteristic of PD. Neuronal toxins such as 6-hydroxyl dopamine (6-OHDA), 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine (MPTP), and rotenone are inducing parkinsonism in animals (William et al., 2003).

Dopamine is produced by dopaminergic neuronal cells and is one of three main neurotransmitters (dopamine, norepinephrine and epinephrine) called "catecholamines". Neurotransmitters are chemical "messengers" that transmit impulses across the synapses between neurons in the brain and onward to the muscles (Fig. 2). Dopaminergic neurons form a network of small clusters, found in several regions of the brain. The main concentration of dopaminergic neurons

responsible for controlling muscle movement, emotional, motivational behavioral mechanisms is located in substantia nigra (Bear, Connors and Paradiso, 1996).

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of catecholamines (Fig. 3), which is neurotransmitter involved in a wide variety of important physiological functions (Milsted et al., 2004). The regulation of TH protein level and activity represents a central means for controlling catecholamine synthesis. Activity of TH can be regulated by two mechanisms: short term direct regulation of enzyme activity (feedback inhibition, allosteric regulation and phosphorylation) and medium to long term regulation of gene expression (enzyme stability, transcription regulation, RNA stability, alternative RNA splicing and translational regulation) (Kumer and Vrana, 1996; Fujisawa and Okuno, 2005). TH is a key molecule of the functioning of the dopaminergic system. Changes in TH expression generally reflect altered activity of dopaminergic neurons in brain. The pathogenesis of some catecholaminergic neurons related disorders such as PD may be related to change in TH. Recently, one current strategy for gene therapy of PD involves local production of dopamine in the striatum achieved by inducing the expression of rate-limiting enzymes involved in the biosynthetic pathway for dopamine (Lu et al., 2005). Therefore, gene therapy strategies for PD have focused on TH gene (Lu et al., 2005).

The aromatic L-amino acid decarboxylase (AADC) (or DOPA decarboxylase) is a homodimeric enzyme belonging to the α-family of pyridoxal 5′-phosphate enzymes. AADC is a non-specific enzyme mainly implicated in the synthesis of dopamine and serotonin through the decarboxylation of substrates L-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan in neuronal and also non-neuronal cells.

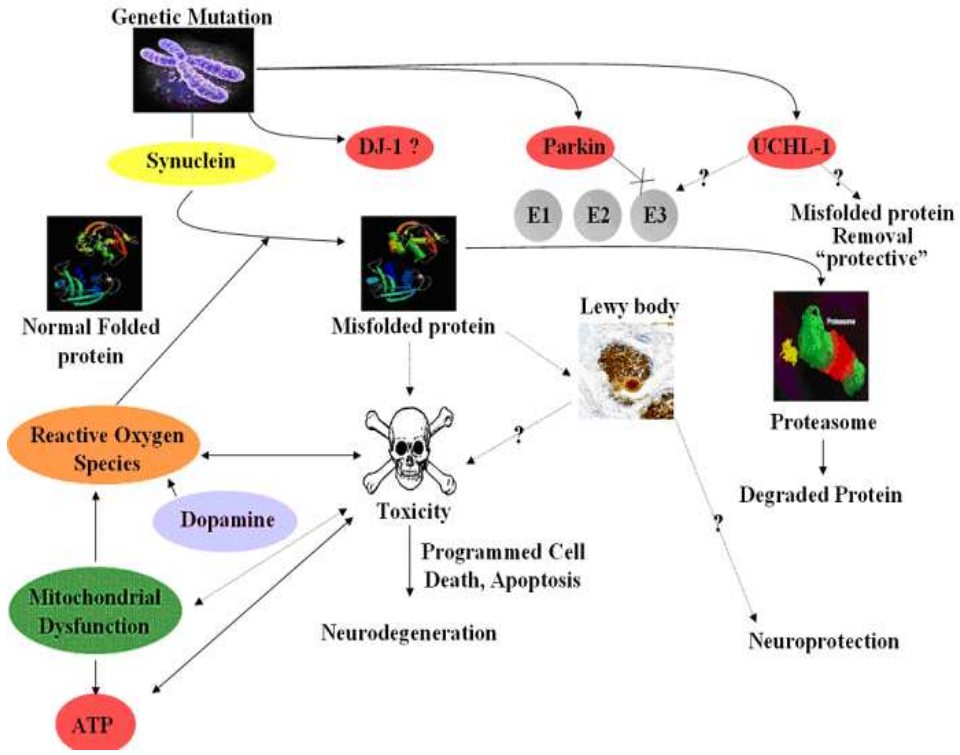

Fig. 1. Mechanisms of neurodegeneration

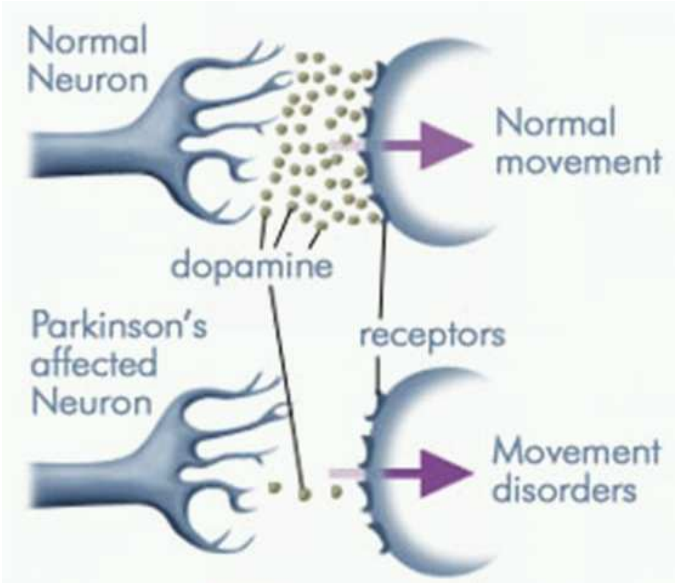

Fig. 2. Dopamine levels in normal and Parkinson's affected neurons.

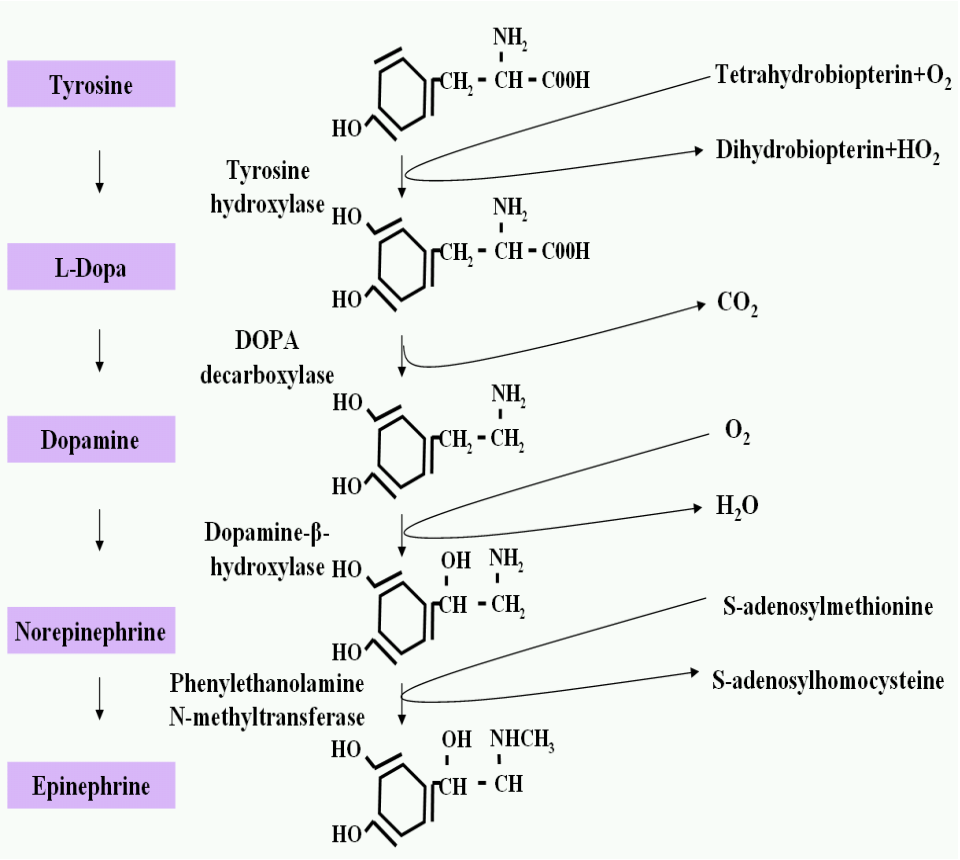

Fig. 3. Catecholamine biosynthesis pathway

The activity of this enzyme has been localized in various areas of the mammalian brain, the sympathetic nervous system, and the adrenal and pineal glands. AADC is also present in the neuroendocrine cells in the tracheobronchial epithelium, neuroepithelial bodies in the broncho pulmonary epithelium, kultschitzky cells in the small intestine and appendix. The essential function of AADC in neuronal cells is to provide the organism with neurohormones and specific neurotransmitters. The physiological function of AADC in non-neuronal organs or tumors mentioned above is not yet well known (Kubovcakova et al., 2004).

Mucuna pruriens (MP) belongs to the family *Leguminosae* and is a twiner with trifoliate leaves, purple flowers and pods covered with hairs (Fig. 4). Seeds of MP have been described as a useful therapeutic agent in various diseases of the human nervous and reproductive system including PD in the ancient Indian medical system (Manyam, 1990; Manyam et al., 2004). MP has been studied for its chemical and pharmacological properties such as analgesic, antipyretic (Iauk et al., 1993), antidiabetic (Akhatar et al., 1990; Rathi et al., 2002). In Nigeria, the seed of MP has been used for antisnake treatment (Aguiyi et al., 1999; Guerranti et al., 2001). Many studies have shown that the raw powder obtained from MP increased dopamine synthesis (Hussain et al., 1997). MP contains L-dopa (5%), 5-indole compounds (tryptamine and 5-hydroxytriptamine), and four tetrahydroisoquinolines alkaloidals (Misra and Wagner, 2004; Tripathi et al., 2001). In addition, MP consists of co-enzyme Q-10 and nicotinamide adenine dinucleotide (NADH), which have neuroprotective activities (Manyam et al., 2004). Other compositions of MP are showed in table 1 (Eustace et al., 2006).

Antioxidants are important in the prevention of various human diseases. Antioxidant compounds may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation. Alcoholic and methanolic extract of the MP seeds were shown to have potential antioxidant activity against in vivo and in vitro models (Tripathi et al., 2001; Yerra et al., 2005). Moreover, methanol extract of MP seeds exhibits potential antitumor and antioxidant activities (Yerra et al., 2005).

Although, many researches have focused on the therapeutic effects of natural plant extracts, but there is no information on genetic analysis of catecholamine biosynthetic enzyme genes induced by MP. Therefore, in this study, we analysed the effects of MP on the expression of TH and AADC genes.

Table 1. Compositions of *Mucuna pruriens.*

Compounds	(g/kg)	Amino acid composition	(g/kg)
Dry matter	905	Alanine	2.74
Crude protein	278	Arginine	4.75
Crude fibre	94	Aspartic acid	9.14
Crude fat	54	Cystine	0.94
Ash	33	Glutamic acid	9.86
Sugar	84	Glycine	3.60
Starch	309	Isoleucine	3.20
Phosphorus	4.0	Leucine	8.39
Calcium	1.4	Lysine	5.72
Total phenols	304	Methionine	0.72
Tannins	71.2	Phenylalanine	3.56
L-DOPA	13.2	Serine	3.53
Phytic acid	12.8	Threonine	2.88
Cyanide	58.4	Valine	3.64

A. **B.**

Fig. 4. ***Mucuna pruriens*** **plant with trifoliate leaves, pods (A) and seeds (B).**

II. MATERIALS AND METHODS

II-A. Materials

Human dopaminergic neuroblastoma cell line SH-SY5Y and human kidney cell line 293 were purchased from the Korea Cell Line Bank (KCLB). Dulbecco's modified Eagle's medium (DMEM), the Ham's F12 medium, fetal bovine serum (FBS), Dulbecco's Phosphate-Buffered Saline (DPBS), Trypsin-EDTA solutions were purchased from the Invitrogen Corporation (USA). Dimethyl Sulfoxide (DMSO) and penicilline were purchased from Sigma–Aldrich Co. (U.S.A). Streptomycin sulfate was purchased from the Calbiochem® (Germany). 3-4, 5-Dimethylthiazol-2-yl-2, 5 diphenyltetrazolium bromide (MTT) was purchased from the Amersham life science (USA). Tissue culture dishes and multiplex 96 wells were purchased from the Nunc (Denmark).

For RT-PCR and Real time PCR, Trizol™ reagent was purchased from the Invitrogen Co. (USA). Isopropanol, chloroform, diethyl pyrocarbonate (DEPC), 3-(N-morpholino) propanesultionic 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, AADC and Actin) and 100bp DNA ladder were purchased from the BioNEER Co. (Korea). Ribonuclease inhibitors, SYBR® Premix Ex Taq™ were purchased from the Takara Bio Inc. (Japan).

For Western blot assay, Trizma®-Base, sodium chloride, glycine, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 2-mercaptoethanol, boric acid, acylamide, bis-acylamide, guanidine hydrochloride, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and ponceau S were purchased from the Sigma-Aldrich Co. (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 Bioscience Rockland Inc. (USA). Bicinchoninic acid (BCA) protein assay kit was purchase from the Cabres Co. (USA).

For determination of free radical activity and antioxidant capacity, 1, 1–diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid and ammonium molybdate were purchased from Sigma-Aldrich Co. (USA). Sulfuric acid and sodium phosphate were purchased from Chemical Co., Ltd (Japan).

II-B. Methods

II-B-1. MP sample preparation

The fresh seeds of MP were collected by removing peel and then ground to a paste by grinder. This powder was kept in deep freezer -70 ℃ and freezing-dry. A total of 30 g of the dry powder was completely dissolved in 30 ㎖ of saline before use.

II-B-2. Determination of DPPH radical scavenging activity

The stable DPPH, molecular formula $C_{18}H_{12}N_5O_6$, was used for determination of free radical-scavenging activity of the MP. Freeze dried MP sample was

dissolved in saline and then filtered through 0.45 µm pore size membrane. Different concentrations (100, 200, 400, 800 ㎍/㎖) of standard (+)-ascorbic acid, BHT and crude extract were added in test tubes. The volume of all tubes was adjusted to 100 ㎕ by addition of distilled water 400 ㎕ solution of DPPH (100 µM) in ethanol was added to all tubes and shaken vigorously. The tubes were allowed to stand at room temperature for 20 min. A control was prepared as described above without samples or standards. Ethanol was used for baseline correction. The changes in the absorbance of the all the samples and standards were measured at 517 nm. The experiment was repeated three times. 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 (%) = $(OD_{control} - OD_{test})/OD_{control} \times 100$.

II-B-3. Cell culture

Two cell lines: SH-SY5Y and 293 cells were used in this study. SH-SY5Y cells were grown in mixture of DMEM and the Ham's F12 medium with ratio 1:1 (DMEM medium contains 1.5 g/ℓ sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. Ham’s F12 (F12) medium contains 1.5 g/ℓ sodium bicarbonate, 15mM HEPES and 2 mM L-glutamine). 293 cells were cultured in DMEM medium. All of these media were supplemented with 10% (v/v) heat- inactivated FBS, 100 units/㎖ penicillin, and 100 ㎍/㎖ streptomycin sulfates. All these cells were maintained in a humidified

incubator under 5% CO2 at 37 ℃. The cells were routinely subcultured twice a week.

II-B-4. Cell viability test

Cell viability was determined with the MTT 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. The cell lines SH-SY5Y and 293 were first cultured in T-75 culture flasks. After cells grown up to 70% confluence, they were seeded into 96-well plates in 100 ㎕ each cell's medium with an initial density of 1×10^4 cells/well. After incubation for 24 hrs at 37℃, cells were treated with different doses of MP (0, 100, 200, 400, 800, 1000 ㎍/㎖) and incubated for 12, 24, and 48 hrs. After medium was removed completely from each well and washed with DPBS, 50 ㎕ of 5 ㎎/㎖ MTT reagent and 200 ㎕ fresh media were added to each well. The plates were covered with aluminum foil and incubated for 4 hrs at 37 ℃. The medium was then discarded. The resulting formazan dye was dissolved in 200 ㎕ DMSO and 25 ㎕ of glycine buffer (0.1M glycine, 0.1 M NaCl, pH 10.5). Immediately, optical density (OD) was read with an ELISA reader at 570 nm. The effects of MP to SH-SY5Y and 293 was determined by IC_{50}

To determine the neuroprotective effect by MP, SH-SY5Y cells were treated with rotenone (10 μM), rotenone (10 μM) plus MP (100 ㎍/㎖) incubated for 12, 24 and 48 hrs. The cell viability was also determined with MTT assay.

II-B-5. Animal experiments

Male Sprague-Dawlay rats, 6 ~ 8 weeks of age and weights from 250~300 g were used in all experiments. Animals were housed four per cage and under controlled environmental conditions (12 hrs light / dark cycle and room temperature 25 ± 2℃). Food and water were supplied all the time. Rats were treated by MP powder dissolved in saline with dose of 100, 200, 300 and 400 ㎎/㎏ each for 2, 4 and 8 hrs (each group = 5 rats). The control groups received the same volume of saline instead of MP.

II-B-6. Total RNA isolation

Total RNA was isolated from frozen brain tissues by using the Trizol™ reagent according to the manufacturer's instructions. Total RNA was precipitated from the aqueous phase by isopropanol. After washing with 70% ethanol, it was dried and dissolved in a small volume of DEPC- treated water. Total RNA then was quantified and purity assessed by spectrophotometry (by measurement of OD 260nm and 280nm). The purity of the RNA was verified by gel electrophoresis of ~ 4 ㎍ RNA on a 0.8 % agarose in 1 X MOPS (3-N-morpholino propanesultionic acid) buffer containing 0.2 ㎍ EtBr. Total RNA was stored at -70 ℃ for following experiments.

II-B-7. Total Protein isolation

Total protein was separated in the organic phase during the preparation of total RNA and subsequently precipitated with isopropanol, and then it was washed three times with 0.3 M guanidine hydrochloride. After washing with 70% ethanol,

protein was dried and dissolved in 1% SDS. Protein concentration was determined by using a BCA protein assay kit. BSA was used as the standard.

II-B-8. Reverse transcriptase polymerase chain reaction (RT-PCR)

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, 10 mM dNTPs, 20 units of RNase inhibitor and 200 units of M-MLV, the volume of reaction is up to 40 $\mu\ell$ volume by DEPC water. The reaction mixture was incubated for 60 min at 42℃, followed by 5 min at 70℃ to inactivate the reverse transcriptase enzyme. Quality of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -20 ℃ for further using.

The cDNA in the reverse transcription product was amplified using Taq DNA polymerase. PCR reaction was performed in 20 $\mu\ell$ of the total volume using 10 pmol of corresponding primers (Table. 2). cDNA was amplified under the following reaction conditions: denaturation at 94℃ for 30s, annealing at 64℃ for TH, 62℃ for AADC and at 56℃ for β-actin for 45s, polymerization at 72℃ for 30s. The cyclic process was performed 35 times for TH and AADC, and 30 times for β-actin. PCR products were analyzed on 1.2% agarose gel in 0.5 X TBE (Tris-Boric acid-EDTA) buffers containing EtBr. Intensity of individual bands was evaluated by Gel Quant software (DNR Bio-Imaging Systems Ltd.).

II-B-9. Real Time PCR

For Smart Cycler® II reaction a mastermix of the following reaction components was prepared to the indicated end-concentration: 9.5 $\mu\ell$ water,

0.5 $\mu\ell$ forward primer (10 μM), 0.5 $\mu\ell$ reverse primer (10 μM) (table 1), 12.5 $\mu\ell$ SYBR® Premix Ex Taq™ (2X) and 2 $\mu\ell$ cDNA. Tubes were closed, centrifuged and placed into the Smart Cycler® rotor. The following Smart Cycler® experimental run protocol was used: denaturation program (95℃ for 5 min), amplification and quantification program repeated 40 times (95℃ for 30s, 60℃ for 30s, 72℃ for 30 s with a single fluorescence measurement), melting curve program (72 ~ 95℃ with a heating rate of 0.1℃ per second and a continuous fluorescence measurement) and finally a cooling step to 40℃.

II-B- 10. Western blot analysis

Total 10 μg of protein isolated from brain tissue was separated by sodium dodecyl sulphate-polyarylamide gel (SDS-PAGE; 5% stacking gel and 10.5% separating gel) and then transfered to a polyvinylidene fluoride (PVDF) membrane. Blocking was carried out with 3% BSA in TBS-T solution (0.2 M Tris-base, 1.37 M NaCl with 0.1% Tween-20, pH 7.6) overnight at 4℃. After membranes washed several times with washing buffer TBS-T, the membranes were incubated with primary anti-TH antibody (1:2000) and primary anti-AADC antibody (1:1000) for 2hrs at 4℃, followed by incubation with secondary antibodies (1:1000) for 2hrs at room temperature. The same amount of protein was loaded and reacted with anti-mouse actin antibody (1:2000), which was used for normalization of protein loading. All primary antibodies and secondary antibodies were diluted in TBS-T solution. To reveal the reaction bands, the membrane was reacted with WEST-ZOL (plus) Western blot detection system (Intron Biotechnology, Inc.) and exposed on

X-ray film (BioMax MS-1, Eastman Kodak). A digital image system was used to determine the density of the bands (Gel Quant, DNR Bio-Imaging Systems Ltd.

II-B-11. Statistical analysis

Data are presented as mean ± S.E.M. Results are evaluated by Student's test and by one- way analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered statistically significant.

Table 2. List of primers used for RT-PCR and real time PCR

Genes	Primer sequences (sense/antisense)	Nucleotide Position	Product size	Gene reference (GenBank #)
TH (for real time PCR)	5' CAGTGCCAGAGAGGACAAG 3' 3' CTCTTCCGCTGTGTATTCC 5'	No.482-698	217bp	M10244
TH (for RT-PCR)	5' GCTGTCACGTCCCCAAGGTT 3' 3' TCAGACACCCGACGCACAGA 5'	No.93-472	380bp	M10244
AADC (for real time and RT-PCR)	5' CTTCAGATGGCAACTACTCC 3' 3' CTTCGGTTAGGTCAGTTCTC 5'	No.684-1028	345bp	U31884
β-actin	5' CCTCTATGCCAACACAGT 3' 3' AGCCACCAATCCACACAG 5'	No.957-1111	155bp	BC063166

III. RESULTS

III-A. Effects of MP on cell viability

Cell viability was tested in different cell lines with different doses of MP (0, 100, 200, 400, 800, 1000 ㎍/㎖) treatment using by MTT assay, which a colorless tetrazolium salt is cleaved and converted to a blue formazan by mitochondrial dehydrogenases of living cells (Fig.5 and Fig. 6). As shown in Fig. 5, there is no significant effect of MP on SH-SY5Y cell viability below 400 ㎍/㎖ doses of MP treatment, compared with untreated cells. In contrast, the living percentage of 293 cells increased sharply above 400 ㎍/㎖ dose of MP treatment for 48 hrs incubation. Following 12 and 24 hrs incubation, cell viability increased steadily to approximately 200 ± 10% at the high dose 1000 ㎍/㎖ (Fig. 6). In addition, MP did not cause any interfering effects on MTT reduction under the concentration used in this study. The result revealed that MP has no cell cytoxicity to below 400 ㎍/㎖ dose treatment for longer time (48hrs).

As shown in Fig. 7, the cell viability of SH-SY5Y was decreased by rotenone treatment. However, the cell viability was increased when MP was added together with rotenone. This result indicates that MP has neuroprotective effect.

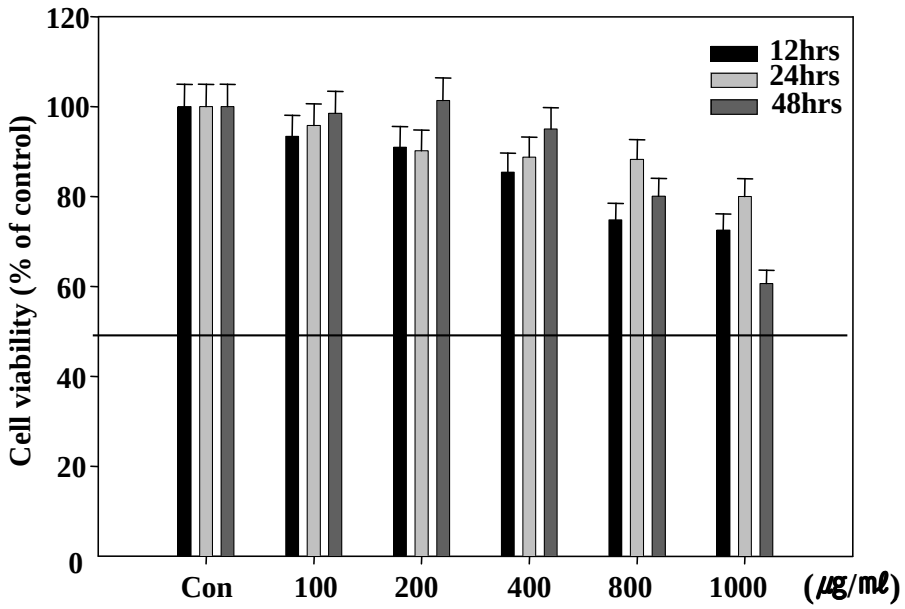

Fig. 5. Effects of MP on cell viability in SH-SY5Y cells. Cells were treated with the different doses of MP for 12, 24, and 48hrs and then processed for MTT assay. Con: untreated control

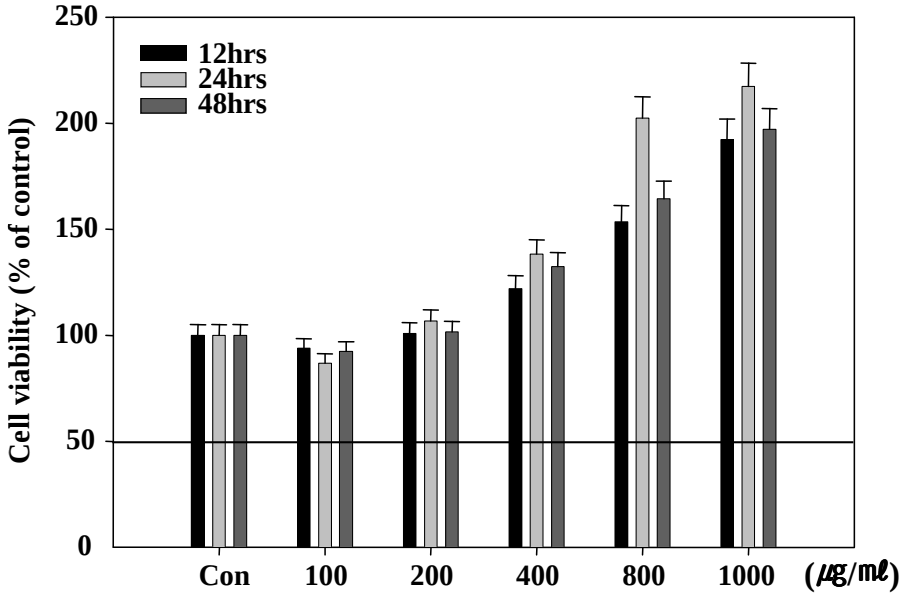

Fig. 6. Effects of MP on cell viability in 293 cells. Cells were treated with the different doses of MP for 12, 24 and 48 hrs then processed for MTT assay. Con: untreated control

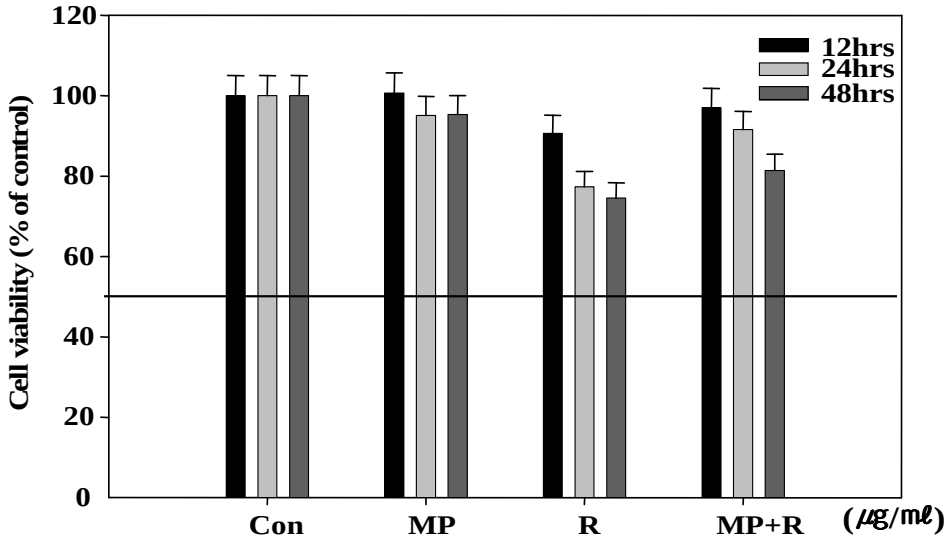

Fig. 7. Effects of MP on cell viability in SH-SY5Y cells treated by rotenone. Cells were treated with 100 ㎍/㎖ of MP (MP), 10 μM of rotenone (R) and 100 ㎍/㎖ of MP plus 10 μM of rotenone (MP+R) for 12, 24, 48hrs and then processed for MTT assay. Con: untreated control.

III-B. Free radical and Antioxidant activity

Free radicals are involved in many disorders such as neurodegenerative diseases, cancer and acquired immune deficiency syndrome (AIDS). Therefore, antioxidants through their scavenging power are useful for the management of those diseases. DPPH is stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It has been widely used to measure free radical scavenging activity. The reduction capability of DPPH radicals can be determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. As shown in Fig. 8, percentage of free radical scavenging activity of MP was increased dramatically from 44% to 91% between doses of 100 to 800 ㎍/㎖ of MP, while ascorbic acid exhibited 92% free radical scavenging activity. In contrast, the proportion radical scavenging activity of BHT was lower than MP and ascorbic acid (between 40 to 68% from doses of 100 and 800 ppm).

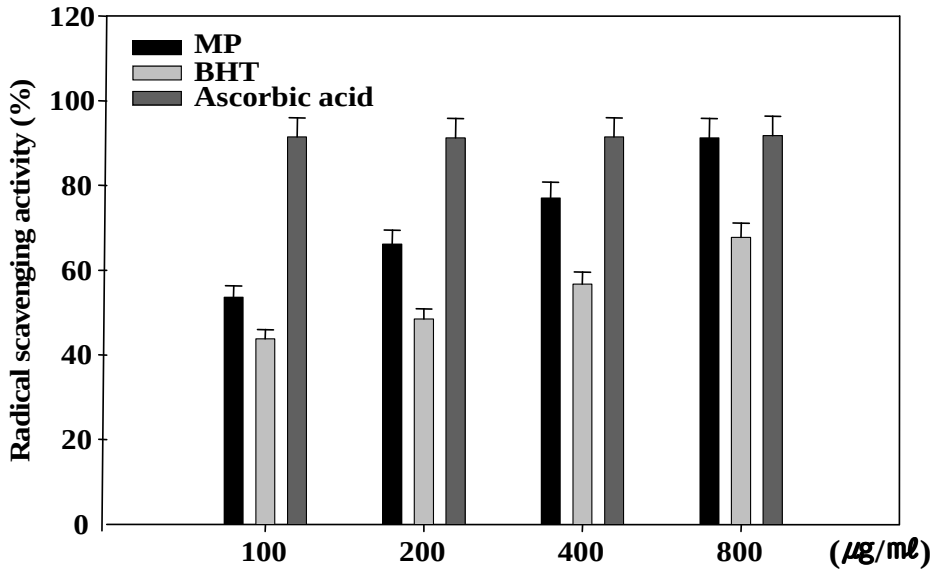

Fig. 8. DPPH free radical-scavenging activity of MP seed. The activity of MP extract was compared with BHT.

III-C. Catecholamine genes expression

III-C-1. Effects of MP on TH and AADC mRNA levels expression in rat brain tissue

To determine the effects of MP on the catecholamine biosynthetic enzyme genes, we determined changes in mRNA level of TH and AADC, the first rate-limiting and the second enzyme in this pathway. TH and AADC mRNA levels were examined by RT-PCR and real time PCR. Rats were treated with different doses of MP (0, 100, 200, 300, and 400 ㎎/㎏) each for 2, 4 and 8 hrs. As shown in Fig. 9, 10, 11 and table 3, TH and AADC mRNA levels increased in dose-dependent manner. However, the AADC mRNA level was less than TH mRNA level. In particular, TH mRNA increased by 2.2 ±3 folds ($P<0.001$), while AADC mRNA level rose to 1.86 and 2 folds with doses 300 ㎎/㎏, 400 ㎎/㎏, respectively. At 4h, TH mRNA level increased sharply and was higher than AADC mRNA level, the highest expression (TH mRNA 7.4 times ($P<0.0001$) and AADC mRNA 1.3 times ($P<0.05$)) was observed with dose 200 ㎎/㎏. However, both TH and AADC mRNA levels were not significantly changed at different doses for 8hrs in comparison with control. The expression of TH and AADC genes depend on MP doses and duration of administration. Thus, MP has a complex and different effects on TH and AADC gene expression in the rat brain tissue.

Table 3. Relative expression levels of TH and AADC mRNA in rat brain tissue by real time PCR after treatment with different doses of MP.

MP (㎎/㎏)	Relative concentration					
	TH			AADC		
	2hrs	4hrs	8hrs	2hrs	4hrs	8hrs
0	1	1	1	1	1	1
100	1.11	7.1	15.44	0.56	8.48	9.7
200	1.27	19.8	4.33	1.83	31.57	4.9
300	4.46	13.59	0.9	2.47	22.23	0.5
400	6.12	8.6	2.44	7.87	21.25	1.39

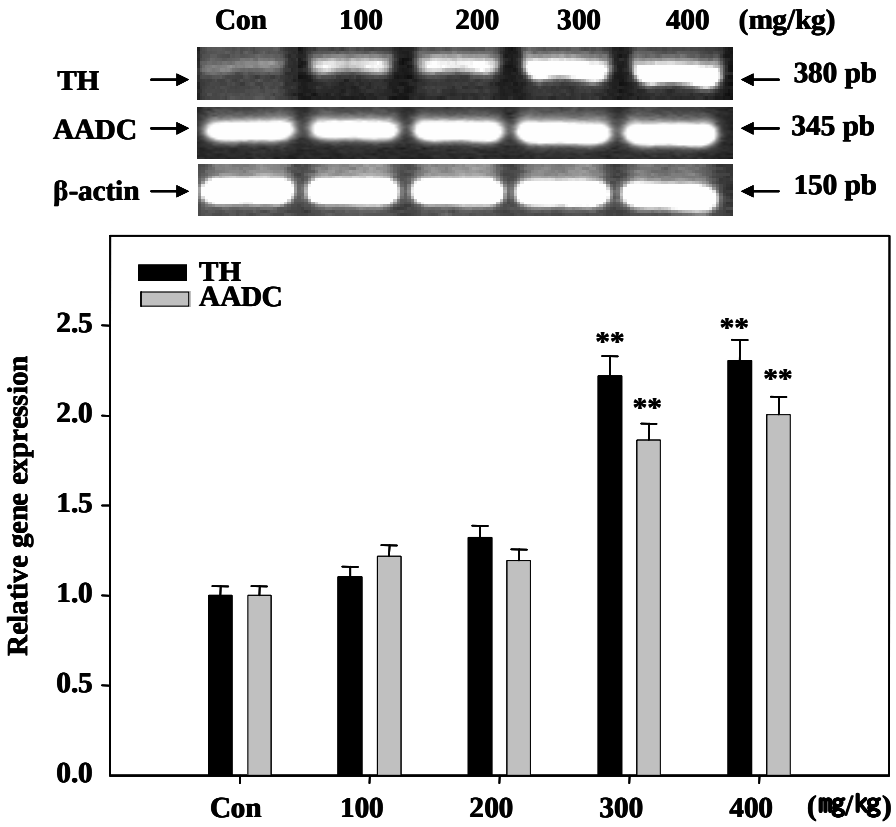

Fig. 9. The effects of MP on the TH and AADC mRNA levels in rat brain tissue at after 2hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one-way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

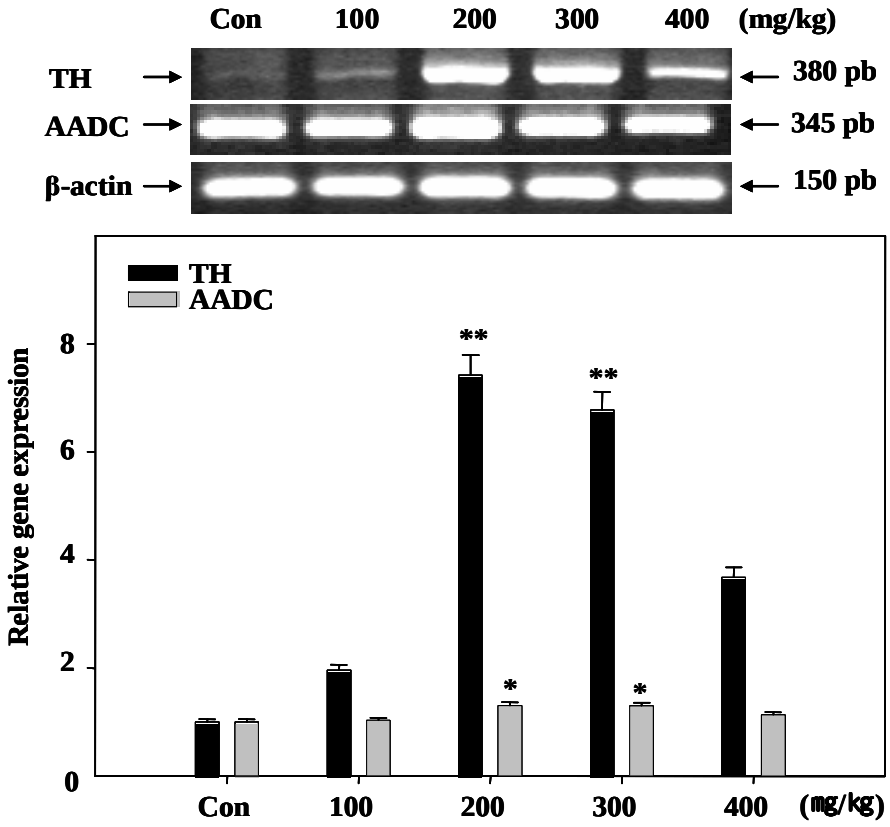

Fig. 10. The effects of MP on the TH and AADC mRNA levels in rat brain tissue at after 4hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one- way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

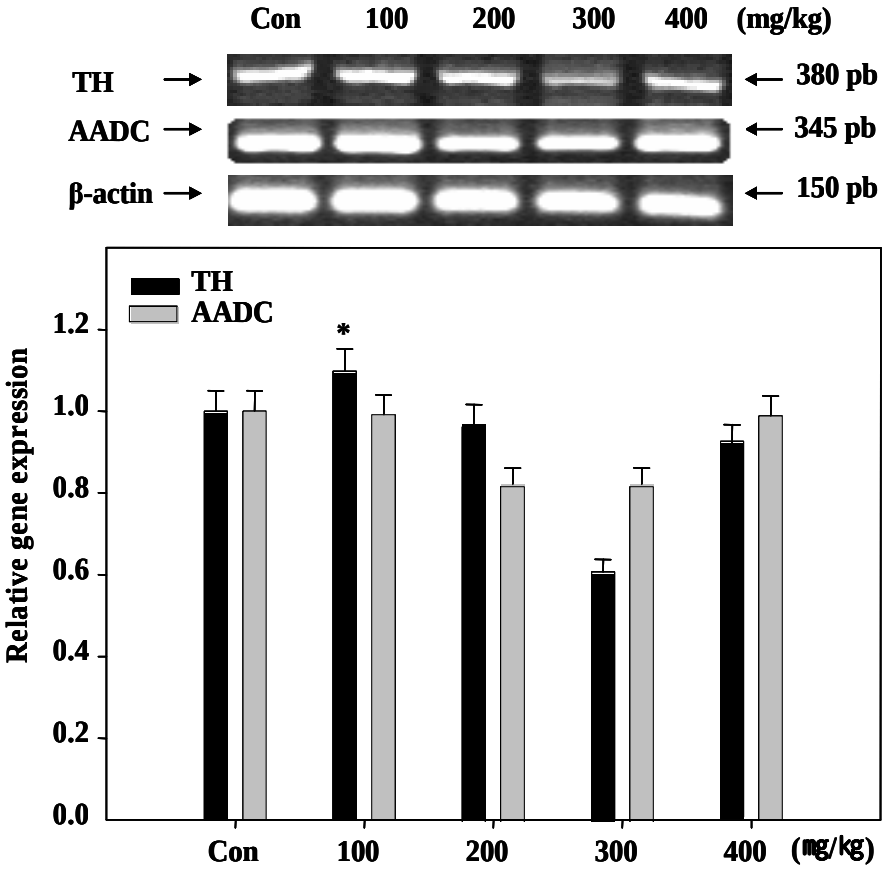

Fig. 11. The effects of MP on the TH and AADC mRNA levels in rat brain tissue at after 8hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one- way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

III-C-2. Effect of MP on TH and AADC protein level expression in rat brain tissue.

The protein levels of TH and AADC were determined by western blot analysis at 2, 4 and 8hrs after treatment. All different doses of MP induced TH and AADC protein levels at 2hrs. TH protein levels increased significantly by 1.34, 5.62, 6.92 and 9.74 folds with the doses of 100, 200, 300, 400 mg/kg, respectively (Fig. 12), AADC protein levels increased highly significantly at a dose of 100 mg/kg by 2.53 times. But only at doses of 200 mg/kg and 300 mg/kg have significantly up regulate the TH protein for 4hrs, but at doses of 100 mg/kg and 400 mg/kg shown the down regulation (Fig. 13). AADC protein levels did not change at different doses of MP at 4hrs after treatment. For 8hrs, AADC protein levels increased considerably at a dose 300 mg/kg by 1.7 times, whereas TH protein levels did not change at all (Fig. 14). The above results indicate that TH and AADC protein levels were significantly up regulated with the treatment of MP

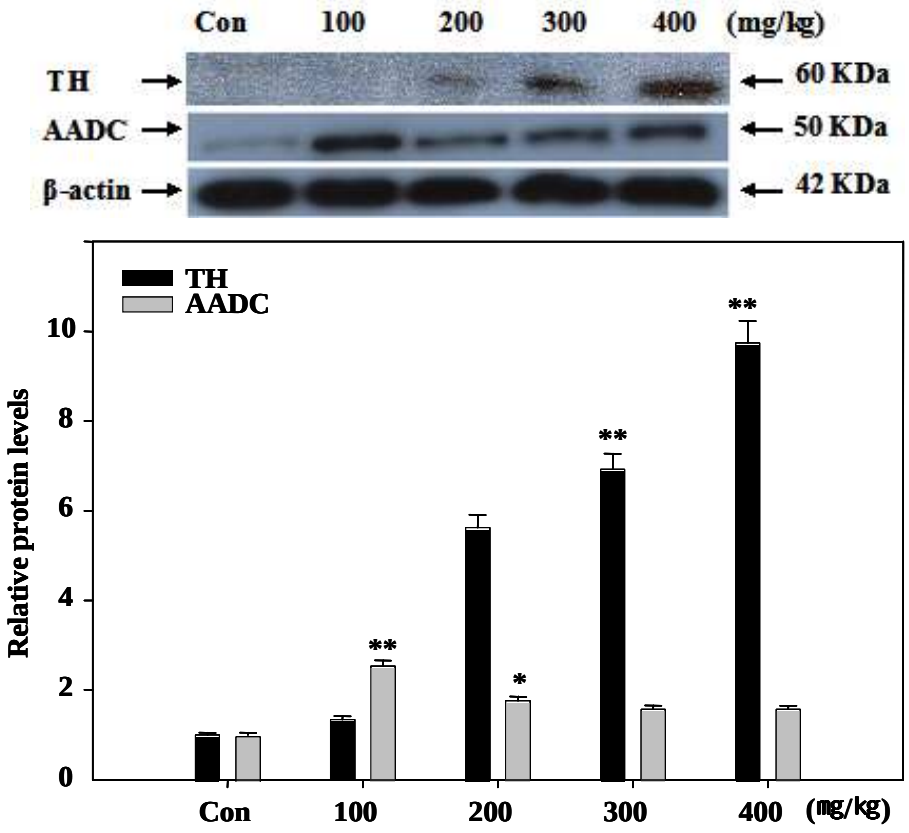

Fig. 12. The effects of MP on the TH and AADC protein levels in rat brain tissue at after 2hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one- way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

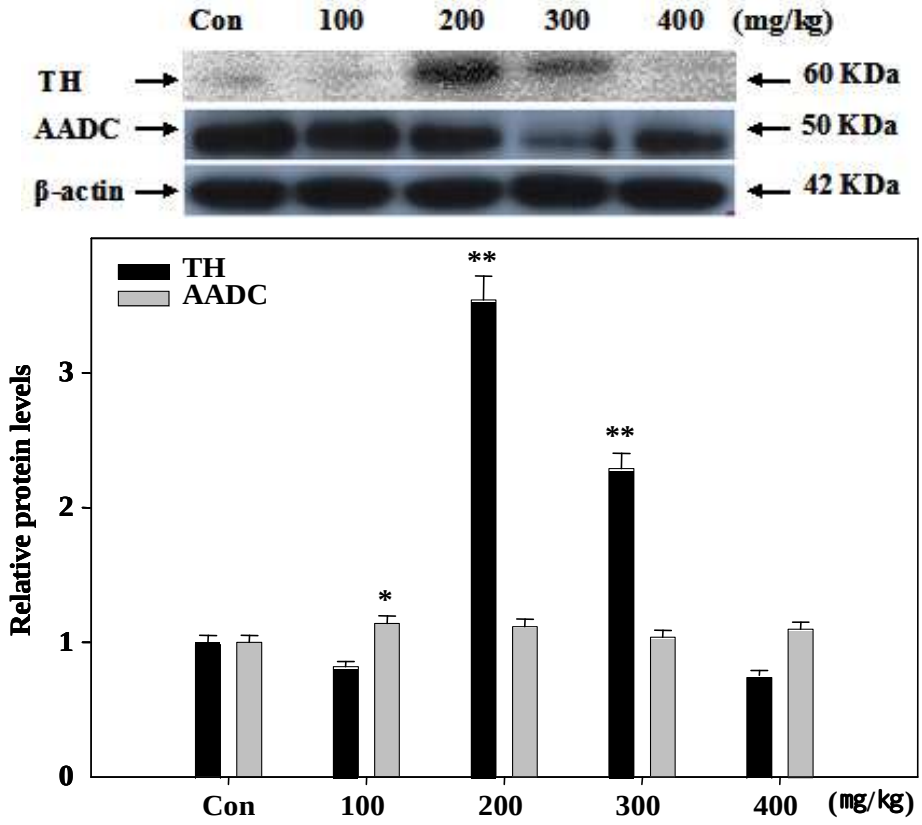

Fig. 13. The effects of MP on the TH and AADC protein levels in rat brain tissue at after 4hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one- way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

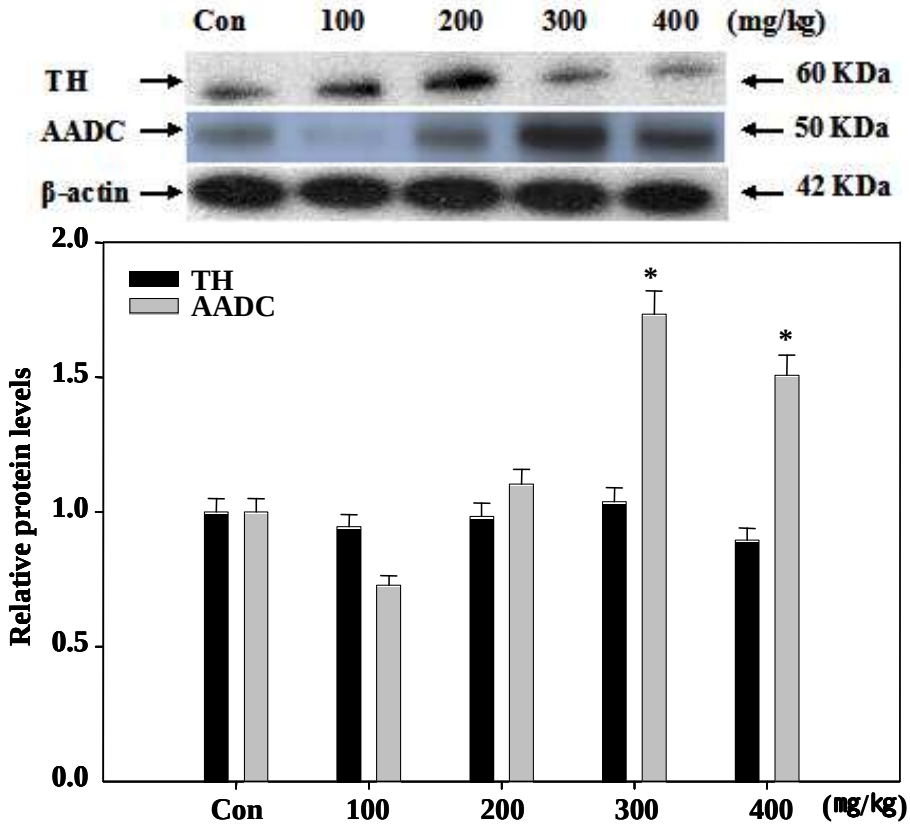

Fig. 14. The effects of MP on the TH and AADC protein levels in rat brain tissue at after 8hrs treatment. Values (n=5/group) are presented as mean ± S.E and compared by one- way ANOVA and Tukey's test: *P<0.05 and ** P< 0.001 versus control

IV. DISCUSSION

Herbal medicines are therapeutically potent, considered to possess fewer side effects, and may be cost-effective as compared to the other synthetic drugs. However, the specific mechanism of action of these plant products is not always easy to establish due to plant extracts or derivatives contain a mixture of several compounds some identified and many unidentified. In spite of this, the therapeutic effects of many plants are established. For example, datura seeds are known to contain anticholinergic alkaloids and have been used for therapeutic purposes in ancient times (Manyam et al., 2004).

The present investigation was carried out to evaluate the effects of MP on the cell viability, neuroprotective effect, its antioxidant status, free radical scavenging activity and inducible effects on the catecholamine biosynthetic enzyme genes particularly TH and AADC. The results showed that MP significantly upregulate both the TH and AADC mRNA levels (Fig. 9, 10 and 11) and protein levels (Fig. 12, 13 and 14). In addition, MP did not induce any cell toxicity to the neuroblastoma cell line below 400 ㎍/㎖ dose treatment and increased cell proliferation in human kidney cell line (Fig.5 and 6). MP also increased cell viability of neuroblastoma cell treated rotenone (Fig. 7).

One of the major finding of this study is the critical role for TH and AADC expression-related signals contributing to the DA neuronal survival by MP. Several studies have been published documenting the growth factors, neurotrophic factors and bioactive peptides stimulated the TH expression and supported the survival and differentation of midbrain DA neurons (Theofilopoulos et al., 2001; Lopez et al.,

2004). We found the novel effect of MP on the TH and AADC induction in rat brain tissue. The regulation of TH and AADC expression has attracted much attention in the field of neurology. Indeed, the biological function of TH and AADC is very improtant for the dopamine biosynthesis as well as for brain function under physiological and pathological conditions.

The AADC is a homodimeric enzyme belonging to the α-family of pyridoxal 5′-phosphate enzymes (Bertoldi and Voltattorni, 2003). AADC is a non-specific enzyme mainly implicated in the synthesis of dopamine and serotonin (Christenson et al., 1972) through the decarboxylation of a substrates (L-DOPA) and L-5-hydroxytryptophan in neuronal and also non-neuronal cells (Nagatsu, 1991) the AADC is responsible not only for the decarboxylation of L-DOPA but also for the decarboxylation of other amino acid, such as phenylalanine, 5-hydroxytryptophan, 3,4 dihydroxyphenylserine, histidine or tryptophan. Unlike other enzymes which are involved in catecholamine biosynthetic pathway, AADC is still generally considered not to be limiting in regulating of catecholamine biosynthesis. Nevertheless, some recent studies concerning the regulation of AADC in vivo and in vitro through phosphorylation indicate that this enzyme may play the role in regulation of catecholamine biosynthesis (Waymire and Haycock, 2002).

MP treatment is known to increase the dopamine content in the rat brain (Manyam et al., 2004). MP exhibited twice the antiparkinsonian activity compared with synthetic levodopa suggesting that MP may contain unidentified antiparkinsonian compounds in addition to levodopa, or that it may have adjuvants that enhance the efficacy of levodopa. The medication for PD has mainly focused on alleviating motor symptoms. However, the current major focus of research is the

development of drugs to slow the degeneration of the dying dopaminergic neurons or their regeneration. Coenzyme Q 10 and NADH has been used in an open label trial as a novel medication in patients with PD, using a parenteral administration technique (Birkmayer et al., 1989). Support for using NADH in PD treatment includes claims that NADH stimulates TH and dopamine biosynthesis in tissue culture and in humans. A beneficial clinical effect was observed. Concomitantly with the improvement in disability, urine homovanillic acid level increased significantly, indicating a stimulation of endogenous levodopa biosynthesis (Birkmayer et al., 1990). The orally applied form of NADH also yielded an overall improvement and was comparable to that of the parenterally applied form (Birkmayer et al., 1993). Most PD treatments palliate symptoms by increasing nigrostriatal dopaminergic tone (Birkmayer and Birkmayer, 1989). This may be due to the presence of NADH. MP powder also has a significant amount of NADH and hence it can possibly increase the mitochondrial complex-I activity and dopamine synthesis benefiting PD patients. The antioxidant and neuroprotective activity of coenzyme Q-10 was also proved in animal models where coenzyme Q-10 administered orally prior to treatment with MPTP was found to protect the nigrostriatal dopaminergic system (Beal et al., 1998). Coenzyme Q- 10 also can protect against striatal lesions produced by both malonate and nitropropionic acid (Matthews et al., 1998). MP powder contains natural coenzyme Q-10. Despite smaller quantities, it may have an adjuvant effect and thus enhance the therapeutic effect of MP powder in PD. MP powder, due to its antioxidants and increased complex-I activity, might have a protective effect on the 6-hydroxydopamine damaged nigral neurons. Another possible mechanism could be monoamine

oxidase inhibition providing a neuroprotective effect. MP did not have any effect on the mitochondrial monoamine oxidase activity, thus eliminating the possibility of monoamine oxidase inhibition leading to neuroprotection. There is evidence that MP seeds have an antioxidant effect (Tripathi and Upadhyay, 2002).

In conclusion, MP did not show any cell toxicity even with high dose (400㎍/㎖) and longer time (48hrs) treatment to human neuroblastoma cell and human kidney cells. MP showed the neuroprotective effect against rotenone. Also, MP have up-regulated both TH and AADC genes. Therefore, MP could have therapeutic value in the improvement and treatment of PD.

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Gwangju, 6th, December, 2006

Nguyen Thi Hiep

저작물 이용 허락서

학 과	Dept.of Biotechnology	학 번	20057812	과 정	석사
성 명	한글: Nguyen Thi Hiep		영문 : Nguyen Thi Hiep		
주 소	Khoa Che Bien, Truong Dai hoc Nha trang, so 2-Nguyen Dinh Chieu, Thanh pho Nha trang, Khanh hoa,Vietnam				
연락처	E-MAIL : bluebio203@yahoo.com				
논문제목	한글 : *Mucuna pruriens*에 의한 카테콜아민 생합성 효소유전자 유도발현에 대한 분자생물학적 분석				
	영문 : Molecular Analysis of Catecholamine Biosynthesis Enzyme Genes Induced by *Mucuna pruriens*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(O) 반대( )

2006 년 12 월 6 일

저작자: Nguyen Thi Hiep (서명 또는 인)

조선대학교 총장 귀하